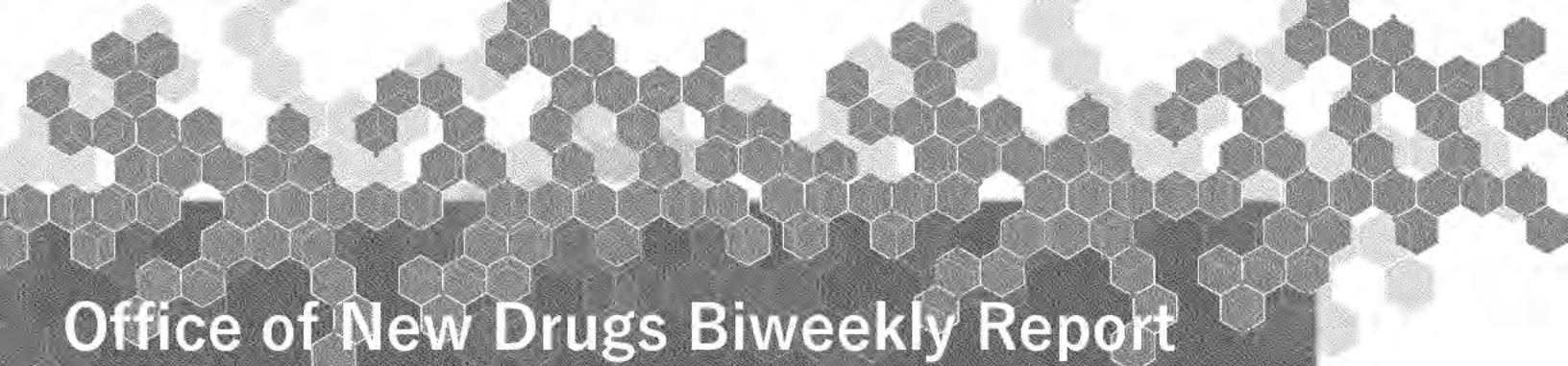




Office of New Drugs Biweekly Report

October 1, 2021

Reporting Period: September 20th to October 29th

Key Issues

NONRESPONSIVE



Recent and Upcoming NDA/BLA Actions

New Molecular Entities (NDA/BLA)						
Drug Name	App. No./ Filing Status	Division	Notable Issue	Goal Date (PDUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

New Molecular Entities (NDA/BLA)						
Drug Name	App. No./ Filing Status	Division	Notable Issue	Goal Date (PDUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

505(b)(2) Applications

Drug Name	App. No./ Filing Status	Division	Notable Issue	Goal Date (PDUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

505(b)(2) Applications

Drug Name	App. No./ Filing Status	Division	Notable Issue	Goal Date (PDUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

505(b)(2) Applications

Drug Name	App. No./ Filing Status	Division	Notable Issue	Goal Date (PDUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

Biosimilar Applications

Drug Name	App. No./ Filing Status	Division	Notable Issue	Goal Date (BsUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

Efficacy Supplements

Drug Name	App. No./ Filing Status	Division	Notable Issue	Goal Date (PDUFA/ BsUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

Efficacy Supplements						
Drug Name	App. No./ Filing Status	Division	Notable Issue	Goal Date (PDUFA/ BsUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE



Efficacy Supplements

Drug Name	App. No./ Filing Status	Division	Notable Issue	Goal Date (PDUFA/ BsUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

Efficacy Supplements						
Drug Name	App. No./ Filing Status	Division	Notable Issue	Goal Date (PDUFA/ BsUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

Other Applications						
Drug Name	App. No./ Filing Status	Division	Notable Issue	Goal Date (PDUFA)	Planned Action Date	Application Information Planned Action
NONRESPONSIVE						

New Applications Received

Notable IND Applications							
Drug Name	App. No./	Division	Notable Issue	Received Date	Goal Date	Planned Action Date	Application Information Planned Action
NONRESPONSIVE							

New Molecular Entities (NDA/BLA)							
Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA)	Planned Action Date	Application Information Planned Action
NONRESPONSIVE							

New Molecular Entities (NDA/BLA)							
Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE



New Molecular Entities (NDA/BLA)	
1	2
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97	98
99	100

Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

505(b)(2) Applications

Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

505(b)(2) Applications

Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

505(b)(2) Applications

Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

Biosimilar Applications

Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA/ BsUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

Efficacy Supplements

Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA/ BsUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

Efficacy Supplements							
Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA/ BsUFA)	Planned Action Date	Application Information Planned Action

Efficacy Supplements

Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA/ BsUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

Lupron Depot 7.5 mg; 3M 22.5 mg, 4M 30 mg, and 6M 45 mg	NDA 019732/ S-045 NDA 020517/ S-043 Unfiled	DO 1		8/6/2021	6/6/2022	5/6/2022	AbbVie; palliative treatment of advanced prostatic cancer; supplement is requesting to remove "palliative" from palliative treatment of advanced prostatic cancer. Filing goal date: 10/5/2021.

NONRESPONSIVE

Efficacy Supplements							
Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA/ BsUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

Efficacy Supplements							
Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA/ BsUFA)	Planned Action Date	Application Information Planned Action
NONRESPONSIVE							

Efficacy Supplements							
Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA/ BsUFA)	Planned Action Date	Application Information Planned Action
NONRESPONSIVE							

Other Applications							
Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA)	Planned Action Date	Application Information (Applicant/Sponsor name, indication) Planned Action
NONRESPONSIVE							

Other Applications							
Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA)	Planned Action Date	Application Information (Applicant/Sponsor name, indication) Planned Action
NONRESPONSIVE							

Notable End-of-Phase 2 Meetings

Notable Type A/Type B						
Meeting Type	Drug Name	App. No.	Division	Notable Issue	Meeting Dates (Internal/Sponsor)	Application Information
NONRESPONSIVE						

Notable Type A/Type B					
Meeting Type	Drug Name	App. No.	Division	Notable Issue	Meeting Dates (Internal/Sponsor) Application Information

NONRESPONSIVE

Notable Type A/Type B						
Meeting Type	Drug Name	App. No.	Division	Notable Issue	Meeting Dates (Internal/Sponsor)	Application Information

NONRESPONSIVE

Other Notable End-of-Phase Meetings						
Drug Name	App. No.	Division	Notable Issue	Meeting Dates (Internal/Sponsor)	Application Information	

NONRESPONSIVE

Other Notable End-of-Phase Meetings					
Drug Name	App. No.	Division	Notable Issue	Meeting Dates (Internal/Sponsor)	Application Information
NONRESPONSIVE					

Other Notable End-of-Phase Meetings					
Drug Name	App. No.	Division	Notable Issue	Meeting Dates (Internal/Sponsor)	Application Information
NONRESPONSIVE					

Clinical Holds					
Drug Name	App. No.	Division	Notable Issue	Date Issued	Application Information
NONRESPONSIVE					

Clinical Holds				
Drug Name	App. No.	Division	Notable Issue	Date Issued
Application Information				
NONRESPONSIVE				

Advisory Committee Meetings

Drug Name or Meeting Title	Division	Date	Summary
NONRESPONSIVE			

Drug Name or Meeting Title	Division	Date	Summary
NONRESPONSIVE			

Other Public Meetings

Drug Name or Meeting Title	Office or Division	Date	Summary
NONRESPONSIVE			

Drug Name or Meeting Title	Office or Division	Date	Summary
NONRESPONSIVE			

Other Meetings

Drug Name or Meeting Title	Division	Date	Summary
NONRESPONSIVE			

Drug Name or Meeting Title	Division	Date	Summary

NONRESPONSIVE

Drug Name or Meeting Title	Division	Date	Summary

NONRESPONSIVE

Medical Policy and Program Review Council

Office/ Division	Drug Name or Meeting Topic	Briefing Date	Summary of Recommendations (if available)

NONRESPONSIVE

Office/ Division	Drug Name or Meeting Topic	Briefing Date	Summary of Recommendations (if available)
NONRESPONSIVE			

Office/ Division	Drug Name or Meeting Topic	Briefing Date	Summary of Recommendations (if available)
NONRESPONSIVE			

Office/ Division	Drug Name or Meeting Topic	Briefing Date	Summary of Recommendations (if available)
NONRESPONSIVE			

Breakthrough Therapy Designations

Drug Name/ App. No./	Sponsor	Division	Indication	Decision (Grant/Deny/Pending)
NONRESPONSIVE				

Drug Name/ App. No./	Sponsor	Division	Indication	Decision (Grant/Deny/Pending)
NONRESPONSIVE				

Formal Dispute Resolution Requests

Office/Division	Deciding Authority/ Appeal level	Sponsor	Drug Name/ Application No.	Description	Status or Outcome
NONRESPONSIVE					

Safety Issues

Newly Identified Safety Signals				
Drug Name	SSID No.	Division	Planned or Actual Date of Action	Description
NONRESPONSIVE				

Newly Identified Safety Signals				
Drug Name	SSID No.	Division	Planned or Actual Date of Action	Description
NONRESPONSIVE				

Newly Identified Safety Signals				
Drug Name	SSID No.	Division	Planned or Actual Date of Action	Description

NONRESPONSIVE

Newly Identified Safety Signals				
Drug Name	SSID No.	Division	Planned or Actual Date of Action	Description
NONRESPONSIVE				

Safety Labeling Change				
Drug Name	App. No.	Division	Planned or Actual Date of Action	Description

NONRESPONSIVE

Safety Labeling Change				
Drug Name	App. No.	Division	Planned or Actual Date of Action	Description
NONRESPONSIVE				

Publications and External Presentations

Publications				
Office/ Division	FDA Authors	Citation with Link		Publication Date
NONRESPONSIVE				

External Presentations					
Office/ Division	FDA Presenters	Conference/Workshop/Mtg	Title/Topic	Location	Date and Time
NONRESPONSIVE					

External Presentations					
Office/ Division	FDA Presenters	Conference/Workshop/Mtg	Title/Topic	Location	Date and Time
NONRESPONSIVE					

Leave and Coverage

NONRESPONSIVE

Guidances and Noteworthy Policy Documents Recently Published or In Clearance

In Clearance, OND-Authored			
Authoring Div. or Office	Title of Guidance/Document	Status (Draft, Final or Revision)	OND Clearance Due Date
NONRESPONSIVE			

In Clearance, Authored Outside of OND			
Authoring Div. or Office	Title of Guidance/Document	Status (Draft, Final or Revision)	Clearance Due Date
NONRESPONSIVE			

In Clearance, Authored Outside of OND			
Authoring Div. or Office	Title of Guidance/Document	Status (Draft, Final or Revision)	Clearance Due Date
NONRESPONSIVE			

Recently Published, OND-Authored			
Authoring Div. or Office	Title of Guidance/Document	Status (Draft, Final or Revision)	Publication Date
NONRESPONSIVE			

Government Accountability Office (GAO) Study Status Update

NONRESPONSIVE

Regulatory Science Research Program Update

NONRESPONSIVE

NONRESPONSIVE

Modernization Update

Workstream	Current Status	Upcoming Milestones
NONRESPONSIVE		

Workstream	Current Status	Upcoming Milestones

NONRESPONSIVE

Office of New Drugs Biweekly Report

August 5, 2022

Reporting Period: July 25th to September 2nd

Key:

Blue text indicates new information or new applications that were not on the previous report.

Red text indicates an application that did not receive action on the PDUFA/goal date.

Key Issues

NONRESPONSIVE

NONRESPONSIVE

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NONRESPONSIVE

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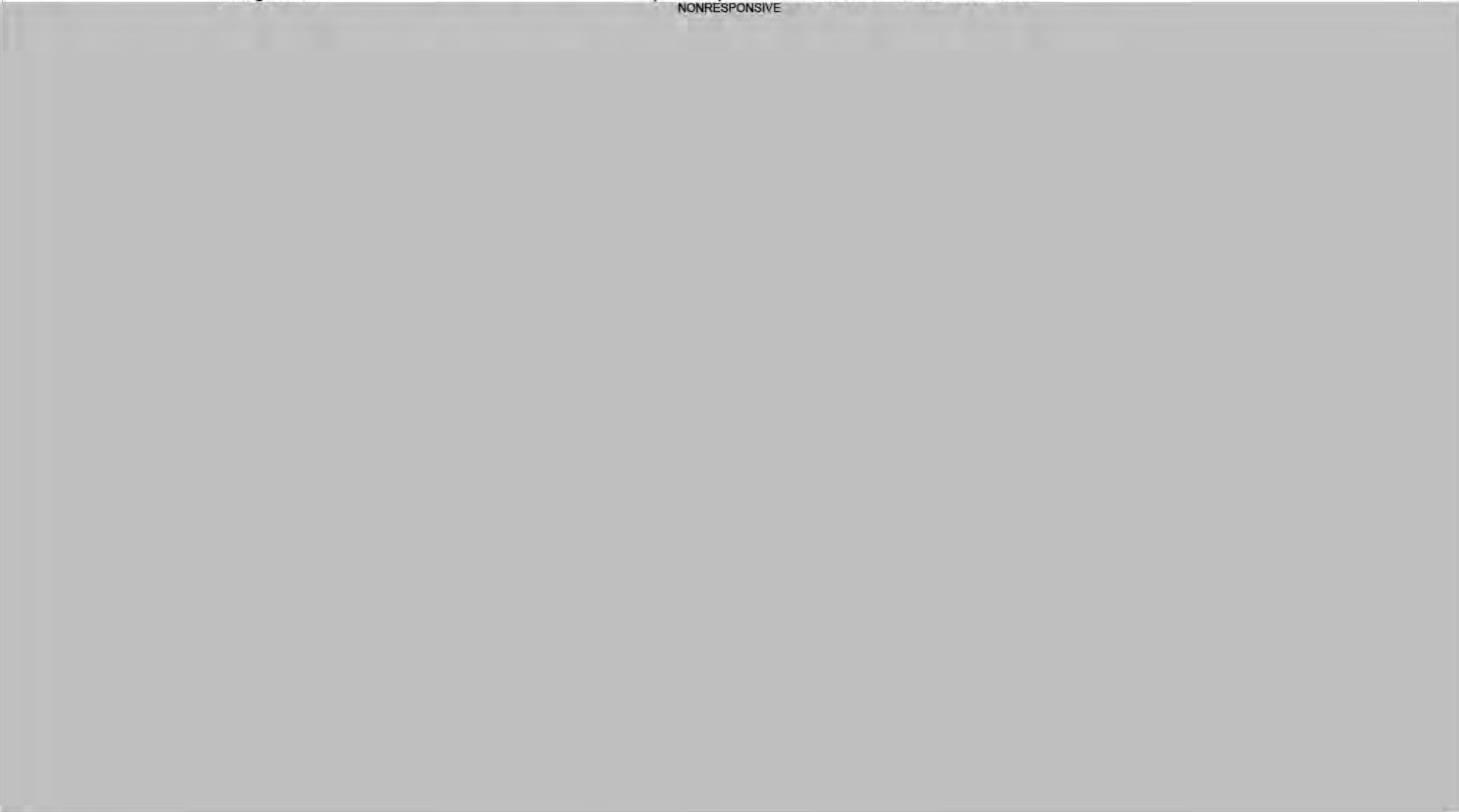
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NONRESPONSIVE

Recent and Upcoming NDA/BLA Actions

New Molecular Entities (NDA/BLA)						
Drug Name	App. No./ Filing Status	Division	Notable Issue	Goal Date (PDUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE



New Molecular Entities (NDA/BLA)						
Drug Name	App. No./ Filing Status	Division	Notable Issue	Goal Date (PDUFA)	Planned Action Date	Application Information Planned Action
NONRESPONSIVE						

505(b)(2) Applications

Drug Name	App. No./ Filing Status	Division	Notable Issue	Goal Date (PDUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

Biosimilar Applications						
Drug Name	App. No./ Filing Status	Division	Notable Issue	Goal Date (BsUFA)	Planned Action Date	Application Information Planned Action
NONRESPONSIVE						
Filing Date: 2/20/2020						

Efficacy Supplements						
Drug Name	App. No./ Filing Status	Division	Notable Issue	Goal Date (PDUFA/ BsUFA)	Planned Action Date	Application Information Planned Action
NONRESPONSIVE						

Efficacy Supplements						
Drug Name	App. No./ Filing Status	Division	Notable Issue	Goal Date (PDUFA/ BsUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

Efficacy Supplements						
Drug Name	App. No./ Filing Status	Division	Notable Issue	Goal Date (PDUFA/ BsUFA)	Planned Action Date	Application Information Planned Action
NONRESPONSIVE						

Efficacy Supplements						
Drug Name	App. No./ Filing Status	Division	Notable Issue	Goal Date (PDUFA/ BsUFA)	Planned Action Date	Application Information Planned Action
NONRESPONSIVE						

Other Applications						
Drug Name	App. No./ Filing Status	Division	Notable Issue	Goal Date (PDUFA)	Planned Action Date	Application Information Planned Action
NONRESPONSIVE						

Other Applications							
Drug Name	App. No./ Filing Status	Division	Notable Issue	Goal Date (PDUFA)	Planned Action Date	Application Information Planned Action	

NONRESPONSIVE

New Applications Received

Notable IND Applications.							
Drug Name	App. No./	Division	Notable Issue	Received Date	Goal Date	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

New Molecular Entities (NDA/BLA)							
Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

New Molecular Entities (NDA/BLA)

Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

New Molecular Entities (NDA/BLA)

Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

505(b)(2) Applications

Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

505(b)(2) Applications

Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

Biosimilar Applications

Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (BsUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

Efficacy Supplements							
Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA/ BsUFA)	Planned Action Date	Application Information Planned Action
NONRESPONSIVE							
Lupron Depot-PED (leuprolide acetate for depot suspension)	NDA 020263/ S-053 Unfiled	DGE		6/14/2022	4/14/2023	4/14/2023	AbbVie Endocrinology; additional 45 mg 6-month dosing regimen to the approved indication for pediatric patients with central precocious puberty. Filing goal date: 8/13/2022.
NONRESPONSIVE							

Efficacy Supplements							
Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA/ BsUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

Efficacy Supplements

Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA/ BsUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

Efficacy Supplements							
Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA/ BsUFA)	Planned Action Date	Application Information Planned Action
NONRESPONSIVE							

Efficacy Supplements							
Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA/ BsUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

Other Applications							
Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA)	Planned Action Date	Application Information Planned Action
NONRESPONSIVE							

Notable End-of-Phase 2 Meetings

Notable Type A/Type B						
Meeting Type	Drug Name	App. No.	Division	Notable Issue	Meeting Dates (Internal/Sponsor)	Application Information
NONRESPONSIVE						

Notable Type A/Type B					
Meeting Type	Drug Name	App. No.	Division	Notable Issue	Meeting Dates (Internal/Sponsor) Application Information

NONRESPONSIVE

Other Notable End-of-Phase Meetings

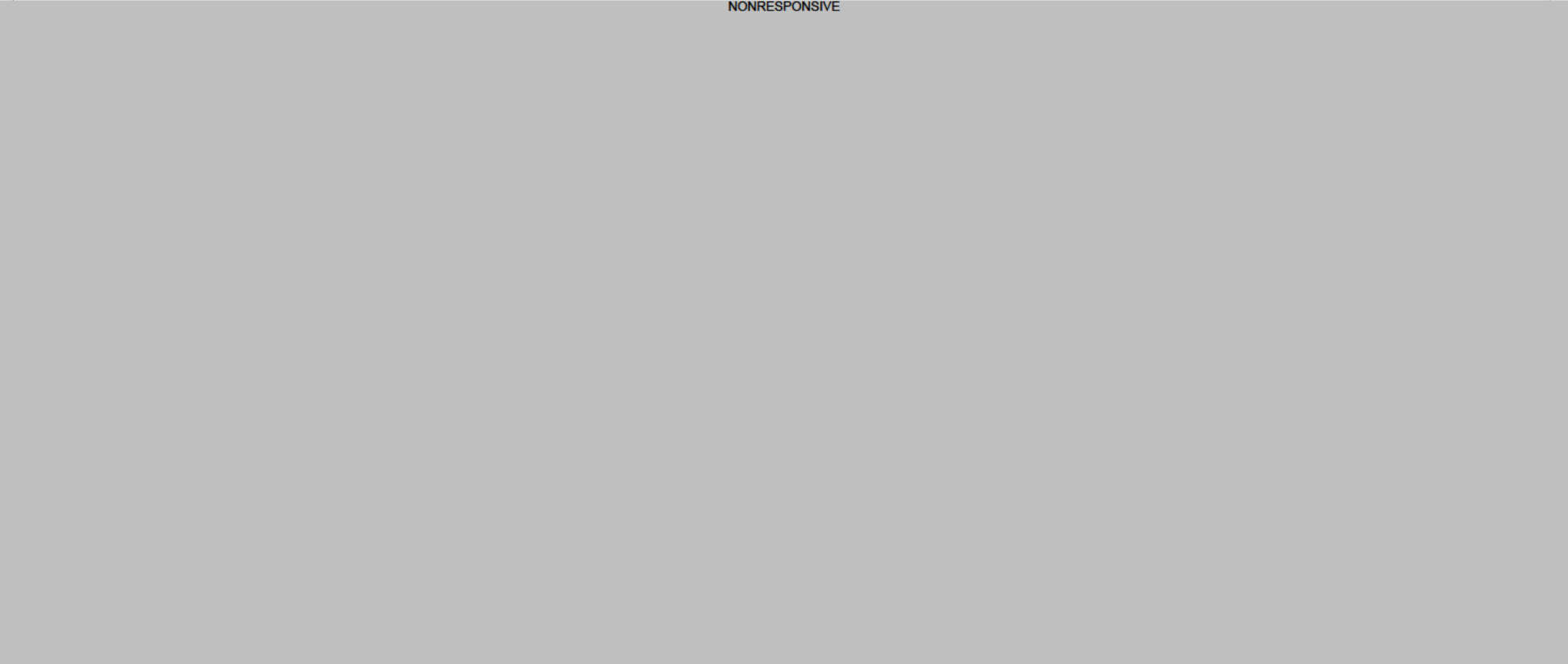
Drug Name	App. No.	Division	Notable Issue	Meeting Dates (Internal/Sponsor) NONRESPONSIVE	Application Information

Other Notable End-of-Phase Meetings					
Drug Name	App. No.	Division	Notable Issue	Meeting Dates (Internal/Sponsor)	Application Information
NONRESPONSIVE					

Clinical Holds					
Drug Name	App. No.	Division	Notable Issue	Date Issued	Application Information
NONRESPONSIVE					

Clinical Holds					
Drug Name	App. No.	Division	Notable Issue	Date Issued	Application Information

NONRESPONSIVE



Clinical Holds					
Drug Name	App. No.	Division	Notable Issue	Date Issued	Application Information
NONRESPONSIVE					

Other Public Meetings

Drug Name or Meeting Title	Office or Division	Date	Summary
NONRESPONSIVE			

Drug Name or Meeting Title	Office or Division	Date	Summary

NONRESPONSIVE

Other Meetings

Drug Name or Meeting Title	Division	Date	Summary

NONRESPONSIVE

Drug Name or Meeting Title	Division	Date	Summary
NONRESPONSIVE			

Medical Policy and Program Review Council

Office/ Division	Drug Name or Meeting Topic	Briefing Date	Summary of Recommendations (if available)

NONRESPONSIVE

Breakthrough Therapy Designations

Drug Name/ App. No./	Sponsor	Division	Indication	Decision (Grant/Deny/Pending)

NONRESPONSIVE

Formal Dispute Resolution Requests

Office/ Division	Deciding Authority/ Appeal level	Sponsor	Drug Name/ Application No.	Description	Status or Outcome
NONRESPONSIVE					

CDER Biosimilar Review Committee, CDER/CBER Biosimilar Policy Council, and CDER/CBER Biologicals Product Classification Subcommittee

Meeting Type	App. No.	Date	Office/ Division	Topic
NONRESPONSIVE				

Safety Issues

Newly Identified Safety Signals				
Drug Name	SSID No.	Division	Planned or Actual Date of Action	Description

NONRESPONSIVE

Newly Identified Safety Signals				
Drug Name	SSID No.	Division	Planned or Actual Date of Action	Description
NONRESPONSIVE				

Drug Safety Communications				
Drug Name	App. No.	Division	Planned or Actual Date of Action	Description

NONRESPONSIVE

Safety Labeling Change				
Drug Name	App. No.	Division	Planned or Actual Date of Action	Description

NONRESPONSIVE

Safety Labeling Change				
Drug Name	App. No.	Division	Planned or Actual Date of Action	Description

NONRESPONSIVE

Publications and External Presentations

External Presentations					
Office/ Division	FDA Presenters	Conference/Workshop/Mtg	Title/Topic	Location	Date and Time

NONRESPONSIVE

Leave and Coverage

NONRESPONSIVE

NONRESPONSIVE

Guidances and Noteworthy Policy Documents Recently Published or In Clearance

In Clearance, Authored Outside of OND			
Authoring Div. or Office	Title of Guidance/Document	Status (Draft, Final or Revision)	OND Clearance Due Date

NONRESPONSIVE

In Clearance, Authored Outside of OND			
Authoring Div. or Office	Title of Guidance/Document	Status (Draft, Final or Revision)	OND Clearance Due Date

NONRESPONSIVE

MAPPs and Noteworthy Process Documents In Clearance

In Clearance				
Authoring Div. or Office	Title of Guidance/Document	Status	OND Clearance Due Date	CDER Clearance Due Date

NONRESPONSIVE

Working Groups and Standing Sub-Committees

Project	Purpose	Risk/Mitigation	Due Date

NONRESPONSIVE

Modernization Update

Workstream	Current Status	Upcoming Milestones

NONRESPONSIVE

Workstream	Current Status	Upcoming Milestones
NONRESPONSIVE		

Workstream	Current Status	Upcoming Milestones
NONRESPONSIVE		

Office of New Drugs Biweekly Report

August 19, 2022

Reporting Period: August 8th to September 16th

Key:

Blue text indicates new information or new applications that were not on the previous report.

Red text indicates an application that did not receive action on the PDUFA/goal date.

Key Issues

NONRESPONSIVE

NONRESPONSIVE

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NONRESPONSIVE

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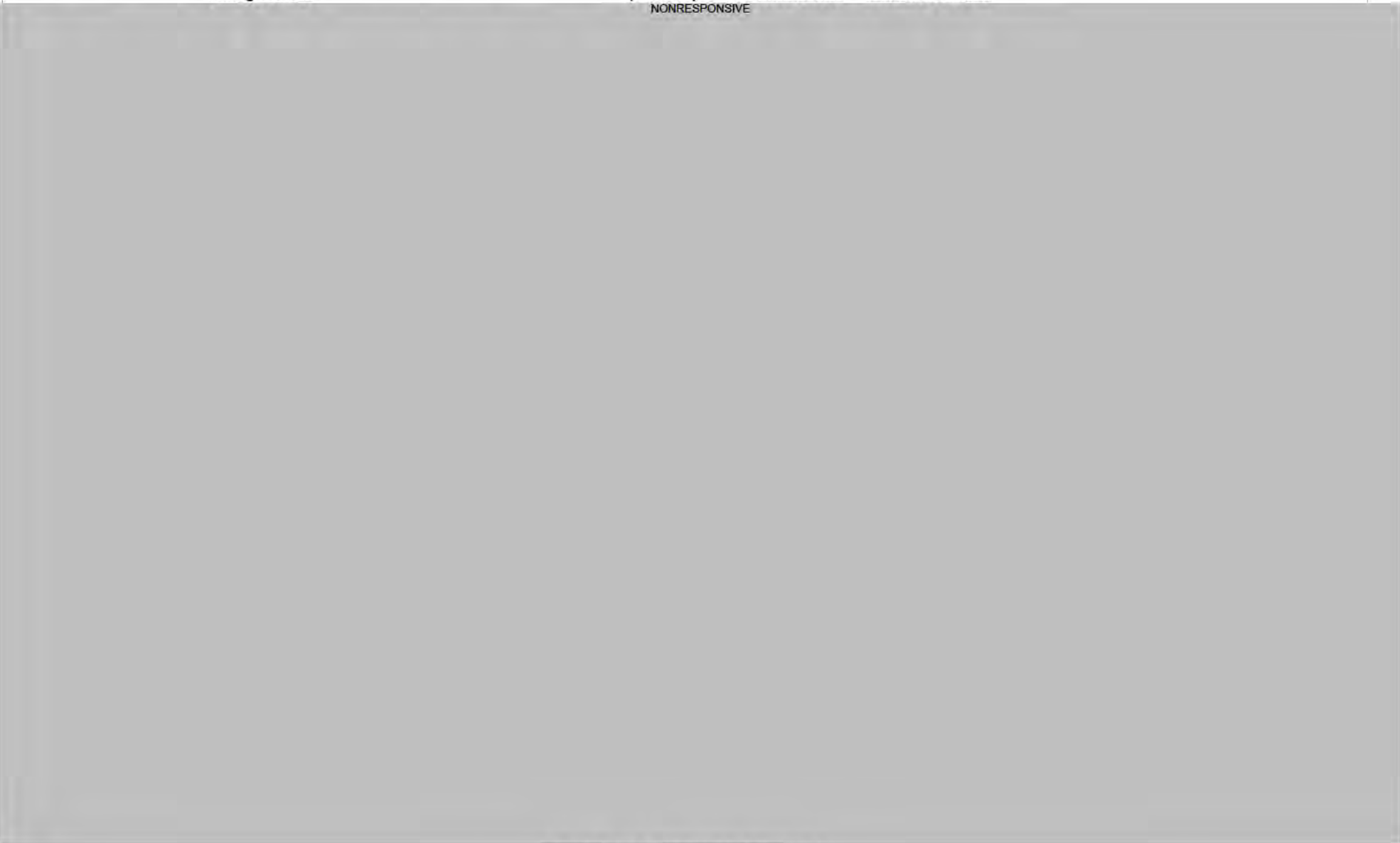
FDACDER000591

NONRESPONSIVE

Recent and Upcoming NDA/BLA Actions

New Molecular Entities (NDA/BLA)						
Drug Name	App. No./ Filing Status	Division	Notable Issue	Goal Date (PDUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE



New Molecular Entities (NDA/BLA)

Drug Name	App. No./ Filing Status	Division	Notable Issue	Goal Date (PDUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

505(b)(2) Applications

Drug Name	App. No./ Filing Status	Division	Notable Issue	Goal Date (PDUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

505(b)(2) Applications						
Drug Name	App. No./ Filing Status	Division	Notable Issue	Goal Date (PDUFA)	Planned Action Date	Application Information Planned Action
NONRESPONSIVE						

Biosimilar Applications						
Drug Name	App. No./ Filing Status	Division	Notable Issue	Goal Date (BsUFA)	Planned Action Date	Application Information Planned Action
NONRESPONSIVE						

Biosimilar Applications						
Drug Name	App. No./ Filing Status	Division	Notable Issue	Goal Date (BsUFA)	Planned Action Date	Application Information Planned Action
NONRESPONSIVE						

Efficacy Supplements						
Drug Name	App. No./ Filing Status	Division	Notable Issue	Goal Date (PDUFA/ BsUFA)	Planned Action Date	Application Information Planned Action
NONRESPONSIVE						

Efficacy Supplements						
Drug Name	App. No./ Filing Status	Division	Notable Issue	Goal Date (PDUFA/ BsUFA)	Planned Action Date	Application Information Planned Action
NONRESPONSIVE						

Efficacy Supplements						
Drug Name	App. No./ Filing Status	Division	Notable Issue	Goal Date (PDUFA/ BsUFA)	Planned Action Date	Application Information Planned Action
NONRESPONSIVE						

Other Applications						
Drug Name	App. No./ Filing Status	Division	Notable Issue	Goal Date (PDUFA)	Planned Action Date	Application Information Planned Action
NONRESPONSIVE						

Other Applications						
Drug Name	App. No./ Filing Status	Division	Notable Issue	Goal Date (PDUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

New Applications Received

Notable IND Applications.							
Drug Name	App. No./	Division	Notable Issue	Received Date	Goal Date	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

Notable IND Applications.

Drug Name	App. No./	Division	Notable Issue	Received	Goal Date	Planned	Application Information
	Filing Status			Date	(PDUFA)	Action	Planned Action
NONRESPONSIVE							

New Molecular Entities (NDA/BLA)

Drug Name	App. No./	Division	Notable Issue	Received	Goal Date	Planned	Application Information
	Filing Status			Date	(PDUFA)	Action	Planned Action
NONRESPONSIVE							

New Molecular Entities (NDA/BLA)							
Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA)	Planned Action Date	Application Information Planned Action
NONRESPONSIVE							

505(b)(2) Applications							
Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA)	Planned Action Date	Application Information Planned Action
NONRESPONSIVE							

505(b)(2) Applications

Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

Biosimilar Applications							
Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (BsUFA)	Planned Action Date	Application Information Planned Action
NONRESPONSIVE							

Efficacy Supplements							
Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA/ BsUFA)	Planned Action Date	Application Information Planned Action
NONRESPONSIVE							

Efficacy Supplements							
Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA/ BsUFA)	Planned Action Date	Application Information Planned Action
Lupron Depot-PED (leuprolide acetate) depot suspension	NDA 020263/ S-053 Filed	DGE		6/14/2022	4/14/2023	4/14/2023	AbbVie Endocrinology; additional 45 mg 6-month dosing regimen to the approved indication for pediatric patients with central precocious puberty. Filing date: 8/13/2022.

NONRESPONSIVE

Efficacy Supplements

Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA/ BsUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

Efficacy Supplements

Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA/ BsUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

Efficacy Supplements							
Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA/ BsUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

Efficacy Supplements							
Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA/ BsUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

Efficacy Supplements							
Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA/ BsUFA)	Planned Action Date	Application Information Planned Action

NONRESPONSIVE

Other Applications							
Drug Name	App. No./ Filing Status	Division	Notable Issue	Received Date	Goal Date (PDUFA)	Planned Action Date	Application Information Planned Action
NONRESPONSIVE							

Notable End-of-Phase 2 Meetings

Notable Type A/Type B						
Meeting Type	Drug Name	App. No.	Division	Notable Issue	Meeting Dates (Internal/Sponsor)	Application Information
NONRESPONSIVE						

Notable Type A/Type B					
Meeting Type	Drug Name	App. No.	Division	Notable Issue	Meeting Dates (Internal/Sponsor) Application Information

NONRESPONSIVE

Other Notable End-of-Phase Meetings					
Drug Name	App. No.	Division	Notable Issue	Meeting Dates (Internal/Sponsor) NONRESPONSIVE	Application Information

Clinical Holds					
Drug Name	App. No.	Division	Notable Issue	Date Issued	Application Information

NONRESPONSIVE

Clinical Holds	
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Drug Name	App. No.	Division	Notable Issue	Date Issued	Application Information

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Drug Name	App. No.	Division	Notable Issue	Date Issued	Application Information

Drug Name	App. No.	Division	Notable Issue	Date Issued	Application Information

Drug Name	App. No.	Division	Notable Issue	Date Issued	Application Information

Drug Name	App. No.	Division	Notable Issue	Date Issued	Application Information

NONRESPONSIVE

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Advisory Committee Meetings

Drug Name or Meeting Title	Division	Date	Summary	Potential Controversial Issues
NONRESPONSIVE				

Other Public Meetings

Drug Name or Meeting Title	Office or Division	Date	Summary
NONRESPONSIVE			

Drug Name or Meeting Title	Office or Division	Date	Summary
NONRESPONSIVE			

Other Meetings

Drug Name or Meeting Title	Division	Date	Summary
NONRESPONSIVE			

Drug Name or Meeting Title	Division	Date	Summary

NONRESPONSIVE

Drug Name or Meeting Title	Division	Date	Summary
NONRESPONSIVE			

Medical Policy and Program Review Council

Office/ Division	Drug Name or Meeting Topic	Briefing Date	Summary of Recommendations (if available)

NONRESPONSIVE

Breakthrough Therapy Designations

Drug Name/ App. No./	Sponsor	Division	Indication	Decision (Grant/Deny/Pending)
NOWRESPONSIVE				

Formal Dispute Resolution Requests

Office/ Division	Deciding Authority/ Appeal level	Sponsor	Drug Name/ Application No.	Description	Status or Outcome

NONRESPONSIVE

CDER Biosimilar Review Committee, CDER/CBER Biosimilar Policy Council, and CDER/CBER Biologicals Product Classification Subcommittee

Meeting Type	App. No.	Date	Office/ Division	Topic

NONRESPONSIVE

Safety Issues

Newly Identified Safety Signals				
Drug Name	SSID No.	Division	Planned or Actual Date of Action	Description
NONRESPONSIVE				

Newly Identified Safety Signals				
Drug Name	SSID No.	Division	Planned or Actual Date of Action	Description
NONRESPONSIVE				

Safety Labeling Change				
Drug Name	App. No.	Division	Planned or Actual Date of Action	Description
NONRESPONSIVE				

Publications and External Presentations

External Presentations					
Office/ Division	FDA Presenters	Conference/Workshop/Mtg	Title/Topic	Location	Date and Time
NONRESPONSIVE					

Leave and Coverage

NONRESPONSIVE	

Guidances and Noteworthy Policy Documents Recently Published or In Clearance

In Clearance, OND-Authored			
Authoring Div. or Office	Title of Guidance/Document	Status (Draft, Final or Revision)	OND Clearance Due Date
NONRESPONSIVE			

In Clearance, Authored Outside of OND			
Authoring Div. or Office	Title of Guidance/Document	Status (Draft, Final or Revision)	OND Clearance Due Date
NONRESPONSIVE			

In Clearance, Authored Outside of OND			
Authoring Div. or Office	Title of Guidance/Document	Status (Draft, Final or Revision)	OND Clearance Due Date
NONRESPONSIVE			

MAPPs and Noteworthy Process Documents In Clearance

In Clearance				
Authoring Div. or Office	Title of Guidance/Document	Status	OND Clearance Due Date	CDER Clearance Due Date
NONRESPONSIVE				

Working Groups and Standing Sub-Committees

Project	Purpose	Risk/Mitigation	Due Date
NONRESPONSIVE			

Modernization Update

Workstream	Current Status	Upcoming Milestones
NONRESPONSIVE		

Workstream	Current Status	Upcoming Milestones
NONRESPONSIVE		

Workstream	Current Status	Upcoming Milestones



Welcome to the 2022 Stanford Drug Discovery Symposium

This symposium provides a valuable and important platform for inspiring interdisciplinary exchange at the forefront of drug research and will support a fantastic networking experience. It provides a great resource for researchers, pharmaceutical companies, investment groups, and those in the wider biomedical community interested in discovering new drugs and improving patient care. We look forward to an exciting two days of talks and discussion.



Joseph C. Wu, MD, PhD
Director, Cardiovascular Institute

Participant Interaction Platform

SDDS will be using **Slido** as a platform to send questions to the speakers. *Please note that the speakers may not have time to answer all questions during the panel sessions.*

Slido is accessible using the following formats:

Webpage: Simply type your question in the Slido interface on the viewing webpage.

New browser window: Open the Slido website in a new browser window and enter participant code **#SDDS2022** in the "Joining as a Participant" field at the top of the page.

Cellphone: You can use Slido on your mobile phone (no app download required). Just scan the QR code below.



REGISTRATION
tinyurl.com/SDDS2022

DAY-OF VIEWING PAGE
tinyurl.com/SDDS2022-livestream



SOCIAL MEDIA
#SDDS2022

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Time (PDT) **WELCOME TO DAY 1**

- 9:00 am **Joseph C. Wu, MD, PhD**
Director, Stanford Cardiovascular Institute
Simon H. Stertzer, MD, Professor of Medicine and Radiology
- 9:05 am **Lloyd Minor, MD**
Dean, Stanford School of Medicine
- 9:10 am **Marc Tessier-Lavigne, PhD**
President, Stanford University

SESSION I: PRESENTATION OF LIFETIME ACHIEVEMENT AWARD

Moderator: Randy Schekman, PhD; Co-Moderator: Joseph C. Wu, MD, PhD

- 9:15 am *Introduction of Katalin Karikó, PhD*
Randy Schekman, PhD
Professor of Molecular and Cell Biology, University of California, Berkeley; 2013 Nobel Laureate
- 9:20 am **AWARDEE LECTURE: Katalin Karikó, PhD**
Senior Vice President, BioNTech
Developing mRNA for therapy
- 9:40 am Question & Answer
- 9:55 am **BREAK**

SESSION II: RESEARCH DEVELOPMENT I

Moderator: Myrtle Davis, DVM, PhD; Co-Moderator: Mark Mercola, PhD

- 10:05 am **Claude Bertrand, PhD**
Executive Vice President R&D, Chief Scientific Officer, Servier
Servier: A New Paradigm for R&D through a significant Transformation Journey
- 10:18 am **Dean Li, MD, PhD**
Executive VP and President of Merck Research Laboratories
The Balance of Computing Power and Human Intelligence
- 10:30 am **Ray Deshaies, PhD**
Sr. Vice President, Global Research, Amgen
Multispecific Molecular Medicines
- 10:43 am **Carlos O. Garner, PhD**
Vice President, Global Regulatory Affairs, Eli Lilly and Company
How COVID Will Reshape Clinical Research
- 10:57 am Panel Discussion
- 11:20 am **BREAK**

Time (PDT) **SESSION III: PATENT LAW**

Moderators: Karin Immergluck, PhD; Co-Moderator: Kuldev Singh, MD

- 11:20 am **Madhuri Roy, JD, PhD**
Partner, Patent Counseling & Prosecution, Cooley
Getting your IP House In Order

Time (PDT) **SESSION III: PATENT LAW *Con't***
Moderators: Karin Immergluck, PhD; Co-Moderator: Kuldev Singh, MD

- 11:33 am **Vern Norviel, JD**
Partner, Patents and Innovations, Wilson Sonsini
Drug Label Patent Extension
- 11:46 am **Hogene Choi, JD**
Partner, Morrison & Foerster
Strategies for Protecting Software Innovation in Drug Discovery
- 12:00 pm Panel Discussion
- 12:20 pm **BREAK**

SESSION IV: PUBLIC POLICY

Moderators: Steven Artandi, MD, PhD; Co-Moderator: Mark Mercola, PhD

- 12:45 pm **Michelle McMurry-Heath, MD**
President & Chief Executive Officer, Biotechnology Innovation Organization
The Distribution of Scientific Progress: The Social Justice Issue of our Age
- 1:00 pm **Gary Gibbons, MD**
Director, NHLBI
Accelerating the Cycle of Innovation for Drug Discovery to Prevent and Preempt Chronic Disease
- 1:16 am **Jacqueline Corrigan-Curay, JD, MD**
Principal Deputy Center Director, FDA, CDER
Evidence Generation – Enhancing Access and Equity
- 1:29 pm **Maria Millan, MD**
President and Chief Executive Officer, CIRM
CIRM: Building California's Public-Private Partnerships to Accelerate the Development of Genes and Therapies
- 1:47 pm Panel Discussion
- 2:05 pm **BREAK**

Time (PDT) **SESSION V: RESEARCH DEVELOPMENT II**
Moderator: Sandra Horning, MD; Co-Moderator: Sanjay Malhotra, PhD

- 2:10 pm **Alfred Sandrock, MD, PhD**
CEO, Voyager Therapeutics
The Discovery and Development of Aducanumab for Alzheimer's Disease
- 2:30 pm **Lisa Coussens, PhD**
President, AACR; Associate Director for Basic Research, Knight Cancer Institute, Oregon Health & Science University
Neutralizing Protumor Inflammation: Lessons Learned from Preclinical Mouse Models
- 2:47 pm **Randy Schekman, PhD**
Professor of Molecular and Cell Biology, University of California, Berkeley; 2013 Nobel Laureate
Engineering a Membrane-Mediated Delivery of Genome Editing Functions
- 2:59 pm Panel Discussion
- 3:20 pm **Joseph Wu, MD, PhD**
Day 1 Closing Remarks

Time (PDT) **WELCOME TO DAY 2**

9:00 am **Joseph Wu, MD, PhD**

Director, Stanford Cardiovascular Institute

Simon H. Stertz, MD, Professor of Medicine and Radiology

SESSION VI: RESEARCH DEVELOPMENT III

Moderators: Kathleen Giacomini, PhD; Co-Moderator: Kuldev Singh, MD

9:05 am **Aida Habtezion, MD**

Chief Medical Officer and Head of Worldwide Medical & Safety, Pfizer

Translating Lessons from the Covid-19 Programs

9:22 am **Mathai Mammen, MD, PhD**

Executive Vice President, Pharmaceuticals, R&D, Johnson & Johnson

Raising the Bar for Innovation

9:42 am **Peter Marks, MD, PhD**

Director, Center for Biologics Evaluation and Research, U.S. Food and Drug Administration

Facilitating the Development and Manufacturing of COVID-19 Vaccines

9:52 am Panel Discussion

10:10 am **BREAK**

SESSION VII: FIRESIDE CHAT WITH KATALIN KARIKÓ, PHD

Moderator: Dean Lloyd Minor, MD; Co-Moderator: Sanjay Malhotra, PhD

10:20 am **Katalin Karikó, PhD**

Senior Vice President, BioNTech

Lloyd Minor, MD

Dean, Stanford School of Medicine

Time (PDT) **SESSION VIII: INVESTING IN DISCOVERY**

Moderators: Amy L. Chang, MSEE; Co-Moderator: Sanjay Malhotra, PhD

10:50 am **Joe Jimenez, MBA**

Aditum Ventures

The Evolving Role of Venture Capital in the Pharma Ecosystem

11:01 am **David Goel**

Founder & Managing General Partner, Matrix Capital Management

The Convergence of Technology and the Life Sciences

11:22 am **Jonathan MacQuitty, PhD**

Sector Head for Life Sciences, Lightspeed Ventures

A New Approach to Healthcare Investing

11:36 am **Margarita Chavez, JD**

Managing Director, AbbVie Ventures

11:46 am Panel Discussion

12:05 pm **BREAK**

SESSION IX: ARTIFICIAL INTELLIGENCE TECHNOLOGIES

Moderators: James Zou, PhD; Co-Moderator: Mark Mercola, PhD

- 12:35 pm **Christopher Gibson, PhD**
Co-Founder, Chief Executive Officer, Recursion
Mapping and Navigating Biology at Scale
- 12:48 pm **Daphne Koller, PhD**
Founder & Chief Executive Officer, Insitro
Transforming Drug Discovery using Digital Biology
- 1:00 pm **Karen Akinsanya, PhD**
President, R&D Therapeutics, Schrödinger
Transitioning from Computer-aided and Empirical Drug Discovery to Computer-driven and Structure-based Drug Discovery
- 1:14 pm Panel Discussion
- 1:35 pm **BREAK**

SESSION X: RESEARCH DEVELOPMENT IV

Moderator: Peter Kim, PhD; Co-Moderator: Kuldev Singh, MD

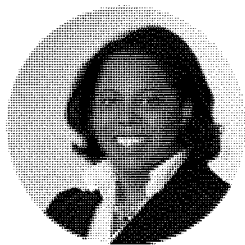
- 1:45 pm **Andrew Plump, MD, PhD**
President, Research & Development, Takeda Pharmaceutical
Creating a Patient-Drive, Science-First R&D Organization
- 1:58 pm **Aviv Regev, PhD**
Executive Vice President, Research and Early Development, Genentech
Multiplicative Levers for Drug Discovery and Early Development
- 2:13 pm **James Bradner, MD**
President, Novartis Institutes of BioMedical Research
New Paradigms in Therapeutics
- 2:29 pm **Merdad Parsey, MD, PhD**
Chief Medical Officer, Gilead
Development of Treatments for the Globe: from HIV to COVID-19
- 2:41 pm Panel Discussion
- 3:00 pm **Joseph Wu, MD, PhD**
Day 2 Closing Remarks



Katalin Karikó, PhD

Lifetime Achievement Award

Katalin Karikó is Senior Vice President of BioNTech SE, Professor of University of Szeged, and Adjunct Professor of Neurosurgery, Perelman School of Medicine, University of Pennsylvania. Dr. Karikó graduated from University of Szeged, Hungary in 1978, and received a doctoral degree in biochemistry from the same university in 1982. She continued her research at the Biological Research Centre in Szeged, at the Temple University in Philadelphia and at the Medical School of the University of Pennsylvania where she worked for 24 years. From 2013, Karikó is a senior vice president at BioNTech located in Mainz, Germany, where she is leading the mRNA-based protein replacement programs. For four decades, her research has been focusing on RNA-mediated mechanisms with the ultimate goal of developing in vitro-transcribed mRNA to treat acquired and genetic diseases. She was perfecting the therapeutic mRNA for years, and in 1997, joined forces with Drew Weissman and together they made mRNA suitable for medical use by replacing the uridine with pseudouridine, thus making the mRNA noninflammatory. They—together with their team—further demonstrated that such modified mRN formulated with LNP can be a potent vaccine. This technology ultimately became the basis for the FDA approved COVID-19 mRNA vaccine that is used to combat the current global pandemic. Their pioneering work fueled a number of advances and has opened the door for future therapeutics. Karikó has received prestigious awards, including the Széchenyi Prize, Rosenstiel Award, Reichstein Medal, Horwitz Prize, Breakthrough Prize and Lasker Award.



Karen Akinsanya, PhD

Karen Akinsanya, President of R&D, Therapeutics, joined Schrödinger in 2018. Karen leads the company's therapeutics group which is responsible for preclinical drug discovery, translational research, and early clinical development, in addition to drug discovery business development and collaborations. She has more than 25 years of experience in academia, pharmaceutical R&D, partnerships, and licensing. Karen joined Merck Research Labs in 2005 and held positions of increasing responsibility in clinical pharmacology as a development team leader working on first-in-human studies through late-stage label studies before joining Discovery Preclinical & Early Development as a therapeutic area lead and then a search and evaluation lead in business development. Karen received her Ph.D. from the Royal Postgraduate Medical School at Imperial College in London, in endocrine physiology. Dr. Akinsanya is currently a member of the Board of Directors at Nautilus Biotechnology, Board of Directors of the Imperial College Foundation, the Board of Trustees of The Rockefeller University, the Scientific Advisory Boards of Variant Bio and Thermo Fisher Scientific.



Steven Artandi, MD, PhD

Steven Artandi, MD, PhD is the Laurie Kraus Laboc Director of the Stanford Cancer Institute and the Jerome and Daisy Low Gilbert Professor of Medicine and Biochemistry at Stanford University. He also serves as the inaugural Senior Associate Dean for Cancer Programs for Stanford School of Medicine and the Chief Cancer Officer for Stanford Health Care. He received his undergraduate degree from Princeton University, and MD and PhD degrees from Columbia University. He trained in Internal Medicine at Massachusetts General Hospital and in Oncology at Dana-Farber Cancer Institute before joining the Stanford faculty in 2000. Dr. Artandi is an oncologist and cancer biologist whose research work has focused on the role played by the enzyme telomerase in cancer, aging and stem cell function. His work has produced new insights into the origins of cancer, revealing how telomerase endows cells with immortal growth properties and how aspiring cancers circumvent critical bottlenecks encountered during carcinogenesis. He has received a number of awards including an Outstanding Investigator Award from the National Cancer Institute and is an elected member of the American Association for the Advancement of Science, the American Society for Clinical Investigation and the Association of American Physicians. He serves on the Editorial Boards of the journals Molecular Cancer Research and Stem Cells.



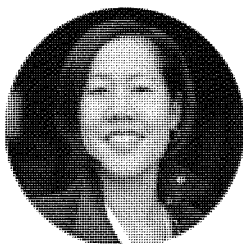
Claude Bertrand, PhD

Dr. Claude Bertrand graduated in pharmacy (PharmD) from Strasbourg University, France, and obtained his PhD in Strasbourg with research in the fields of immunopharmacology and neurogenic inflammation. After a 2-year post-doctoral appointment at University of California, San Francisco, Claude joined the allergy and asthma unit at Ciba-Geigy (later Novartis) in Basel. In 1996, he moved to the Inflammatory disease Unit at Roche Bioscience in Palo Alto, CA, where he became head of the in vivo pharmacology group and was responsible for supporting projects in rheumatology and respiratory diseases. In 1999, he was recruited as Director of Biology for Inflammation, GI and Pain at Pfizer. In 2004, Dr. Bertrand joined AstraZeneca as Vice-President Discovery for Respiratory & Inflammation Research at Alderley Park, UK and in 2005 was appointed Global Senior Vice President for Respiratory & Inflammation Research Area overseeing R&D activities at three sites in the UK and Sweden. In 2009, Dr. Bertrand joined Ipsen, France, as Executive Vice-President, Chief Scientific Officer and was since June 2011, Executive Vice-President for Research & Development, Chief Scientific Officer with a focus on Oncology, Neurology and Endocrinology. In March 2017, Claude was appointed General Director R&D, Chief Scientific Officer at Servier and then promoted to EVP R&D joining the EXCOM on November 1, 2018. Claude sits on at the Board of Directors of Eclon2, MAAT Pharma and he is Advisor to Abivax Board.



James Bradner, MD

James Bradner, M.D., joined Novartis on January 1, 2016 and became President of the Novartis Institutes for BioMedical Research (NIBR) on March 1, 2016. He is a member of the Executive Committee of Novartis. Prior to joining Novartis, Dr. Bradner was on the faculty of Harvard Medical School in the Department of Medical Oncology at the Dana-Farber Cancer Institute in the United States from 2005 through 2015. Dr. Bradner is a co-founder of five biotechnology companies and has authored more than 250 scientific publications and 50 US patent applications. Dr. Bradner is a graduate of Harvard University and the University of Chicago Medical School in the US. He completed his residency in medicine at Brigham and Women's Hospital and his fellowship in medical oncology and hematology at the Dana-Farber Cancer Institute. He has been honored with many awards and was elected into the American Society for Clinical Investigation in 2011 and the Alpha Omega Alpha Honor Medical Society in 2013.



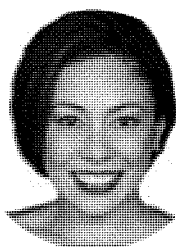
Amy Chang, MSEE

Amy serves on the board of directors for Disney, Procter & Gamble, Marqeta, SambaNova, Pragma and has previously served on the boards of Cisco, Splunk and Informatica, which encompass nearly a trillion dollars in total market cap. Post-acquisition of her startup Accompany by Cisco for \$270M, she led Cisco's multi-billion dollar Collaboration business. As EVP and GM of this 6000 person team, she led the videoconferencing, cloud calling, contact center, video device and phones businesses. Accompany was an AI/ML-based relationship intelligence platform serving Fortune 500 companies.

Prior to Accompany, Amy was at Google, where she led the teams for Google Analytics, Website Optimizer, Trends, and multichannel attribution for over 7 years, growing Google Analytics to serve over 86% of the entire web. She previously led product for the paid search and affiliates channels at eBay, as well as worked in the semiconductor and software industries at McKinsey. She started her career in hardware with Intel, AMD and Motorola.

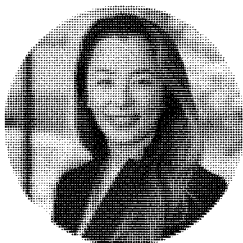
She serves on the UCSF Hospital Executive Committee, the Stanford School of Engineering Dean's Advisory Board, and as an advisor to Panther Labs, DataRobot, Greenlight, DataChat, Snorkel.ai, PerfectDay, KatanaGraph, Galileo, Proximo.io, NoviConnect, Hubspot (now public), Datorama (acquired by Salesforce), Skyhigh Networks (acquired by McAfee), Optimizely (acquired by Episerver), BloomReach, Origami Logic (acquired by Intuit).

Amy holds a BS in Electrical Engineering with a hardware subspecialty and an MS in Electrical Engineering with a network systems subspecialty, both from Stanford University.



Margarita Chavez, JD

Margarita is Managing Director at AbbVie Ventures. Margarita has lead investments in over a dozen biotech companies in the US and Europe and is responsible for AbbVie's investments in Morphic Therapeutics, Palleon Pharmaceuticals, eFFECTOR Therapeutics, CARISMA Therapeutics, Jnana Therapeutics and Paragen Bio. Margarita brings over 20 years of dealmaking experience, with over a decade in biotech M&A, licensing, and venture. Most recently, Margarita was a Director with Abbott's Global Pharmaceutical Licensing & Acquisitions. Among other deals, Margarita was involved in the in-licensing of Elagolix, the acquisition of Immuven, and the acquisition of the Lupron franchise. Before joining Abbott, Margarita practiced as a corporate and securities lawyer in Silicon Valley with the firm of Brobeck Phleger & Harrison, advising in equity financings, M&A and IPOs. Margarita currently serves as a Board Member of the New England Venture Capital Association and the MidAmerica Healthcare Investors Network and on the Advisory Board of the Santa Clara University School of Law.



Hogene Choi, JD

Hogene Choi works with clients at the forefront of the life sciences, healthcare, and technology industries on a range of intellectual property matters, focusing on patent prosecution, transactions, and counseling.

She works closely with her clients to understand their business objectives and develop strategies to protect and defend their intellectual property throughout the product life cycle. Her patent prosecution and transaction experience covers technologies related to machine learning and artificial intelligence, bioinformatics, blockchain, computer vision, cloud infrastructure and services, internet applications and server-side architecture, desktop applications and operating systems, graphics and audio/video, as well as semiconductors, medical devices, electronics, nanotechnology, and the mechanical arts. She also has extensive due diligence experience and regularly advises clients on patent procurement transactions, licensing, and acquisitions. She provides counsel on patentability and freedom-to-operate issues as well as patent portfolio evaluations for business transactions.

Hogene received her B.A. in computer science from University of California, Berkeley and her J.D. from University of California, Hastings College of the Law.



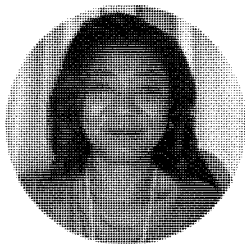
Jacqueline Corrigan-Curay, JD, MD

Jacqueline Corrigan-Curay, JD, MD, is the Principal Deputy Center Director in FDA's Center for Drug Evaluation and Research (CDER). Most recently, she served as the Acting Center Deputy Director for Operations, directing center and agency-level priority and initiative programs and leading GDUFA III reauthorization negotiations. Previously, Dr. Corrigan-Curay was director of CDER's Office of Medical Policy (OMP). In that role, she led the development, coordination, and implementation of medical policy programs and strategic initiatives. Dr. Corrigan-Curay brings to the position a unique legal, scientific policy, and clinical background with expertise in risk and scientific assessment, and clinical trial design and oversight. Before joining FDA, she served as supervisory medical officer with the Immediate Office of the Director, National Heart, Lung and Blood Institute (NHLBI) at the National Institutes of Health (NIH). She also served in director and acting director roles with the Office of Biotechnology Activities (OBA), Office of Science Policy at NIH, where she was executive secretary of the NIH Recombinant DNA Advisory Committee. She has held positions as an attending physician with the VA Medical Center, a policy analyst with the Congressional Office of Technology Assessment, and as a practicing attorney in Washington, D.C. Dr. Corrigan-Curay earned her law degree from Harvard Law School, her medical degree from University of Maryland School of Medicine, and a bachelor's degree in history of science from Harvard/Radcliffe College in Cambridge, MA.



Lisa M. Coussens, PhD

Dr. Coussens is Chairwoman of the Department of Cell, Developmental & Cancer Biology, and Associate Director for Basic Research in the Knight Cancer Institute at Oregon Health & Sciences University, and holds the Hildegard Lamfrom Endowed Chair in Basic Science. Dr. Coussens's research focuses on dissecting the roles of normal immune cells in regulating various facets of solid tumor development, identifying leukocyte activities that are co-opted by early tumors to support ongoing cancer development, and in understanding the role leukocytes play in regulating responses to cytotoxic, targeted and immune-based therapies. Her research has identified critical immune-regulated pathways for therapeutic targeting that are being clinically translated in combination with chemotherapy in women with metastatic triple negative breast cancer, pancreas cancer, and head & neck squamous cancer. In recognition of her research contributions Dr. Coussens' has been acknowledged with multiple awards in recognition of her scientific contributions including the 13th Rosalind E. Franklin Award from the National Cancer Institute (2015), the 12th AACR-Princess Takamatsu Memorial Lectureship (2018), the 2018 Susan G. Komen Brinker Award for Scientific Distinction in Basic Science, and was elected as Fellow of the American Association for Advancement of Science (AAAS; 2018) and Fellow of the AACR Academy (2019), and most recently elected as AACR President-elect (2021) and President (2022).



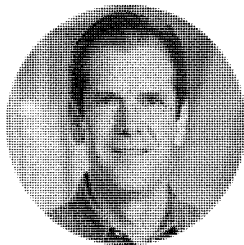
Myrtle Davis, DVM, PHD

Dr. Myrtle Davis is the Vice President of Discovery Toxicology at Bristol Myers Squibb. Myrtle joined BMS from the National Cancer Institute where she was the Chief of the Toxicology and Pharmacology Branch of the Developmental Therapeutics Program. Myrtle has previous experience as a Research Advisor in the Drug Safety group of Lilly Research Laboratories. In both roles, she contributed critical expertise to the advancement of several drugs candidates and to the understanding of toxicological mechanisms. She also has several years of academic experience as an Associate Professor in the Department of Pathology in the School of Medicine at the University of Maryland.

Myrtle is currently responsible for leading the scientific efforts in Discovery Toxicology to provide target and molecular hazard identification and risk assessments for issues identified in discovery research. She also leads and oversees the investigative toxicology efforts needed to support mechanistic understanding of compound- or target-mediated toxicities in discovery and development.

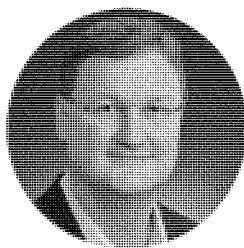
Myrtle is a Fellow of the Academy of Toxicological Sciences, an active member of the Society of Toxicology (recently elected as Vice President elect for the Society), and a member of the Society of Toxicologic Pathology. She is currently serving on the Board of Scientific Councilors of the National Toxicology Program, and she is a reviewer for the Assay Development and Screening Technologies Laboratory of the National Center for Advancing Translational Sciences (NCATS). She is an Associate Editor for Toxicological Sciences and Toxicologic Pathology, and she is Editor-in-Chief of the ILAR Journal (Institute for Laboratory Animal Research of the National Academy of Sciences).

Myrtle attended Tuskegee University where she pursued a BS degree in Chemistry and Mathematics followed by a Doctorate of Veterinary Medicine. She then received her Ph.D. in Toxicology from the University of Illinois and obtained post-doctoral training in Toxicologic Pathology at the University of Maryland before starting her academic career.



Ray Deshaies, PhD

Prior to joining Amgen, Deshaies served as a professor at the California Institute of Technology (Caltech) and an executive officer in Caltech's Division of Biology and Biological Engineering. He was also an investigator at the Howard Hughes Medical Institute. He has published over 150 papers on various subjects including discoveries of Sec61 translocon, cullin-RING ubiquitin ligases, and proteolysis-targeting chimeric molecules (ProTacs). In addition to his academic work, Deshaies co-founded Proteolix in 2003. In 2011, he cofounded Cleave Biosciences. Deshaies holds a bachelor's degree in biochemistry from Cornell University and a Ph.D. in biochemistry from the University of California, Berkeley. He is also a member of the National Academy of Sciences, and American Academy of Arts and Sciences.



Carlos Garner, PhD

Dr. Garner joined Eli Lilly and Company in 1997 as a senior scientist where he led a laboratory investigating the drug metabolism, pharmacokinetics and pharmacodynamics of new chemical entities in animal models and human. His work in these areas supported the advancement of many innovative molecules into human testing, late clinical development, and the commercialization of innovative new medicines treating schizophrenia and cancer. Dr. Garner subsequently served as senior director of project management and research strategy overseeing the development of more than 50 programs in discovery and development and providing portfolio strategy and management to Lilly Research Laboratories.

Dr. Garner move into regulatory sciences in 2011 where he led the North American regulatory affairs support of Lilly's Biomedicines development and product portfolio across neuroscience, musculoskeletal, urology, men's health, cardiovascular, and immunology diseases, where his team brought a number of NMEs and NBE to market and supported the broad portfolio of marketed products. Dr. Garner currently leads the broader regulatory function for Eli Lilly and Company supporting discovery, medical devices, all human health business units, and global manufacturing. Dr. Garner has published numerous scientific articles on his research and in health policy and has been invited to provide national and international lectures on his research, drug discovery, drug development and regulatory sciences.



Kathleen Giacomini, PhD

Kathleen Giacomini, a professor at the University of California, San Francisco, is a leader in the field of membrane transporters with a focus on genetic polymorphisms. She cloned, characterized and discovered the endogenous role of the human xenobiotic transporter, OCT1 (SLC22A1), and recently de-orphaned SLC22A24 and SLC22A15, discovering physiologic and pharmacologic substrates of both transporters. Together with others, she co-founded the International Transporter Consortium, which has published highly impactful papers informing regulatory policy, and the Pharmacogenomics Global Research Network, an independent scientific society focused on research in pharmacogenomics. She is the Co-Principal Investigator of the UCSF-Stanford Center of Excellence in Regulatory Sciences and Innovation, funded by the Food and Drug Administration. She has received numerous awards including an honorary doctorate degree from Uppsala University, and is an elected member of the National Academy of Medicine.



Gary H. Gibbons, MD

Gary H. Gibbons, MD, is Director of the National Heart, Lung, and Blood Institute (NHLBI) at the National Institutes of Health (NIH), where he oversees the third largest institute at the NIH, with an annual budget of approximately \$3 billion and a staff of nearly 2,100 federal employees, contractors, and volunteers. NHLBI provides global leadership for research, training, and education programs to promote the prevention and treatment of heart, lung, and blood diseases and enhance the health of all individuals so that they can live longer and more fulfilling lives. Since being named Director of the NHLBI, Dr. Gibbons has enhanced the NHLBI investment in fundamental discovery science, steadily increasing the pipeline and number of awards for established and early stage investigators. His commitment to nurturing the next generation of scientists is manifest in expanded funding for career development and loan repayment awards as well as initiatives to facilitate the transition to independent research awards. Dr. Gibbons has made many scientific contributions in the fields of vascular biology, genomic medicine, and the pathogenesis of vascular diseases. His research focuses on investigating the relationships between clinical phenotypes, behavior, molecular interactions, and social determinants on gene expression and their contribution to cardiovascular disease. Dr. Gibbons earned his undergraduate degree from Princeton University in Princeton, NJ, and graduated magna cum laude from Harvard Medical School in Boston. He completed his residency and cardiology fellowship at the Harvard-affiliated Brigham and Women's Hospital in Boston. Dr. Gibbons has been a faculty member at Stanford University, Harvard Medical School, and Morehouse School of Medicine in 1999, where he served as the founding director of its Cardiovascular Research Institute.



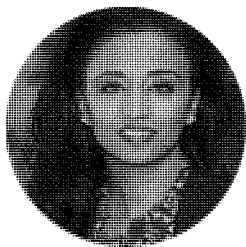
Christopher Gibson, PhD

Chris Gibson, PhD, is the Co-Founder and CEO of Recursion (NASDAQ:RXRX), a clinical-stage pharma tech company leveraging the latest in automation and artificial intelligence to map and navigate biology and chemistry to discover and develop new medicines at scale. He developed the technology and approach that seeded Recursion as part of his MD/PhD work in the lab of Co-Founder Dr. Dean Li (currently President of Merck Research Labs) while at the University of Utah. After completing his PhD, he left medical school to build Recursion into the rapidly growing company it is today. Dr. Gibson is a graduate of Rice University with degrees in bioengineering and management. He also serves on the Board of BioUtah and is the Chair of BioHive, the public-private partnership driving expansion of Utah's life-science ecosystem. Dr. Gibson is also active as an advisor and mentor, both formally and informally, of many young biotech founders.



David E. Goel

David Goel is Co-founder and Managing General Partner of Matrix Capital Management, a \$10 billion investment firm focused on technology and the life sciences. Mr. Goel is responsible for the firm's day-to-day management. Prior to forming Matrix in 1999, he was a Member and Technology Research Analyst at Tiger Management. Previously, Mr. Goel was an Associate at General Atlantic Partners and an Investment Banking Analyst at Morgan Stanley. He is a member of the Harvard Medical School Board of Fellows, the Board of Trustees of the American Repertory Theater (A.R.T.) at Harvard University, the Harvard Quantum Initiative (H.Q.I.) Founders Group, the Harvard Corporation Committee on Finance, the Advisory Council of Harvard Kennedy School's Mossavar-Rahmani Center for Business and Government, the Harvard Faculty of Arts and Sciences (F.A.S.) Boston Major Gifts Committee, and Assistant Treasurer of the Board of Trustees of the Winsor School. He is a Director of nference, Inc., Anumana, Inc., Pramana, Inc., AltPep Corporation, and Bento Dental. Mr. Goel is a magna cum laude graduate of Harvard University and a cum laude graduate of Phillips Exeter Academy.



Aida Habtezion, MD, MSc, FRCPC

As Chief Medical Officer of Pfizer, Aida Habtezion leads Pfizer's Worldwide Medical & Safety organization responsible for ensuring that patients, physicians, and regulatory agencies are provided with information on the safe and appropriate use of Pfizer medications. Prior to joining Pfizer, Dr. Habtezion was a practicing physician and scientist at Stanford University's School of Medicine, Division of Gastroenterology and Hepatology. She led a large translation research lab funded by multiple NIH, DOD, and foundation grants focused on understanding disease mechanisms and identifying potential immune-based therapeutic targets for pancreatic and intestinal inflammatory diseases and their long term complications such as cancer. Her research also included understanding the effect of environmental factors such as cigarette smoke and interaction of immune-enteric nervous system in GI motility disorders. She also served as an Associate Dean for Academic Affairs at Stanford. Dr. Habtezion served in several national and international study sections, including six years in NIH study section, American Gastroenterological Association (AGA) Institute Research Awards and AGA Research Policy Committee. Dr. Habtezion is the recipient of numerous awards and recognitions including: the Robert Wood Johnson Harold Amos Medical Faculty Development Award, being an elected member of the American Society for Clinical Investigation (ASCI), the Association of American Physicians (AAP) and named the 2020 Allen Distinguished Investigator. She currently serves in The New York Academy of Sciences Board of Governors, as Executive Board member for the International Science Reserve, and is the American Pancreas Association (APA) President. Dr. Habtezion is a tenured and endowed Professor of Medicine, currently on a leave of absence from Stanford University. Dr. Habtezion obtained her Bachelor of Science in Chemistry from the University of Alberta and Master of Science in Nutritional Sciences from the University of Guelph. She completed her medical degree from McMaster University. Dr. Habtezion completed her Internal Medicine residency at the University of Western Ontario and Gastroenterology & Hepatology clinical fellowship at the University of Toronto in Canada. Following her clinical fellowship training, she obtained postdoctoral research training in Immunology at Stanford University.



Sandra Horning, MD

Sandra J. Horning, MD currently serves on the Board of Directors of Moderna, Gilead Sciences, Olema Oncology, and EQRx, for which she is also a co-founder. Dr. Horning was Chief Medical Officer and Head of Global Product Development for Roche/Genentech from 2014 until her retirement in 2019. During her tenure, she oversaw the successful development of 15 new molecular entities (NMEs), numerous new indications of marketed products, and 28 FDA Breakthrough Designations in oncology, hematology, neuroscience, ophthalmology, immunology and infectious disease. The Healthcare Businesswomen's Association named Dr. Horning 2020-21 Woman of the Year. She was a practicing oncologist, investigator and tenured professor at Stanford University School of Medicine for 25 years, where she remains a professor of medicine emerita.



Karin Immergluck, PhD

Dr. Karin Immergluck is the Executive Director of the Office of Technology Licensing (OTL) at Stanford University where she leads a large, internationally renowned team of technology development, licensing and industry contracting professionals. The Stanford OTL handles inventions that come out of Stanford's research facilities, evaluating their licensing potential and fostering partnerships to make use of that research. Dr. Immergluck is leading several new initiatives within the OTL to increase faculty and investor engagement, and to ensure that the OTL continues to be an active facilitator and promoter of Stanford's highly successful entrepreneurial ecosystem, which has led to the founding of over 40,000 companies.

Prior to joining Stanford, Dr. Immergluck worked for three years at the UC Office of the President and then for 14 years at UCSF, where she eventually led the Office of Technology Management as Executive Director for five years. Under her leadership, UCSF became a pioneering national leader in digital health licensing and collaboration, faculty were more actively engaged resulting in a 25% increase in invention disclosures per \$100M in research funding, and overall licensing productivity was increased by nearly 40%. She also taught several IP-related classes and workshops at UCSF. Karin received her M.S. equivalent in Biochemistry and her Ph.D. in Developmental Molecular Genetics from the University of Zurich in Switzerland. She conducted her doctoral research at UCSF under the dual tutelage of Nobel Laureate and former Chancellor Dr. J. Michael Bishop, and Professor and former President of the Federal Institute of Technology and former Professor of University of Zurich, Dr. Ernst Hafen.



Joe Jimenez, MBA

Joe Jimenez is Co-Founder and Managing Partner at Aditum Bio, a biotech venture firm. From 2010 to 2018, Mr. Jimenez held the position of Chief Executive Officer (CEO) of Novartis, one of the world's leading pharmaceutical companies. Under his leadership, Novartis developed one of the largest pipelines of self-originated drugs in the industry, driven by a strong commitment to R&D. Mr. Jimenez is currently a member of the Board of Directors of General Motors Company, The Procter & Gamble Company, Century Therapeutics, and Graphite Bio. Additionally, he served on the Board of Directors of Colgate-Palmolive Company from 2009 to 2015, and of AstraZeneca PLC, from 2002 to 2007. He graduated in 1982 with a bachelor's degree from Stanford University and in 1984 with a Master of Business Administration from the University of California, Berkeley.



Peter Kim, PhD

Peter S. Kim is the Virginia & D.K. Ludwig Professor of Biochemistry at Stanford University School of Medicine and an Institute Scholar of Stanford ChEM-H. He is also the Lead Investigator of the Infectious Disease Initiative at the Chan Zuckerberg Biohub. He was President of Merck Research Laboratories from 2003–2013 and oversaw development of more than 20 new medicines and vaccines, including JANUVIA, GARDASIL, ISENTRESS, ZOSTAVAX, and KEYTRUDA. Earlier, he was Professor of Biology at MIT, Member of the Whitehead Institute and an HHMI Investigator. He is known for discovering a salient component of how proteins cause viral membranes to fuse with cells and has pioneered efforts to create an AIDS vaccine based on inhibiting the HIV-1 membrane-fusion process. He is a member of the National Academy of Sciences, the National Academy of Medicine and the National Academy of Engineering.



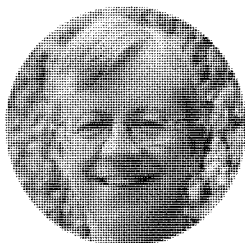
Daphne Koller, PhD

Daphne Koller is CEO and Founder of insitro, a machine-learning enabled drug discovery company. Daphne is also co-founder of Engageli, was the Rajeev Motwani Professor of Computer Science at Stanford University, where she served on the faculty for 18 years, the co-CEO and President of Coursera, and the Chief Computing Officer of Calico, an Alphabet company in the healthcare space. She is the author of over 200 refereed publications appearing in venues such as Science, Cell, and Nature Genetics. Daphne was recognized as one of TIME Magazine's 100 most influential people in 2012. She received the MacArthur Foundation Fellowship in 2004 and the ACM Prize in Computing in 2008. She was inducted into the National Academy of Engineering in 2011 and elected a fellow of the American Association for Artificial Intelligence in 2004, the American Academy of Arts and Sciences in 2014, and the International Society of Computational Biology in 2017.



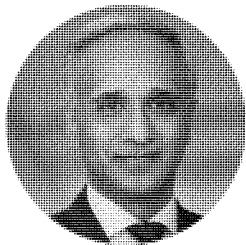
Dean Y. Li, MD, PhD

Dean Li serves as executive vice president and president of Merck Research Laboratories. He leads the company's worldwide human vaccines and therapeutics research and development organization. Since joining Merck in 2017 Dean has held leadership roles in the Translational Medicine and Discovery functions and was appointed to President, Merck Research Laboratories in January 2021. Prior to joining Merck, Dean held positions of increasing responsibility in translational medical research at the University of Utah. Most recently he served as the H.A. & Edna Benning Professor of Medicine and Cardiology, chief scientific officer, associate vice president and vice dean at the University of Utah Health System. From 2015 to 2016, he also served as interim CEO of Associated Regional University Pathologists, one of the United States' largest clinical reference laboratories. During his tenure at the University of Utah, he co-founded several biotechnology companies based upon research conducted in his laboratory, including Recursion Pharmaceuticals, Hydra Biosciences and Navigen Pharmaceuticals. Dean received his Bachelor's degree in Chemistry from the University of Chicago and his graduate and clinical training at Washington University School of Medicine in St. Louis. Dean is a board-certified cardiologist, a member of the American Society for Clinical Investigation and the Association of American Physicians.



Jonathan MacQuitty, PhD

Jonathan joined Lightspeed in 2016 with over three decades of operating and investing experience in healthcare and life sciences. Jonathan has been instrumental in life science investing for Lightspeed including D2G Oncology, Forty Seven (acquired by Gilead for \$4.9B), Orca Bio, Personalis (NASDAQ: PNL) and TeneoBio (acquired by Amgen for up to \$2.5B). Jonathan is currently serving as CEO of D2G Oncology, a biotechnology company spun out of Stanford focused on using AI & data driven approaches for translational oncology. He is also serving as Chairman of the Board at Personalis, a cancer genomics company providing advanced molecular data for developing and using novel cancer therapies. Previously Jonathan had served as Founding CEO of Forty Seven, an immunoncology company also spun out of Stanford. Prior to Lightspeed, Jonathan served as a partner and head of the West Coast office of Abingworth, a UK based venture capital firm focusing on life sciences. He was also founding CEO of GenPharm International, a Bay Area biotech company specializing in human sequence antibody platforms. Jonathan holds a PhD in Chemistry from the University of Sussex, an MBA from the Stanford Graduate School of Business, and a BA and MA in Chemistry from Oxford University.



Mathai Mammen, MD, PhD

As Executive Vice President, Pharmaceuticals, R&D, Johnson & Johnson (J&J), Mathai's mission is to focus the energy of the best research and development teams in the world at the intersection of unmet medical need and breakthroughs in science and technology to make medicines with unequivocal benefit for patients worldwide. His team works across a wide range of therapeutic areas and biological pathways to deliver that impact, including: Oncology, Cardiovascular, Metabolic and Retinal Disease, Pulmonary Hypertension, Immunology, Neuroscience, and Infectious Disease and Vaccines.

Mathai is redefining how drugs are discovered and developed, investing deeply in Data Science throughout every aspect of R&D and in immunology across all disease areas. He is also strongly committed to improving access to make medicines and vaccines for all parts of the globe.

Since joining Janssen in June 2017, Mathai built on an already strong foundation to create a truly world-class portfolio, and under his leadership, the organization has advanced seven new FDA-approved prescription medicines and 30 additional approvals for expanded indications or new product formulations to benefit hundreds of millions of patients. Agnostic to the source of innovation, Janssen R&D has also executed more than 40 acquisitions and licenses, and more than 350 strategic partnerships and collaborations to advance its industry-leading portfolios.

Mathai is an inclusive leader, passionate about talent development, and a champion of diversity. He is the executive sponsor of J&J's Data Science Council, and his patient-first approach has helped fuel Janssen to be one of the largest pharmaceutical companies in the world.

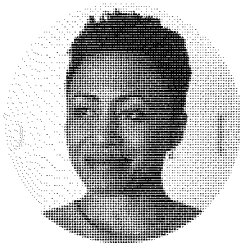
Prior to joining Janssen, Mathai held senior leadership positions at Merck Research Laboratories. He previously led R&D at Theravance, a company he co-founded in the San Francisco Bay Area, which created 31 development candidates and five approved products. Mathai and the team later created Innoviva, a second successful publicly traded company focused on royalty management.

Mathai has more than 150 peer-reviewed publications and patents, and serves on various boards and advisory committees, including 10x Genomics, MIT Abdul Latif Jameel Clinic for Machine Learning in Health, Institute for Medical Engineering and Science and MIT, President's Advisory Committee for Dalhousie University, and the Vagelos Life Science Management Program at the University of Pennsylvania. He received his M.D. from Harvard Medical School/Massachusetts Institute of Technology (HST program) and his Ph.D. in Chemistry from Harvard University Department of Chemistry, working with George Whitesides. He received his BSc in Chemistry and Biochemistry from Dalhousie University in Halifax, Nova Scotia.



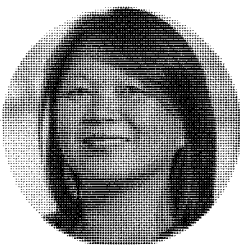
Peter Marks, MD, PhD

Peter Marks received his graduate degree in cell and molecular biology and his medical degree at New York University and completed Internal Medicine residency and Hematology/Medical Oncology training at Brigham and Women's Hospital in Boston. He has worked in academic settings teaching and caring for patients and in industry on drug development. He joined the FDA in 2012 as Deputy Center Director for CBER and became Center Director in January 2016.



Michelle McMurry-Heath, MD, PhD

BIO represents nearly 1,000 life sciences companies and organizations from 30 countries. The organization's mission is to support companies that discover and deploy scientific breakthroughs that improve human health, environmental stewardship, and sustainable agriculture. Since assuming leadership of BIO on June 1, 2020, Dr. McMurry-Heath has positioned BIO as a leading champion of scientific innovation and the bio-revolution, which aims to use technological breakthroughs to cure patients, protect our climate, and nourish humanity. A common thread throughout McMurry-Heath's career has been her focus on broadening access to scientific progress so more patients from diverse backgrounds can benefit from cutting-edge advancements. She calls the distribution of scientific progress “the social justice issue of our age.” Before coming to BIO, Dr. McMurry-Heath worked at Johnson & Johnson, where she served as Global Head of Evidence Generation for Medical Device Companies and then Vice President of Global External Innovation and Global Leader for Regulatory Sciences. Prior to her time at J&J, Dr. McMurry-Heath was a key science policy leader in government, including serving as the associate science director of the FDA’s Center for Devices and Radiological Health. McMurry-Heath was also the founding director of the Aspen Institute’s Health, Biomedical Science, and Society Policy Program. McMurry-Heath received her MD/PhD from Duke’s Medical Scientist Training Program, becoming the first African American to graduate from the prestigious program.



Maria Millan, MD

Dr. Maria Millan is a physician-scientist who has devoted her career to treating and developing innovative solutions for children and adults with debilitating and life-threatening conditions. After obtaining her MD, she completed her surgical training and post-doctoral research at Harvard Medical School – Beth Israel Deaconess Medical Center. After a transplant surgery fellowship at Stanford, she began her academic career with a busy pediatric and adult transplant surgery practice focused on technical advancements and optimization of patient outcomes. In parallel, she was promoted to Associate Professor and the Director of the Pediatric Organ Transplant Program at Stanford. She also served on the Medical School’s Faculty Senate and the Lucile Packard Children’s Hospital operations committee. In 2006, Maria joined StemCells, Inc., and joined the California Institute for Regenerative Medicine in 2012 where she led the formation of the Alpha Stem Cell Clinics Network. This clinical network successfully supports over 45 clinical trials and was recently expanded to include 5 programs composed of 7 medical centers and affiliated hospitals. In July 2017, Dr. Millan took on the role as President and CEO of CIRM. Under her leadership, CIRM continues to drive the mission of accelerating stem treatments to patients with unmet medical needs.



Lloyd B. Minor, MD

Lloyd B. Minor, MD, is a scientist, surgeon, and academic leader. He is the Carl and Elizabeth Naumann Dean of the Stanford University School of Medicine, a position he has held since December 2012. He is also a professor of Otolaryngology – Head and Neck Surgery and a Professor of Bioengineering and of Neurobiology, by courtesy, at Stanford University. As Dean, Dr. Minor plays an integral role in setting strategy for the clinical enterprise of Stanford Medicine, an academic medical center that includes the Stanford University School of Medicine, Stanford Health Care, and Stanford Children's Health and Lucile Packard Children's Hospital Stanford. He also oversees the quality of Stanford Medicine's physician practices and growing clinical networks. With Dr. Minor's leadership, Stanford Medicine has established a strategic vision to lead the biomedical revolution in Precision Health. The next generation of health care, Precision Health, is focused on keeping people healthy and providing care that is tailored to individual variations. It is predictive, proactive, preemptive, personalized, and patient-centered. An advocate for innovation, Dr. Minor has provided significant support for fundamental science and for clinical and translational research at Stanford. Through bold initiatives in medical education and increased support for PhD students, Dr. Minor is committed to inspiring and training future leaders. Among other accomplishments Dr. Minor has led the development and implementation of an innovative model for cancer research and patient care delivery at Stanford Medicine and has launched an initiative in biomedical data science to harness the power of big data and create a learning health care system. Committed to diversity, he has increased student financial aid and expanded faculty leadership opportunities.



Vern Norviel, JD

Vern Norviel is a partner at Wilson Sonsini Goodrich & Rosati and a senior practitioner in the firm's patents and innovations counseling practice. Vern has three decades of experience formulating successful strategies for life science companies and helping them develop IP programs. He represents a wide variety of companies, as well as venture capital firms, in areas such as therapeutics, diagnostics, nanotechnology, genomics, proteomics, and personalized medicine. In fact, Vern's interest in the field of personalized medicine prompted him to become the first attorney to have had his or her entire genome sequenced and made available in a public database. Before joining the firm in 2003, Vern was the general counsel and corporate secretary of Perlegen Sciences, Inc., a start-up biotechnology company that scans the entire human genome for important therapeutic and diagnostic products. Previously, as senior vice president and general counsel, he was an early employee of Affymetrix, the biotechnology company that pioneered and developed DNA chip technology. He also had been a partner at Townsend and Townsend and Crew in Palo Alto. During his career, Vern has authored or prosecuted dozens of patents that have been litigated in the U.S. and abroad; and has overseen intellectual property lawsuits throughout the world. Vern previously served as a member of Wilson Sonsini's board of directors and currently serves on the board of the Wilson Sonsini Foundation. In addition, he is a lecturer in biotechnology law at UC Berkeley School of Law.



Merdad Parsey, MD, PhD

Merdad Parsey, MD, PhD is Gilead Sciences' chief medical officer, responsible for overseeing the company's global clinical development and medical affairs organizations. With an unrelenting commitment to create a healthier world for all people, Merdad supervises all clinical trials and development operations. Together with the leadership team, he works to advance clinical development strategies and programs with the goal of changing the trajectory of disease, and transforming care for the patients of today and tomorrow. Merdad joined Gilead in 2019, after serving as senior vice president of Early Clinical Development at Genentech, where he led clinical development for areas including inflammation, oncology and infectious diseases. Prior to Genentech, Merdad served as President and CEO of 3-V Biosciences (now Sagimet BioSciences), held development roles at Sepracor, Regeneron and Merck, Inc. and was Assistant Professor of Medicine and Director of Critical Care Medicine at the New York University School of Medicine. He completed his MD and Ph.D. at the University of Maryland, Baltimore, his residency in Internal Medicine at Stanford University and his fellowship in Pulmonary and Critical Care Medicine at the University of Colorado. Merdad currently serves on the Board of Directors for Sagimet BioSciences.



Andrew Plump, MD, PhD

Andrew Plump, MD, PhD, is the president of Research & Development at Takeda Pharmaceutical Company Limited and serves as a member of the company's board of directors. His career spans nearly 30 years in the pharmaceutical industry and academia and his experience encompasses early research through regulatory approval and patient access.

Dr. Plump oversees Takeda's global R&D organization of more than 5,000 employees. Early in his tenure he orchestrated a multi-year strategic and cultural transformation that has resulted in a renewed focus on innovation, streamlined global footprint, prioritized therapeutic areas of focus, a rich network of partnerships and a modality-diverse portfolio of newly approved and promising late-stage development experimental therapies. He also championed efforts to build an exciting and sustainable early development and research pipeline, founded on strong translational science as well as platform capabilities in cell therapy, gene therapy and data sciences. Over the last several years, Dr. Plump has substantially increased the company's investment in research and partnerships and the launch and growth of medicines that provide meaningful benefit to society.

Dr. Plump has been recognized for his contributions to the healthcare industry, education and the arts. He serves on several not-for-profit boards including the board of directors of the PhRMA Foundation, the Sarnoff Cardiovascular Research Foundation and the Biomedical Science Careers Program. He also serves in advisory board positions at BioCentury and the Boston Symphony Orchestra.

Prior to Takeda, Dr. Plump served as head of Research & Translational Medicine, deputy to the president of R&D at Sanofi, based in Paris, France. Prior to Sanofi, Dr. Plump served as worldwide cardiovascular (CV) research head at Merck.

Dr. Plump received his M.D. from the University of California, San Francisco (UCSF), his Ph.D. in cardiovascular genetics with Dr. Jan Breslow at Rockefeller University and his B.S. from the Massachusetts Institute of Technology (MIT). He completed a residency in internal medicine and a fellowship in medical genetics at UCSF. Following his clinical training, Dr. Plump trained as a Howard Hughes and Stanley J. Sarnoff postdoctoral fellow with Dr. Marc Tessier-Lavigne at UCSF, concurrently assuming faculty responsibilities as an adjunct clinical instructor in the department of medical genetics.

Dr. Plump and his wife, Suzanne Driscoll Plump, PhD, reside in Chestnut Hill, MA, with their three children.



Aviv Regev, PhD

As Executive Vice President, Genentech Research and Early Development (gRED), Aviv Regev is responsible for the management of all aspects of Genentech's drug discovery and drug development activities. She is a member of the Genentech Executive Committee and the expanded Corporate Executive Committee for Roche. Prior to Genentech, Regev served as Chair of the Faculty, Core Institute Member, founding director of the Klarman Cell Observatory, and member of the Executive Leadership Team of the Broad Institute of MIT and Harvard, as well as Professor of Biology at MIT and Investigator at the Howard Hughes Medical Institute. She is a founding co-chair of the Human Cell Atlas. Regev has served on multiple corporate advisory, scientific advisory, and journal editorial boards, including the advisory committee to the National Human Genome Research Institute at the National Institutes of Health. Regev is a leader in deciphering molecular circuits that govern cells, tissues and organs in health and their malfunction in disease. Her lab has pioneered foundational experimental and computational methods in single-cell genomics, working toward greater understanding of the function of cells and tissues in health and disease, including autoimmune disease, inflammation and cancer. She is a member of the National Academy of Sciences and National Academy of Medicine, and she is also a Fellow of the International Society of Computational Biology. Regev has a PhD in computational biology and a Master of Science from Tel Aviv University.



Madhuri Roy, JD, PhD

Dr. Madhuri (Mani) Roy focuses her practice in developing and implementing comprehensive patent portfolio strategies for biotechnology, pharmaceutical and chemical companies. This work includes patent preparation and prosecution, strategic patent counseling and intellectual property due diligence. She also has carried out investor-side and company-side due diligences, freedom-to-operate analyses, patentability analyses and prelitigation work involving questions of validity and non-infringement. Mani brings 10+ years of integrative biotechnology experience to her practice. She has industry experience in mid-size and large biotech companies in the areas of discovery, target validation, screening and in vivo pharmacology. She has also conducted academic research in the molecular and cellular aspects of neurodegenerative and neuropsychiatric diseases. She has authored numerous scientific publications. Prior to joining Cooley, Mani was an associate at Morrison & Foerster and prior to law school, she worked as a patent agent at Wilson Sonsini Goodrich & Rosati. In these previous roles, she specialized in strategic patent counseling, prosecution and pre-litigation analyses for biotechnology clients. Mani has an active pro bono practice focusing on providing legal services for those in need through the Pro Bono Project's Lawyers in the Library program and working on Immigration and Naturalization matters. Her doctoral research at Stanford University focused on the molecular and cellular aspects of gene therapy for the central nervous system.



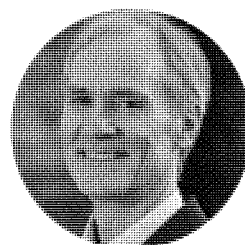
Alfred Sandrock, MD, PhD

Dr. Sandrock has been CEO of Voyager and a Board member since March of 2022. Dr. Sandrock joined Voyager following a 23-year career at Biogen where he identified and developed novel therapies for a variety of serious diseases. While at Biogen, Dr. Sandrock served in positions of increasing responsibility, culminating in his service as Executive Vice President, Research and Development. He also served as Chief Medical Officer and held a seat on the Biogen Executive Committee. Over the course of his tenure, he led the discovery and development, and regulatory approval of several medicines for multiple sclerosis, spinal muscular atrophy, and Alzheimer's Disease. Dr. Sandrock earned a B.A. in human biology from Stanford University, an M.D. from Harvard Medical School, and a Ph.D. in neurobiology from Harvard University. He completed an internship in medicine, a residency and chief residency in neurology, and a clinical fellowship in Neuromuscular Disease and Clinical Neurophysiology at Massachusetts General Hospital. He was Assistant Professor in Neurology at Harvard Medical School and an Associate in Neurology at Massachusetts General Hospital prior to joining Biogen.



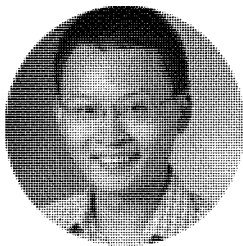
Randy Schekman, PhD

Dr. Randy Schekman is a Professor in the Department of Molecular and Cell Biology, University of California, Berkeley, and an Investigator of the Howard Hughes Medical Institute. He studied the enzymology of DNA replication as a graduate student with Arthur Kornberg at Stanford University. His current interest in cellular membranes developed during a postdoctoral period with S. J. Singer at the UC Diego. Among his awards are the Gairdner International Award, the Albert Lasker Award in Basic Medical Research and the Nobel Prize in Physiology or Medicine, which he shared with James Rothman and Thomas Südhof. Beginning in 2018, Schekman has served as the Scientific Director of "Aligning Science Across Parkinson's Disease" a major philanthropic effort organized along with The Michael J. Fox Foundation to identify molecular and cellular mechanisms in the initiation and progression of Parkinson's Disease (<https://parkinsonsroadmap.org>). Schekman's laboratory investigates the mechanism of vesicular traffic in the secretory pathway in eukaryotic cells. Currently the lab investigates the mechanism of biogenesis of extracellular vesicles including how small RNAs are sorted for secretion in exosomes and the means by which these vesicles are internalized and function in target cells.



Marc Tessier-Lavigne, PhD

Pioneering neuroscientist Marc Tessier-Lavigne became Stanford University's 11th president on September 1, 2016. He returned to Stanford after serving as President of The Rockefeller University, a graduate biomedical research university in New York City. From 2001 to 2005, he was a Professor of biological sciences at Stanford, where he held the Susan B. Ford Professorship in the Humanities and Sciences. He has also held faculty positions at the University of California, San Francisco, and executive positions, including Chief Scientific Officer at Genentech.



James Zou, PhD

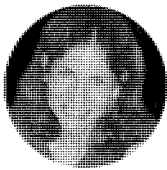
Dr. James Zou is an Assistant Professor of Biomedical Data Science and, by courtesy, of Computer Science and Electrical Engineering at Stanford University. He works on making machine learning more reliable, human-compatible and statistically rigorous, and is especially interested in applications in human disease and health. Dr. Zou received his Ph.D from Harvard in 2014, and was at one time a member of Microsoft Research, a Gates Scholar at Cambridge and a Simons fellow at U.C. Berkeley. He joined Stanford in 2016. Dr. Zou develops novel machine and deep learning algorithms that have a strong statistical guarantee, and several of his methods are currently being used by biotech companies. Dr. Zou has received several awards, including Google Faculty Award, a Tencent AI award, and is a Chan-Zuckerberg Investigator.

DAY 1

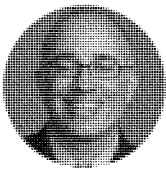
DAY 2

BIOS

Organizing Committee



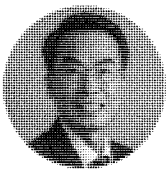
Amanda Chase, PhD
Associate Director of Strategic
Research Development
Cardiovascular Institute
Stanford University School of
Medicine



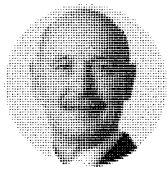
Kuldev Singh, MD
Professor of Ophthalmology
Stanford University School of Medicine



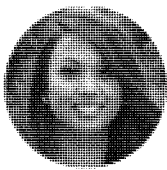
Sanjay V. Malhotra, PhD
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Joseph C. Wu MD, PhD
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Sent: 9/10/2021 9:39:46 AM
To: Jungman, Elizabeth [/o=ExchangeLabs/ou=Exchange Administrative Group (FYDIBOHF23SPDLT)/cn=Recipients/cn=5238a0caec064ba8b5d598115bc4f99f-Elizabeth.J]
Subject: RE: CDER Trade Press News Update September 10, 2021

Could we speak about Propecia at the next opportunity?

Patrizia

From: CDER Trade Press <CDERTradePress@fda.hhs.gov>
Sent: Friday, September 10, 2021 9:29 AM
To: CDER Trade Press <CDERTradePress@fda.hhs.gov>
Subject: CDER Trade Press News Update September 10, 2021



Today's Headlines (click to go directly to the article):

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[Biogen CEO blames Aduhelm's rocky launch on 'too much confusion, misinformation and controversy'](#)

[Despite mixed phase 3 showing, Apellis hopes to push eye disorder drug past FDA's finish line](#)

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[Room Available In US Pharma Industry For OTC Switch Leader: Has Perrigo Made A Reservation?](#)

(BioCentury)

[Sept. 9 Quick Takes: Obsidian readies for IPO with \\$115M crossover round](#)

[Cancer leads way to routine use of accelerated approval: Data Byte](#)

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Biogen Declines To Say If It Seeks IG Advice On Aduhelm Giveaway

By [Gabrielle Wanneh](#) / September 9, 2021 at 10:42 AM

Biogen is giving free Aduhelm to patients at Florida-based First Choice Neurology, and that arrangement is at risk of being considered a kickback, a former HHS Inspector General official said. However, the company said its program is common practice and declined to say whether it seeks an advisory opinion from the IG that would help shield it from enforcement action.

“Biogen is committed to ensuring that appropriate patients have access to any Biogen medicines prescribed by their physicians,” Biogen said. “It is common for pharmaceutical companies, including Biogen, to offer patient assistance programs designed to help remove non-clinical barriers to access to care, and Biogen designs and administers its access programs to comply with applicable laws and regulations.”

Reuters reported that Biogen is providing its Alzheimer’s drug for free while Medicare determines whether, and under what circumstances, to cover Aduhelm. FDA granted Aduhelm an accelerated approval in June against the advice of independent agency advisers. It’s the first new Alzheimer’s treatment in nearly two decades, but many insurers and doctors don’t believe there is sufficient evidence that the drug works to outweigh the risk of serious side effects.

It’s common for companies to offer free medications, and the legality of patient assistance programs depends on their details. Companies may seek advice from the IG on their programs, but they aren’t required to get advisory opinions, and advisory opinions are not legally binding, according to Jennifer Michael, former chief of the Industry Guidance Branch at the IG.

Based on the description of Biogen’s program in the *Reuters* article, Michael says there’s a chance the IG would consider the arrangement to be a ‘seeding’ program. Seeding refers to the practice of giving free drugs to a subset of eligible patients to induce those patients to use drugs that federal health programs will eventually pay for.

“The government might be concerned that those types of seeding programs could result in the potential for overutilization or inappropriate utilization,” Michael told *Inside Health Policy*. -- [Gabrielle Wanneh \(gwanneh@iwppnews.com\)](#)

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Premier Acquires Stake In Specialty Drug Maker To Ensure Supply Of At-Risk Drugs

By [Gabrielle Wanneh](#) / September 9, 2021 at 2:39 PM

The group purchasing organization Premier and 11 leading health systems have invested a small stake in specialty drug maker Exela Pharma Sciences to ensure the supply of essential drugs that face possible shortages due to sourcing problems spurred by the COVID-19 pandemic.

The participating health systems are expected to secure an uninterrupted supply of 19 pharmaceutical products, including generic injectables that often appear on the FDA drug shortage list and 503B compounded pharmaceutical products provided by Exela, which primarily sources active ingredients domestically and from Europe, through its manufacturing facility in North Carolina.

The move is a part of a longtime initiative of Premier’s ProvideGx and is similar to actions by other hospital groups like CivicaRx that aim to avert drug shortages and prevent price gouging that often follows a drug scarcity. For more than a decade, several drug shortages have contributed to \$230 million in additional drug costs as well as poorer health outcomes for patients, according to a 2018 study conducted by researchers at the University of Pittsburgh.

“Leveraging Exela’s significant production capacity, this collaboration is expected to give Premier’s participating members uninterrupted access to high-quality, U.S.-made pharmaceutical products and safety stock,” Premier President and CEO Michael Alkire said in a statement Wednesday (Sept. 8). “With this investment, we are committing to support the domestic production of vital medicines for our members and the market and to bring much-needed solutions to help eliminate drug shortages.”

The participating health systems have signed a multi-year commitment to buy a portion of their drugs from Exela through Premier’s GPO. They include Baystate Health of Springfield, MA; Genesis Health System of Davenport, IA; Henry Ford Health System of Detroit, MI; McLaren Health Care of Grand Blanc, MI; Memorial Healthcare System of Hollywood, FL; OSF HealthCare of Peoria, IL; Riverside Health System of Newport News, VA; Saint Francis Health System of Tulsa, OK; Texas Health Resources of Arlington, TX; and Universal Health Services, Inc. Of King of Prussia, PA. -- [Gabrielle Wanneh \(gwanneh@iwppnews.com\)](#)

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FDA updates guidance on generic drug development during COVID

Posted 09 September 2021 | By [Michael Mezher](#)

The US Food and Drug Administration (FDA) on Wednesday revised its questions and answers guidance on generic drug development and abbreviated new drug application (ANDA) submissions during the COVID-19 public health emergency.

The revised guidance updates the agency's previous guidance on generic drug development during the COVID-19 pandemic that was released in April 2021. (RELATED: [FDA offers guidance on generic drug development during COVID](#), *Regulatory Focus* 5 April 2021)

The revisions include a clarification about when FDA will accept requests to receive an ANDA with "less than the full complement of recommended stability data," and information on how to handle expired batches of test product in interrupted bioequivalence studies. FDA also says it has made some minor updates throughout the frequently asked questions section.

"FDA recognizes that the COVID-19 public health emergency may impact the development of generic drug products, interrupt or delay ANDA applicants' bioequivalence studies, and impact the submission of ANDAs to FDA for assessment. The ongoing development of generic drugs submitted as ANDAs to the FDA for assessment helps Americans continue to have access to safe, effective generic drugs," FDA says.

In the revised guidance, FDA now explains that it will "generally accept requests to receive an ANDA with less than the full complement of recommended stability data if the drug product is on FDA's Drug Shortage List or FDA confirms that the drug product is vulnerable to shortage." The agency also says it will also generally do so for products that meet its criteria for priority review to help address the COVID-19 pandemic.

FDA is also granting generic drugmakers additional flexibility when it comes to resuming bioequivalence studies that were interrupted due to the COVID-19 public health emergency. The revised guidance now provides recommendations allowing prospective applicants to potentially use a test product batch that is beyond the established expiration dating period to resupply bioequivalence studies, "as long as sufficient evidence can be provided to demonstrate the acceptability of the test product batch."

FDA further explains that applicants who wish to follow this approach "should submit data to ensure the test batch samples stored at the long-term stability conditions beyond the current expiration date are in compliance with the finished drug product shelf-life specifications at the time of conducting the [bioequivalence] BE study."

Kaleido warned; FDA not persuaded by 'medical food' claim

Posted 09 September 2021 | By [Joanne S. Eglovitch](#)

The US Food and Drug Administration (FDA) recently issued a [warning letter](#) to Kaleido Biosciences for administering an investigational new COVID-19 treatment to patients without obtaining an investigational new drug (IND) application.

The company argued that the product under investigation was a food and not a drug, and therefore not subject to IND filing requirements. Yet the agency disagreed, saying that the endpoints used in the clinical trials went beyond 'dietary management' of COVID-19 and were more aligned with drug trials in assessing effectiveness of the treatment.

The 26 August warning letter was sent over "objectionable conditions" observed following a mid-February to early March inspection of Kaleido's Lexington, MA facility, conducted under the auspices of FDA's bioresearch monitoring program. At issue was the firm's clinical investigation of a molecule called KB109 for treating COVID-19. The treatment is a microbiome metabolic therapy, a synthetic glycan that is orally administered for treating COVID-19.

The warning letter focused on two protocols, Protocol K031-120, and Protocol K032-120. The first trial was a randomized, open-label prospective parallel group study to assess the natural history of COVID-19 and compare the effect of the treatment in patients undergoing supportive self-care (SSC). The other trial was an exploratory open-label clinical study to evaluate how the treatment affected the gut microbiota of adult patients with mild to moderate COVID-19.

Between the two multi-site trials, 399 patients were randomized.

Kaleido argued it was not obligated to submit an IND before initiating these clinical investigations because KB109 is a food rather than a drug. Specifically, Kaleido argued that the investigations were conducted "to evaluate the effect of KB109 on the microbiome" and to "determine the safety and tolerability of KB109 in the disease population," according to FDA's characterization of the firm's position.

These arguments failed to persuade FDA, which concluded that the "evidence collected during the inspection showed that KB109 was intended for use as a drug and not as a food."

FDA maintained that "even if there were distinctive nutritional requirements established for COVID-19, Protocols K031-120 and K032-120 went beyond 'dietary management' of COVID-19 to evaluate KB109's ability to treat COVID-19 by measuring the investigational product's effects on fever, oxygen saturation, viral load, and other biomarkers of COVID-19 infection, among other things."

Kaleido's argument that KB109 is a medical food fails on other counts as well, wrote FDA. Since no particular nutritional requirements that might help manage COVID-19 have yet been discovered, KB109 cannot be used in the dietary

management of the disease, as would be required for a product classified as a medical food.

Also, the fact that an article has been established to be generally recognized as safe (GRAS) does not preclude its use as a drug. FDA clarified that “an article may be a drug as used in a clinical trial, even if it is considered a food as used in other contexts.”

The company has 15 days to file a response.

Daniel Menichella, Kaleido’s chief executive officer, told *Focus* in an email that “we are reviewing the letter currently and look forward to providing a response within the given timeframe and continuing our discussions with the FDA.”

[Kaleido warning letter](#)

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FDANEWS

Advocacy Group Files Lawsuit Seeking FDA Action on Propecia

September 10, 2021

A new lawsuit filed against the FDA this week contends that the male pattern baldness drug Propecia (1 mg) should either be taken off the market or its prescribing information should include warnings about the risk of long-term erectile dysfunction, depression and suicide.

The consumer watchdog group Public Citizen filed the suit Sept. 8 in the U.S. District Court for the District of Columbia on behalf of the Post-Finasteride Syndrome Foundation (PFSF). The complaint asks the FDA to act on a petition the foundation filed in 2017, which has gone unanswered.

The case has more merit than it did when originally filed, Philip Roberts, PFSF’s patient manager, told *FDAnews*. “Much more scientific information has come to light since then,” he said, including two studies by Harvard investigators confirming the risk of long-term erectile dysfunction, depression, suicidal ideation and suicide; and epidemiologic data showing that the drug alters the expression of more than 3,700 genes in men who take the drug. The drug mimics a male hermaphroditism gene; people who have this are genetic males who sometimes look female and who never go bald.

The 2017 petition asked FDA to either withdraw 1 mg oral Propecia from the market or include a Black Box warning and send out a “Dear Healthcare Provider” letter, alerting clinicians to the risks associated with the medication. The petition included information on about a dozen men whose suicides were considered related to Propecia use and contraindications for patients with a history of sexual dysfunction and depression.

Since then, PFSF has amassed more data, including 17,953 adverse event reports filed in the International Drug Monitoring database. These include reports of erectile and sexual dysfunction (5,234) and 4,313 reports of psychiatric disorders, including anxiety (1,756) and depression (1,386). The database includes 88 cases of completed suicides, 39 attempts and 384 cases of suicidal ideation.

In light of Propecia's enormous global market (almost 9 million users in 2018, according to the website ClinCalc.com), the numbers are very small. Nevertheless, they represent an unnecessary tragedy that needs to be addressed, said Michael Kilpatrick, the Public Citizen attorney who filed the suit.

"It's a small percentage, but the number of prescriptions is so high that it's still a large number of people," he told FDAnews. "We think patients should know this. It's a drug for a cosmetic issue. If people knew there was even a slight change you could develop a significant, life-altering syndrome and even think about suicide, they might not want to take it."

The FDA has been sitting on the matter far too long, he said.

"A four-year wait is unreasonable. Our goal is to compel FDA to issue a decision on the original petition. We think it's a strong one and that the decision should be for granting a black box warning or even withdrawing the drug. This delay is in violation of the Administrative Procedure Act and given the seriousness of the issue and the quality of the petition, it should be resolved."

Merck didn't reply to a request for comment. The company has moved all of its Propecia patient and prescribing data to a new company spinoff, Organon, launched on June 3.

Read the PFSF complaint here: www.fdanews.com/09-09-21-Complaint.pdf. — Michele G. Sullivan

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Humanigen's Lenzilumab for COVID-19 Draws FDA Complete Response Letter

September 10, 2021

The FDA has declined to grant Humanigen an emergency use authorization (EUA) for its antibody lenzilumab for treatment of hospitalized COVID-19 patients.

The agency instead sent the Burlingame, Calif.-based company a Complete Response Letter (CRL), in which it said it was "unable to conclude that the known and potential benefits of lenzilumab outweigh the known and potential risks of its use as a treatment for COVID-19," Humanigen said.

But the agency also said in the letter that it is committed to working with Humanigen to develop lenzilumab and has invited the company to submit additional data as it becomes available, the company said.

Humanigen CEO Cameron Durrant said the National Institutes of Health's ACTIV-5/BET-B study, which is enrolling up to 500 patients, is expected to provide further data that may support a new EUA request. The company said it is also committed to completing the regulatory processes it has under way to seek a marketing authorization for lenzilumab to treat hospitalized COVID-19 patients in the UK and other territories.

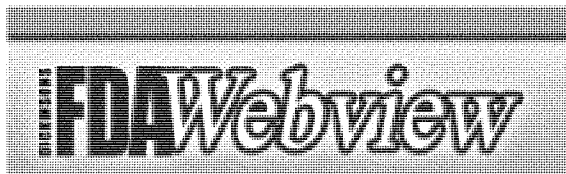
Humanigen is focused on preventing and treating an immune hyper-response called 'cytokine storm,'" which has been a major problem for critically ill COVID-19 patients. Lenzilumab neutralizes granulocyte-macrophage colony-stimulating factor (GM-CSF) that plays a role in inflammation that can lead cytokine storm.

The company said its phase 3 LIVE-AIR study "suggests early intervention with lenzilumab may prevent consequences of a full-blown cytokine storm in hospitalized patients with COVID-19."

Humanigen is also studying the antibody for other inflammatory conditions such as acute graft versus host disease in patients undergoing allogeneic hematopoietic stem cell transplantation, eosinophilic asthma, and rheumatoid arthritis.

— Martin Berman-Gorvine

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Apellis Pharma Plan NDAs for Geographic Atrophy

09/09/2021

Apellis Pharmaceuticals says it plans to file an NDA in the first half of 2022 after reporting top-line results from the Phase 3 DERBY and OAKS studies evaluating intravitreal pegcetacoplan, an investigational targeted C3 therapy, in 1,258 adults with geographic atrophy secondary to age-related macular degeneration. Geographic atrophy is a leading cause of blindness that affects about one million people in the U.S.

In the studies, pegcetacoplan demonstrated a clinically meaningful slowing of disease progression with an even stronger effect in geographic atrophy patients with extrafoveal lesions, the company says. "Monthly and every-other-month treatment with pegcetacoplan met the primary endpoint in OAKS, significantly reducing [geographic atrophy] lesion growth by 22% ($p=0.0003$) and 16% ($p=0.0052$), respectively, compared to pooled sham at 12 months," it says. "DERBY did not meet the primary endpoint, showing a reduction in GA lesion growth of 12% ($p=0.0528$) and 11% ($p=0.0750$) with monthly and every-other-month treatment, respectively, compared to pooled sham at 12 months."

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Myfembree sNDA for Endometriosis Pain

09/09/2021

FDA has accepted for review a Myovant Sciences and Pfizer supplemental NDA for Myfembree (relugolix 40 mg, estradiol 1 mg, and norethindrone acetate 0.5 mg) for managing moderate to severe pain associated with endometriosis. The agency set a user fee review action target date of 5/6/2022.

The submission is based on data from the Phase 3 SPIRIT program, which included two multinational, replicate pivotal clinical studies (SPIRIT 1 and SPIRIT 2) in over 1,200 women with pain associated with endometriosis for 24 weeks, and an open-label extension study for eligible women through one year.

Endometriosis is an estrogen-dependent, inflammatory disease in which tissue similar to the uterine lining is found outside the uterine cavity, which results in chronic inflammation and can cause scarring and adhesions.

Myfembree is currently approved for managing heavy menstrual bleeding associated with uterine fibroids in premenopausal women, with a treatment duration of up to 24 months.

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New FDA Jardiance Breakthrough Therapy Designation

09/09/2021

FDA has granted breakthrough therapy designation to Boehringer Ingelheim and Lilly's Jardiance (empagliflozin) for treating heart failure with preserved ejection fraction. An *American Journal of Managed Care* online [post](#) says Jardiance is the first drug in its class to gain the designation. The post says results from the EMPEROR Preserved trial showed that Jardiance, a sodium glucose co-transporter 2 inhibitor reduced cardiovascular death and hospitalization for heart failure by 21%.

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FDA Rejects Lenzilumab EUA for Hospitalized Covid Patients

09/09/2021

FDA has rejected a Humanigen emergency use authorization (EUA) request for the use of its lenzilumab in newly-hospitalized Covid-19 patients. FDA reportedly told the company it was unable to conclude that the known and potential benefits of lenzilumab outweigh the known and potential risks of its use as a treatment for Covid-19.

The company [says](#) the agency committed to working with it in the development of lenzilumab and invited Humanigen to submit additional data as they become available. Humanigen says the National Institutes of Health ACTIV-5/BET-B study is expected to provide further data that may support a new EUA request.

Humanigen describes lenzilumab as a first-in-class antibody that binds to and neutralizes granulocyte-macrophage colony-stimulating factor.

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Congress Must Regulate CBD Since FDA Won't: Steve Mister

09/09/2021

Responding to an FDA objection to a new dietary ingredient notification submitted by Charlotte's Web to demonstrate that its full-spectrum hemp extract is reasonably expected to be safe, Council for Responsible Nutrition CEO **Steve Mister** says Congress must

step in and regulate the CBD (cannabidiol) marketplace. Mister appeared with Charlotte's Web CEO **Deanie Elsner** in a "Short Legal Briefs" [video](#) posted by *Natural Products Insider*.

In the video, Elsner said she was disappointed in the way the agency made its decision to object to the notification for two reasons: that CBD products are excluded from the definition of a dietary supplement and that there were concerns about the sufficiency of the safety evidence.

She indicated that her company met multiple times with FDA officials before the notification was submitted to share data. The agency "ignored big parts of our application," she said.

Mister said the FDA denial letter showed that the agency is not going to solve the problem with CBD and thus Congress must act. He said the industry should turn its attention to persuading Congress to intervene. Regulation is needed, he said, because reputable companies that are concerned about the quality of their products are stuck in a "quagmire" with other companies that are not as concerned about quality, and consumers are being hurt.

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FiercePharma

No dice: Court tells AbbVie it can't force Takeda to supply a drug that's in shortage worldwide

by [Eric Sagonowsky](#) | Sep 9, 2021 9:28am

Amid the global shortage of prostate cancer drug Lupron, AbbVie took its partner Takeda to court over alleged failures to live up to supply agreements. Now, a court has denied the company's plea to force Takeda to fill orders.

In an [opinion](#) (PDF) this week, Delaware Chancery Court Vice Chancellor Sam Glasscock III denied AbbVie's request to compel Takeda to fill supply orders from its plant in Hikari, Japan.

Under a deal between the companies, AbbVie sells the drug in the U.S. and Canada. Because Takeda is the sole supplier of the drug worldwide, AbbVie relies on the Japanese drugmaker for its product.

Problem is, the med has been in short supply worldwide after the FDA and Takeda in 2019 found "protocol violations" at the plant, Glasscock wrote in this week's opinion. Thanks to "remediation efforts" including a weeks-long production hold global supply hiccups and shortages have persisted to this day, Glasscock wrote.

AbbVie had asked the court to find that Takeda breached its contract plus issue damages and "injunctive relief," or an order forcing Takeda to supply the drug. In his new opinion, Glasscock said an injunction would be "unworkable" because it'd force the court to oversee Takeda's operations and would "inevitably lead to contempt hearings" over Takeda's ability to fulfill orders.

"In other words, even if I find that Takeda has breached its contract with AbbVie and that, as a result, AbbVie faces irreparable harm, the injunctive relief sought is unavailable," Glasscock wrote.

The Lupron saga started in November 2019, when FDA inspectors traveled to Japan to review production processes at the Hikari site. In a June 2020 warning letter, the FDA slapped Takeda for poor equipment maintenance, faulty documentation and quality shortfalls. The company has been working to resolve those concerns, but the Lupron problem has dragged on throughout this year.

Right now, the FDA lists most Lupron Depot dose forms as "available" in the U.S., but there's one dose that's not expected to become available until next January.

While Glasscock decided against an injunction forcing Takeda to fill orders, he hasn't made a ruling on whether the company breached its contract. He plans to issue a decision on that issue in "due course," he said, and another trial phase on damages could follow.

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WCLC: Jazz charts new path for small cell lung cancer drug Zepzelca after late-stage flop

by [Angus Liu](#) | Sep 9, 2021 11:00am

Jazz Pharmaceuticals wasn't able to ride a breakthrough FDA nod to a pivotal trial win for conditionally approved small cell lung cancer (SCLC) drug Zepzelca. But after talking with the FDA, the company and partner PharmaMar see a new path forward.

In previously treated SCLC, the combination of Zepzelca and doxorubicin failed to extend patients' lives over physician's choice of doxorubicin-based chemo or Hycamtin, according to data presented at the 2021 World Conference on Lung Cancer virtual meeting.

Jazz and PharmaMar first unveiled the phase 3 ATLANTIS failure in December. Now we know patients who mostly got the Zepzelca regimen as their second-line treatment lived a median 8.6 months, versus 7.6 months in the control arm.

If successful, the trial could have served as the confirmatory trial to convert Zepzelca's accelerated approval earned last June into a full one. In a previous single-arm study, Zepzelca monotherapy shrunk tumors in 35% of relapsed SCLC patients as measured by investigator assessment. The response lasted a median 5.3 months.

Pascal Besman, chief operating officer at PharmaMar, noted that the ATLANTIS study used a 2-mg/m² dose of Zepzelca, which is lower than the FDA-approved dose of 3.2-mg/m². Although the Zepzelca-doxorubicin combo didn't improve survival, "the overall results support the activity and tolerability of Zepzelca monotherapy in second-line therapy," Besman said in an email interview.

As ATLANTIS investigator Luis Paz-Ares, M.D., Ph.D., noted in a prepared presentation, the research team ran a so-called exposure-response analysis to gauge Zepzelca's contribution on patient survival. The model predicted that single-agent Zepzelca, at the 3.2-mg/m² dose, would have had a statistically significant advantage over the control arm at prolonging the lives of patients in the ATLANTIS study.

Jazz stands "firmly behind the favorable benefit/risk profile of monotherapy Zepzelca" in second-line SCLC as shown in its phase 2 trial, the company's chief medical officer, Rob Iannone, M.D., said in a separate email interview.

For now, Zepzelca will remain on the market. Jazz and PharmaMar have agreed "in principle" with the FDA to run another phase 3 as the confirmatory trial, Besman said.

The three-arm trial will test Zepzelca monotherapy at 3.2 mg/m², and a second experimental arm will combine the drug at 2 mg/m² with Pfizer's Camptosar (irinotecan).

Second-line patients in the control arm will get physician's choice or either Camptosar or Hycamtin. The trial, expected to launch this year, will also support a regulatory filing to the European Medicines Agency, Besman added.

In addition, Jazz is collecting data from an observational phase 4 trial to understand Zepzelca's real-world profile in second-line SCLC, Iannone noted.

Meanwhile, Jazz is also looking to move Zepzelca earlier in the treatment line. Through a partnership with Roche, a phase 3 trial dubbed ImForte will test Zepzelca with immunotherapy as a first-line maintenance therapy for patients with extensive-stage SCLC after induction chemotherapy. If successful, the new regimen could pressure AstraZeneca and its Imfinzi, which is currently dominating the front-line SCLC space.

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WCLC: AstraZeneca's detailed Imfinzi, tremelimumab data in metastatic NSCLC are far from a clear-cut win

by [Angus Liu](#) | Sep 9, 2021 11:00am

After two high-profile late-stage trial flops, AstraZeneca has finally scored a victory for Imfinzi and ill-fated tremelimumab in newly diagnosed metastatic non-small cell lung cancer (NSCLC). But in a lucrative field where immunoncology rivals Merck and Bristol Myers Squibb have established a solid presence, a detailed look at the data reveals a win that's perhaps too little, too late.

A cocktail of Imfinzi, investigational CTLA-4 inhibitor tremelimumab and chemotherapy pared down the risk of death by 23% over chemo alone in previously untreated patients, AZ announced Thursday at the 2021 World Conference on Lung Cancer virtual meeting.

The phase 3 POSEIDON trial came after two prior phase 3 flops for Imfinzi in metastatic NSCLC and marked the first positive pivotal readout for treme after many more failures in multiple indications. But the data don't look favorable compared to Merck's Keytruda or Bristol Myers' Opdivo, and efficacy in some patient subgroups showed imbalances that could limit an eventual FDA approval if AZ gets one.

First off, the combination of Imfinzi and chemo failed to significantly outdo chemo alone at extending patients' lives. That pairing only reduced the risk of death by 14% over chemo alone. That already makes Imfinzi look inferior to current standard-of-care Keytruda.

Keytruda's combo with chemo is well known for its impressive 51% death risk reduction over chemo alone in front-line non-squamous NSCLC. Those were the results at an interim analysis in the phase 3 Keynote-189 trial, while the final analysis at 31 months of follow-up showed a 44% risk decrease. As for front-line squamous cell NSCLC, Keytruda and chemo reduced death risk by 36% at interim and 29% at final analysis in the KEYNOTE-407 trial.

Luckily for AZ, treme finally pulled through to help Imfinzi. The CTLA-4 agent has a bad track record with Imfinzi and has been marred with failures in NSCLC as well as small cell lung, head and neck and bladder cancers. In front-line NSCLC alone, the dual immunotherapy combo of Imfinzi and treme flopped the phase 3 MYSTIC trial in a broad patient group and the NEPTUNE trial in patients with high tumor mutation burden.

Now that the Imfinzi-treme-chemo doesn't appear likely to shake up Keytruda's dominance, what about less competitive Opdivo? Data don't look very promising for AZ.

For one thing, while the chemo-free regimen of Imfinzi and treme failed, Opdivo's own CTLA-4 combo with Yervoy is approved for front-line NSCLC patients whose tumors express PD-L1.

For a better apple-to-apple comparison, the Opdivo-Yervoy-chemo combo won an FDA nod last year for front-line NSCLC patients regardless of PD-L1 status. In the CheckMate 9LA trial, Opdivo, Yervoy and chemo were able to show a survival benefit early, having cut patients' risk of death by 31% over chemo after a minimum follow-up of 8.1 months. After a longer follow-up of at least 24.4 months, the rate stood at 28%, as patients on the Opdivo-Yervoy-chemo regimen lived a median 15.8 months versus 11 months for the solo chemo group.

Imfinzi-treme-chemo's 23% death reduction looks slightly weaker than Opdivo-Yervoy-chemo's 28%, but cross-trial comparisons have their problems. In POSEIDON, patients on the AZ therapy lived a median 14 months, versus 11.7 months for those who took chemo alone.

To further complicate the front-line NSCLC landscape, Roche's PD-L1 inhibitor Tecentriq is allowed as a monotherapy for PD-L1-high patients thanks to data from the IMpower110 trial. And its combo with Avastin and chemo has an approval for non-squamous disease after showing it reduced the risk of death by 22% over Avastin and chemo.

Sanofi and Regeneron's Libtayo also bears a monotherapy nod in front-line PD-L1-high NSCLC. The companies recently found that adding Libtayo on top of chemo could further prolong the lives of first-line patients in the phase 3 EMPOWER-lung 3 trial.

Eli Lilly and Innovent Biologics have a front-line non-squamous NSCLC filing for their China-approved PD-1 inhibitor Tyvyt under review at the FDA. The application includes data from a China trial that showed a combination of Tyvyt, Lilly's Alimta and platinum chemo could stave off tumor progression or death over chemotherapy alone.

For AZ, non-squamous NSCLC might be just what it could eventually reach for with the Imfinzi-treme-chemo pairing.

As POSEIDON lead investigator Melissa Johnson, M.D., noted during a prepared presentation Thursday, among patients with the squamous histology, progression-free survival (PFS) "and overall survival were poor across all treatment arms, with little separation of the curves for PFS or overall survival."

In the squamous subgroup, Imfinzi, treme and chemo only managed to reduce the risk of death by 12% over chemo.

What's more, the AZ therapy also showed some worrisome signs in other patient subgroups. Notably, women and Asian patients didn't seem to experience any additional survival benefits from Imfinzi and treme.

The one upside? The efficacy of Imfinzi, treme and chemo didn't seem to be bound by a patient's PD-L1 expression level.

Still, with the mixed bag of data and a poor track record, AZ could face a limited FDA label and a hard time winning over market share in front-line NSCLC. Imfinzi is currently approved to treat stage 3 NSCLC and extensive-stage small cell lung cancer. In the first half of 2022, AZ plans to read out data from PEARL, an IMpower110 counterpart trial that evaluates Imfinzi monotherapy against chemo as front-line treatment for PD-L1-high patients.

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Biogen CEO blames Aduhelm's rocky launch on 'too much confusion, misinformation and controversy'

by [Eric Sagonowsky](#) | Sep 9, 2021 1:44pm

Before Biogen scored an FDA approval for Alzheimer's drug Aduhelm, the company probably hoped its launch would go much smoother than the last three months have been. It turns out Biogen's CEO Michel Vounatsos is willing to say so publicly.

Speaking at a Morgan Stanley healthcare event Thursday, Vounatsos said there's "clearly too much confusion, misinformation and controversy surrounding our data and the approval process." The launch has been "even more gradual than we had expected," he added.

The company is facing "near-term challenges," Vounatsos told the virtual audience, noting that "everybody can see that." Now, months after the drug's approval, he said it's "imperative" that "society moves its attention [from] the clinical data to the patients in need."

After Aduhelm's accelerated approval in June, a firestorm quickly engulfed the new Alzheimer's drug. Lawmakers and medical experts have questioned the data Biogen used to support an approval plus the FDA's procedures that led to the accelerated nod.

The criticism grew so intense that the FDA and Biogen, in an unusual move, **walked back** Aduhelm's broad label just weeks into the launch. The FDA originally endorsed the drug for all Alzheimer's patients but later restricted the label to those with mild cognitive impairment or mild dementia.

In another big setback for the rollout, the Department of Veteran's Affairs last month decided against covering the drug, citing a "risk of significant adverse drug events" and a "lack of evidence of a positive impact on cognition."

Looking forward, one key question mark for the rollout remains in the Centers for Medicare & Medicaid Services' coverage decision on the drug. That's expected in January and will determine whether federal insurance programs get behind the treatment.

Meanwhile, the company is working to improve the public's understanding of the clinical data and on generating additional data, Vounatsos said. It's also aiming to bring more treatment centers online and negotiate reimbursement deals to "maximize access," he added.

Biogen's drug launched with big sales expectations, but its initial stumbles have dampened some of the enthusiasm on Wall Street. In a note about Vounatsos' Thursday presentation, Jefferies analysts said the Street is "very negative on the early launch." Analysts still expect the drug to bring in multiple billions of dollars per year once it gets rolling.

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Despite mixed phase 3 showing, Apellis hopes to push eye disorder drug past FDA's finish line

by [Noah Higgins-Dunn](#) | Sep 9, 2021 4:05pm

Apellis Pharmaceuticals' pegcetacoplan posted mixed results in two late-stage trials studying the C3 inhibitor in patients living with geographic atrophy (GA). Despite the mixed showing, CEO Cedric Francois thinks the med's performance was strong enough to warrant an FDA nod.

Apellis studied pegcetacoplan, the active ingredient in rare disease drug Empaveli, in two phase 3 trials, known as Oaks and Derby, as a monthly and every-other-month treatment in 1,258 patients with geographic atrophy (GA) secondary to age-related macular degeneration (AMD). For the studies, investigators weighed efficacy by measuring change in the total area of GA lesions from baseline.

In Oaks, pegcetacoplan met its primary endpoint in both dosing groups, significantly reducing GA lesion growth by 22% in the monthly arm and 16% in the every-other-month group compared with sham injections after 12 months, Apellis said.

The drug failed to do the same in Derby, reducing lesions by just 12% and 11%, respectively. The drug didn't produce any significant safety concerns in both trials, although its use was associated with slightly higher rates of exudation, or emitted fluid.

Despite the conflicting readouts, Apellis is "unbelievably happy" with the results given the totality of pegcetacoplan clinical trial performance, CEO Cedric Francois said in an interview. The company plans to file the med with the FDA in the first half of 2022.

There are no FDA-approved treatments for the condition, an advanced form of AMD that eventually leads to vision loss and blindness in older people.

"This is a drug that, in our opinion, has absolutely no doubt around its efficacy and comes out with a safety profile that I think exceeds our own and other people's expectations," Francois said.

Meanwhile, the drug posted another late-stage upside, Francois argued. In a prespecified analysis of the combined trials, pegcetacoplan was more effective in patients with extrafoveal lesions at the start of the trials,

decreasing GA lesion growth in that group by as much as 26% in the monthly arm and 23% in the every-other-month group.

GA patients typically present first with extrafoveal lesions, which then progress toward the fovea where central vision is impacted, Apellis says. That's important because the disease is like a forest fire in the retina, continuously destroying photoreceptor cells until someone's blind with no way to turn it around, Francois said.

There are roughly 1 million Americans living with the condition and 5 million people globally. If Apellis is able to do what others have failed, it could be looking at \$3 billion in peak sales, Jefferies analysts recently **predicted**.

Pharma has tried and failed to push GA treatments over the FDA's finish line in recent years, including an effort by Roche on its complement-inhibiting drug lampalizumab. That drug, however, **crashed** out of two phase 3 trials in 2017.

This has already been a busy year for Apellis, which **scored** its first-ever FDA nod in May for pegcetacoplan in patients with the rare blood disorder paroxysmal nocturnal hemoglobinuria (PNH). That has teed-up a showdown between Apellis' C3 inhibitor and Alexion's heavyweight C5 inhibitors Soliris and its follow-up Ultomiris, which target a different enzyme.

"If this drug gets approved, it will be the first time that complements control would get approved in a large speciality, rather than in a rare disease like PNH," Francois said. "I think it opens up a new era for what a complement can do in many of these conditions across multiple therapeutic areas."

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Room Available In US Pharma Industry For OTC Switch Leader: Has Perrigo Made A Reservation?

09 Sep 2021

ANALYSIS

Malcolm.Spicer@TheTanSheet malcolm.spicer@informa.com

Executive Summary

Acquiring HRA Pharma, Perrigo could take OTC switch lead, likely starting with HRA's daily oral contraceptive before moving to numerous other potential candidates. Pharmas previously busy in switch have other priorities for their consumer health businesses.

The US pharma industry has a vacancy for an OTC switch leader as longtime sponsors of applications to make additional ingredients available nonprescription prioritize reorganizing their consumer health businesses over expanding their product portfolios.

By acquiring HRA Pharma, Perrigo Company PLC could fill the space in the pharma industry. Its switch proposals likely will start with HRA's daily oral contraceptive before moving to numerous other potential candidates.

"There aren't a lot of switch powerhouses out there. I think there's an opportunity for a new player to emerge as a switch powerhouse, given the changing market dynamics," says pharma industry consultant Susan Levy.

"I think this alignment, HRA with Perrigo's scale, is just what the industry needs," said switch consultant Susan Lavine Coleman, president of NCI Consulting in Moorestown, NJ.

Levy and Coleman spoke with HBW Insight following Perrigo's 8 September announcement of an agreement to acquire Paris-based HRA, listed in France as Héra SAS, for €1.8bn (\$2.1bn) in a cash deal.

Perrigo's predominant revenue stream has been private label/store brand OTC drugs, although it has added brands with its 2014 move into Europe's consumer health market and by acquiring the Prevacid 24 HR (lansoprazole/15 mg) proton pump inhibitor in 2019 and licensing the Burt's Bees brand in May to make and market a line of organic baby formulas and supplements for babies and children.

"I think there's an opportunity for a new player to emerge as a switch powerhouse, given the changing market dynamics." – Susan Levy

But while it consistently is first to launch generic equivalents for brand switches, its name hasn't been linked to submitting proposals for making additional drug ingredients available OTC.

Bringing HRA, including management, under its roof changes the switch outlook for Perrigo.

"These are pros. They know what they're doing," Perrigo president and CEO Murray Kessler said about HRA's management during a briefing about the deal. "They've all agreed to stay on with Perrigo."

Slow Time For Switch NDAs

The HRA acquisition isn't the only reason Perrigo could become a top OTC switch sponsor in the US. Other pharmas are leaving the top spot open.

Looking over the current lineup of major OTC marketers in the US, Levy, founder and principal of Susan B. Levy Consulting LLC in Cranford, NJ, doesn't see any interested in sponsoring new drug applications for OTC switches.

"If you look at who has historically been a driver for OTC switches, you've got GSK, Novartis and Pfizer, but they're all one [consumer health] company now, and it's being spun out. It's kind of unclear to what extent they are positioned right now to invest in expensive growth programs," she said.

GlaxoSmithKline plc is the majority owner of a joint venture with Pfizer Inc. marketing the two firms' consumer health product lines. GSK, with products including brands it acquired after previously operating a consumer health JV with Novartis AG, plans to spin out its current consumer health business. (Also see "McNamara To Lead GSK's Standalone Consumer Healthcare Business" - HBW Insight, 23 Jul, 2021.)

"Then you've got Sanofi," Levy added.

The French firm is known to be considering proposing switches for flu treatment Tamiflu (oseltamivir) and erectile dysfunction drug Cialis (tadalafil), but it too looks to separate its consumer health business. (Also see "Sanofi To Divest 60% Of Brands In Consumer Healthcare Overhaul" - HBW Insight, 10 Feb, 2021.)

"It's unclear where they want to put their bets right now as a result of being spun out independently," she said.

Bayer AG earlier in 2021 received Food and Drug Administration approval for OTC sales of Astepro Allergy (azelastine hydrochloride nasal spray, 205.5 mcg per spray). Bayer plans to launch the first-in-class switch, the first ingredient available OTC that is a first-generation H1 (histamine receptor inhibitor) antihistamine, in 2022. (Also see "Market Exclusivity Decision Pending For Bayer's First-In-Class OTC Switch Astepro Allergy" - HBW Insight, 24 Jun, 2021.)

Levy pointed out the previous OTC switch for a product the German firm markets in the US was Oxytrol for Women overactive bladder treatment, a first-in-class switch when it was approved in 2013 when US firm Merck & Co., Inc. owned the brand. After acquiring Merck & Co.'s consumer business in 2014, Bayer relinquished OTC rights to the 3.9mg oxybutynin transdermal patch to Allergan PLC less than a year later.

J&J Large In Consumer, Not In Switch

Johnson & Johnson may be synonymous with OTC drugs due to its market-leading brands, but like Perrigo it hasn't been a switch driver.

"Who could be the powerhouses? ... J&J has not historically been a switch player. They don't have an experienced team of switch professionals," Levy said.

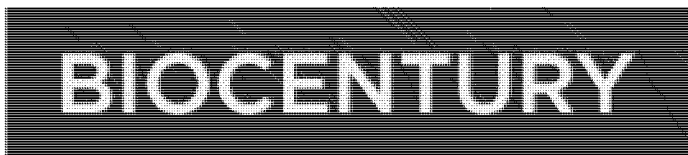
"It speaks to the fact that there is a gap in the marketplace because these switch programs are very expensive for an OTC company. They are somewhat risky, and they're much longer-term than typical OTC innovation. ... If you're heading up a consumer health care company and thinking about where you're going to put your R&D bets, much more incentives are there to do some of the shorter-term, easier innovation."

The FDA is encouraging firms to study making available nonprescription drug ingredients indicated for chronic conditions; in 2012 it opened a docket for recommendations on changing its OTC switch NDA rules to allow for "novel switches" targeting chronic conditions, indications more complicated than most currently available OTC drugs have.

The agency launched the initiative expecting labeling for novel switches to be subject to the same limits as other OTCs, with all necessary information available on a product's Drug Facts label. However, it has since indicated in a 2018 guidance that switch sponsors could tap into digital media and other extra-label tools to help demonstrate that consumers can assess whether an OTC drug is appropriate for them and they can use the product safely. (Also see ["Before Asking FDA For Novel OTC Switch, Be Sure DFL Alone 'Can't Get There'"](#) - HBW Insight, 23 May, 2019.)

In a recent FDA workshop conducted online, industry experts and other stakeholders said some problems with OTC DFLs are worse than others and some likely can be corrected with minor changes to the format, but other changes, notably for supporting OTC labeling for drugs that currently are available Rx-only and are indicated for chronic conditions aren't minor and won't happen within the limits of one-dimensional text media printed on nonprescription drug containers, packages and inserts. (Also see ["Progress On Adding OTC Drugs To US Market? Room To Move But Label Format Limits Options"](#) - HBW Insight, 18 Jun, 2021.)

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Sept. 9 Quick Takes: Obsidian readies for IPO with \$115M crossover round

PLUS: VENTURE ROUNDS FOR LEXEO, EVOMMUNE, ALAMAR, AND SETBACKS FOR HUMANIGEN, SANOFI AND MORE

BY BIOCENTURY STAFF

SEP 9, 2021 | 9:44 PM EDT

Cell and gene therapy play Obsidian Therapeutics Inc. looks set to seek a public listing after raising \$115 million in a series B round led by The Column Group Crossover Fund. Other new investors included RA Capital, Surveyor Capital, Cowen Healthcare Investments, Deep Track Capital, Logos Capital, Pivotal BioVenture Partners, [Samsara BioCapital](#) and Soleus Capital. Existing investors Atlas Venture, Vertex Ventures HC, Amgen Ventures, Bristol Myers Squibb Co. (NYSE:BMJ) and Vertex Pharmaceuticals Inc. (NASDAQ:VRTX) also participated. Obsidian's lead tumor infiltrating lymphocyte (TIL) program, cytoTIL15, is expected to enter the clinic next year.

\$100M SERIES B TO SUPPORT LEXEO'S GENE THERAPIES

Adding to an \$85 million January series A, Lexeo Therapeutics raised \$100 million in a series B led by D1 Capital Partners and Eventide Asset Management. Also backing the round were CAM Capital, Verition Fund Management, Laurion Capital Management, Gray's Creek Capital Partners, and existing investors Longitude Capital, Omega Funds, Lundbeckfonden Ventures, PBM Capital, Janus Henderson Investors, Woodline Partners LP, Invus Capital and Alexandria Venture Investments. The company has three lead AAV-mediated gene therapy programs: LX2006, which is in preclinical testing for cardiomyopathy associated with Friedreich's ataxia, and LX1004 for CLN2 Batten disease and LX1001 for APOE4-associated Alzheimer's disease, both of which are in Phase I/II testing. The company is also developing recently acquired cardiac gene therapies from Stelios Therapeutics Inc. and additional preclinical programs targeting Alzheimer's disease.

EVOMMUNE RAISES \$83M IN SERIES A

Evommune Inc., which was founded in 2020 by Dermira Inc. alums, raised \$83 million in series A round with participation from Andera Partners, LSP, global syndicate of investors and Pivotal BioVenture Partners, the seed investor, to enable development of therapies to treat inflammatory diseases through industry and academic collaborations. Evommune has exclusive rights to three of the four candidates in its pipeline, from Dermira, now a Eli Lilly and Co. (NYSE:LLY) company.

SHERPA LEADS ALAMAR'S \$80M SERIES B

An \$80 million series B round led by Sherpa Healthcare Partners will enable Bay Area-based proteomics company Alamar Biosciences Inc. to accelerate the development of its NULISA and Attobody technology platforms to deliver single-digit attomolar detection sensitivity. NULISA (NUcleic acid-Linked Immuno-Sandwich Assay) seeks to provide "ultra-high sensitivity and massively parallel scale" to proteomic analysis, while Attobody, the company says, yields highly specific picomolar affinity antibodies. Other firms participating in the round included new investors Morningside Ventures and Samsara Biocapital and existing investors Qiming Venture Partners, which lead the series A, and Illumina Ventures. The company has now raised \$110 million.

LILLY TAKES EQUITY STAKE IN LYCIA AS PART OF \$70M SERIES B

New Lycia Therapeutics, Inc. partner Eli Lilly and Co. (NYSE:LLY) has invested in the South San Francisco company's \$70 million series B, which Redmile Group led. Founding investor Versant Ventures and new investors Cowen Healthcare Investments, Invus, RTW Investments and Alexandria Venture Investments also participated. The financing comes two weeks after Lycia picked Lilly as its collaborator to develop LYTACs against targets implicated in the pharma's areas of focus.

FIRST APPROVAL FOR PFIZER'S ORAL ATOPIC DERMATITIS THERAPY COMES IN U.K.

The U.K.'s MHRA approved Cibinqo abrocitinib from Pfizer Inc. (NYSE:PFE) to treat atopic dermatitis, marking the first global approval for the JAK1 inhibitor. FDA pushed back the therapy's PDUFA date due to a class-wide safety review. Last month, the oral therapy demonstrated superiority to leading atopic dermatitis therapy Dupixent dupilumab in the Phase III JADE DARE trial.

CENTESSA REPORTS FIRST POSITIVE DATA READOUT SINCE FORMATION

Asset-centric play Centessa Pharmaceuticals plc (NASDAQ:CNTA) had its first positive data readout since the company was formed this year by Medicxi. Centessa subsidiary ApcinteX Ltd. said that in the Phase IIa portion of a Phase I/IIa trial, the high dose of SerpinPC over six months led to an 88% reduction in annualized bleeding rate compared with a pre-exposure observation period in patients with either hemophilia A or hemophilia B. The PROC inhibitor was well tolerated with no cases of sustained elevation of D-dimer, a marker of excess thrombin generation. Data from an open-label extension of the study are expected next year. Centessa rose \$1.21 to \$22.26

HUMANIGEN SINKS AFTER FDA DECLINES TO ISSUE EUA

Shares of Humanigen Inc. (NASDAQ:HGEN) shed nearly half their value Thursday after the biotech said FDA declined to grant an emergency use authorization for lenzilumab, its mAb targeting GM-CSF, to treat hospitalized patients with COVID-19. Humanigen said FDA could not yet conclude that lenzilumab's benefits outweigh its risks, but the biotech believes the NIH-funded ACTIV-5/BET-B study may generate data sufficient to support the mAb's emergency authorization. Humanigen lost \$7.14 (47%) to \$7.97 on Thursday.

ACQUIRED IN PRINCIPIA DEAL, SANOFI'S BTK MISSES IN PEMPHIGUS

Sanofi (Euronext:SAN; NASDAQ:SNY) said Btk inhibitor rilzabrutinib failed to significantly improve remission rates in the Phase III PEGASUS trial to treat pemphigus, missing the study's primary endpoint. The pharma gained the molecule via its \$3.4 billion buyout of Principia Biopharma Inc. (NASDAQ:PRNB) last year. Rilzabrutinib is also in a Phase III study to treat chronic immune thrombocytopenia.

AZ-AVILLION COMBO MEETS MARK IN ASTHMA READOUTS

AstraZeneca plc (LSE:AZN; NYSE:AZN) and Avillion LLP said PT027, the partners' fixed-dose combination of albuterol and budesonide, met the primary endpoints in the Phase III DENALI and MANDALA trials to treat asthma. The treatment significantly improved lung function compared with each of its components in DENALI, and significantly reduced exacerbations compared with albuterol alone as a rescue medicine in MANDALA. Blackstone Life Sciences, Abingworth and Royalty Pharma plc (NASDAQ:RPRX) are backing the PT027 development program in a risk-sharing arrangement.

POSITIVE READOUT FOR A SUBCUTANEOUS GENMAB BISPECIFIC

Epcoritamab, a subcutaneously administered CD3 x CD20 bispecific antibody from Genmab A/S (CSE:GMAB; NASDAQ:GMAB), led to an 88% ORR in relapsed or refractory diffuse B cell lymphoma patients, providing support that the formulation may compete with more burdensome IV infusions on efficacy. Among eight patients who received the recommended Phase II dose of 48 mg, 38% achieved a complete response. ORR was 90% and CR was 50% in the 10 follicular lymphoma patients treated across dose ranges in the dose-escalation study. There was no Grade 3 or higher cytokine release syndrome.

Odronextamab (REGN1979), a CD3 x CD20 bispecific from Regeneron Pharmaceuticals Inc. (NASDAQ:REGN), led to an ORR of 92.9% in follicular lymphoma patients in a Phase I readout last year, though a partial clinical hold was placed on the program in December due to incidence of Grade 3 and higher CRS during step-up dosing.

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Cancer leads way to routine use of accelerated approval: Data Byte

AN ANALYSIS FROM BIOCENTURY'S 2021 BACK TO SCHOOL PACKAGE

BY SELINA KOCH, EXECUTIVE EDITOR

SEP 9, 2021 | 7:08 PM EDT

Accelerated approval is becoming the *de facto* route for approvals of new molecular entities in cancer.

In three of the past five years, the number of new cancer drugs granted accelerated approval by FDA has outnumbered those given full approval. Halfway through this year, the count stood at eight accelerated to four standard approvals.

BioCentury's annual Back to School project explored cancer as a case study of the promise and problems of accelerated approval, and argued for changes that could usher in routine use of the pathway for all serious diseases with unmet needs.

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From: Cavazzoni, Patrizia [/O=EXCHANGELABS/OU=EXCHANGE ADMINISTRATIVE GROUP (FYDIBOHF23SPDLT)/CN=RECIPIENTS/CN=C42ABD33834044ECBAA03D075CC0A5D2-PATRIZIA.CA]
Sent: 9/10/2021 9:45:27 AM
To: Jungman, Elizabeth [/o=ExchangeLabs/ou=Exchange Administrative Group (FYDIBOHF23SPDLT)/cn=Recipients/cn=5238a0caec064ba8b5d598115bc4f99f-Elizabeth.J]
Subject: RE: CDER Trade Press News Update September 10, 2021

I can speak now, you can call me on mobile

Patrizia

From: Jungman, Elizabeth <Elizabeth.Jungman@fda.hhs.gov>
Sent: Friday, September 10, 2021 9:45 AM
To: Cavazzoni, Patrizia <Patrizia.Cavazzoni@fda.hhs.gov>
Subject: RE: CDER Trade Press News Update September 10, 2021

I am open until 10, between 12 and 2, or at 4 – can send other times if those don't work. I know your day today is nuts.

Elizabeth Jungman, J.D., M.P.H. (she/her)
Director

Office of Regulatory Policy
Center for Drug Evaluation and Research
U.S. Food and Drug Administration
Tel: 240-402-1563 (desk)
Tel: 240-278-6021 (work cell)
EA: Laura DeGrouchy

Elizabeth.Jungman@fda.hhs.gov



From: Cavazzoni, Patrizia <Patrizia.Cavazzoni@fda.hhs.gov>
Sent: Friday, September 10, 2021 9:40 AM
To: Jungman, Elizabeth <Elizabeth.Jungman@fda.hhs.gov>
Subject: RE: CDER Trade Press News Update September 10, 2021

Could we speak about Propecia at the next opportunity?

Patrizia

From: CDER Trade Press <CDERTradePress@fda.hhs.gov>
Sent: Friday, September 10, 2021 9:29 AM
To: CDER Trade Press <CDERTradePress@fda.hhs.gov>
Subject: CDER Trade Press News Update September 10, 2021



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(FDA News)

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(FDA Webview)

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[Biogen CEO blames Aduhelm's rocky launch on 'too much confusion, misinformation and controversy'](#)

[Despite mixed phase 3 showing, Apellis hopes to push eye disorder drug past FDA's finish line](#)

(HBW Insight)

[Room Available In US Pharma Industry For OTC Switch Leader: Has Perrigo Made A Reservation?](#)

(BioCentury)

[Sept. 9 Quick Takes: Obsidian readies for IPO with \\$115M crossover round](#)

[Cancer leads way to routine use of accelerated approval: Data Byte](#)

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Biogen Declines To Say If It Seeks IG Advice On Aduhelm Giveaway

By [Gabrielle Wanneh](#) / September 9, 2021 at 10:42 AM

Biogen is giving free Aduhelm to patients at Florida-based First Choice Neurology, and that arrangement is at risk of being considered a kickback, a former HHS Inspector General official said. However, the company said its program is common practice and declined to say whether it seeks an advisory opinion from the IG that would help shield it from enforcement action.

“Biogen is committed to ensuring that appropriate patients have access to any Biogen medicines prescribed by their physicians,” Biogen said. “It is common for pharmaceutical companies, including Biogen, to offer patient assistance programs designed to help remove non-clinical barriers to access to care, and Biogen designs and administers its access programs to comply with applicable laws and regulations.”

Reuters reported that Biogen is providing its Alzheimer's drug for free [while Medicare determines whether, and under what circumstances,](#) to cover Aduhelm. FDA granted Aduhelm an accelerated approval in June against the advice of independent agency advisers. It's the first new Alzheimer's treatment in nearly two decades, but many insurers and doctors don't believe there is sufficient evidence that the drug works to outweigh the risk of serious side effects.

It's common for companies to offer free medications, and the legality of patient assistance programs depends on their details. Companies may seek advice from the IG on their programs, but they aren't required to get advisory opinions, and advisory opinions are not legally binding, according to Jennifer Michael, former chief of the Industry Guidance Branch at the IG.

Based on the description of Biogen's program in the Reuters article, Michael says there's a chance the IG would consider the arrangement to be a 'seeding' program. Seeding refers to the practice of giving free drugs to a subset of eligible patients to induce those patients to use drugs that federal health programs will eventually pay for.

"The government might be concerned that those types of seeding programs could result in the potential for overutilization or inappropriate utilization," Michael told *Inside Health Policy*. -- [Gabrielle Wanneh \(gwanneh@iwpnews.com\)](mailto:gwanneh@iwpnews.com)

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Premier Acquires Stake In Specialty Drug Maker To Ensure Supply Of At-Risk Drugs

By [Gabrielle Wanneh](#) / September 9, 2021 at 2:39 PM

The group purchasing organization Premier and 11 leading health systems have invested a small stake in specialty drug maker Exela Pharma Sciences to ensure the supply of essential drugs that face possible shortages due to sourcing problems spurred by the COVID-19 pandemic.

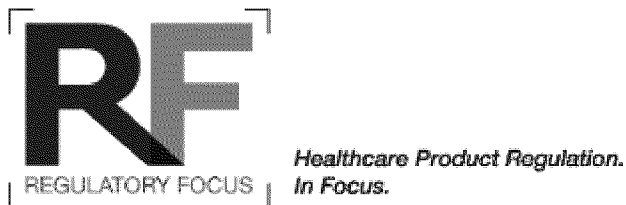
The participating health systems are expected to secure an uninterrupted supply of 19 pharmaceutical products, including generic injectables that often appear on the FDA drug shortage list and 503B compounded pharmaceutical products provided by Exela, which primarily sources active ingredients domestically and from Europe, through its manufacturing facility in North Carolina.

The move is a part of a longtime initiative of Premier's ProvideGx and is similar to actions by other hospital groups like CivicaRx that aim to avert drug shortages and prevent price gouging that often follows a drug scarcity. For more than a decade, several drug shortages have contributed to \$230 million in additional drug costs as well as poorer health outcomes for patients, according to a 2018 study conducted by researchers at the University of Pittsburgh.

"Leveraging Exela's significant production capacity, this collaboration is expected to give Premier's participating members uninterrupted access to high-quality, U.S.-made pharmaceutical products and safety stock," Premier President and CEO Michael Alkire said in a statement Wednesday (Sept. 8). "With this investment, we are committing to support the domestic production of vital medicines for our members and the market and to bring much-needed solutions to help eliminate drug shortages."

The participating health systems have signed a multi-year commitment to buy a portion of their drugs from Exela through Premier's GPO. They include Baystate Health of Springfield, MA; Genesis Health System of Davenport, IA; Henry Ford Health System of Detroit, MI; McLaren Health Care of Grand Blanc, MI; Memorial Healthcare System of Hollywood, FL; OSF HealthCare of Peoria, IL; Riverside Health System of Newport News, VA; Saint Francis Health System of Tulsa, OK; Texas Health Resources of Arlington, TX; and Universal Health Services, Inc. Of King of Prussia, PA. -- [Gabrielle Wanneh \(gwanneh@iwpnews.com\)](mailto:gwanneh@iwpnews.com)

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FDA updates guidance on generic drug development during COVID

Posted 09 September 2021 | By [Michael Mezher](#)

The US Food and Drug Administration (FDA) on Wednesday revised its questions and answers guidance on generic drug development and abbreviated new drug application (ANDA) submissions during the COVID-19 public health emergency.

The revised guidance updates the agency's previous guidance on generic drug development during the COVID-19 pandemic that was released in April 2021. (RELATED: [FDA offers guidance on generic drug development during COVID](#), *Regulatory Focus* 5 April 2021)

The revisions include a clarification about when FDA will accept requests to receive an ANDA with "less than the full complement of recommended stability data," and information on how to handle expired batches of test product in interrupted bioequivalence studies. FDA also says it has made some minor updates throughout the frequently asked questions section.

"FDA recognizes that the COVID-19 public health emergency may impact the development of generic drug products, interrupt or delay ANDA applicants' bioequivalence studies, and impact the submission of ANDAs to FDA for assessment. The ongoing development of generic drugs submitted as ANDAs to the FDA for assessment helps Americans continue to have access to safe, effective generic drugs," FDA says.

In the revised guidance, FDA now explains that it will "generally accept requests to receive an ANDA with less than the full complement of recommended stability data if the drug product is on FDA's Drug Shortage List or FDA confirms that the drug product is vulnerable to shortage." The agency also says it will also generally do so for products that meet its criteria for priority review to help address the COVID-19 pandemic.

FDA is also granting generic drugmakers additional flexibility when it comes to resuming bioequivalence studies that were interrupted due to the COVID-19 public health emergency. The revised guidance now provides recommendations allowing prospective applicants to potentially use a test product batch that is beyond the established expiration dating period to resupply bioequivalence studies, "as long as sufficient evidence can be provided to demonstrate the acceptability of the test product batch."

FDA further explains that applicants who wish to follow this approach "should submit data to ensure the test batch samples stored at the long-term stability conditions beyond the current expiration date are in compliance with the finished drug product shelf-life specifications at the time of conducting the [bioequivalence] BE study."

[FDA](#)

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Kaleido warned; FDA not persuaded by 'medical food' claim

Posted 09 September 2021 | By [Joanne S. Eglovitch](#)

The US Food and Drug Administration (FDA) recently issued a [warning letter](#) to Kaleido Biosciences for administering an investigational new COVID-19 treatment to patients without obtaining an investigational new drug (IND) application.

The company argued that the product under investigation was a food and not a drug, and therefore not subject to IND

filing requirements. Yet the agency disagreed, saying that the endpoints used in the clinical trials went beyond 'dietary management' of COVID-19 and were more aligned with drug trials in assessing effectiveness of the treatment.

The 26 August warning letter was sent over "objectionable conditions" observed following a mid-February to early March inspection of Kaleido's Lexington, MA facility, conducted under the auspices of FDA's bioresearch monitoring program. At issue was the firm's clinical investigation of a molecule called KB109 for treating COVID-19. The treatment is a microbiome metabolic therapy, a synthetic glycan that is orally administered for treating COVID-19.

The warning letter focused on two protocols, Protocol K031-120, and Protocol K032-120. The first trial was a randomized, open-label prospective parallel group study to assess the natural history of COVID-19 and compare the effect of the treatment in patients undergoing supportive self-care (SSC). The other trial was an exploratory open-label clinical study to evaluate how the treatment affected the gut microbiota of adult patients with mild to moderate COVID-19.

Between the two multi-site trials, 399 patients were randomized.

Kaleido argued it was not obligated to submit an IND before initiating these clinical investigations because KB109 is a food rather than a drug. Specifically, Kaleido argued that the investigations were conducted "to evaluate the effect of KB109 on the microbiome" and to "determine the safety and tolerability of KB109 in the disease population," according to FDA's characterization of the firm's position.

These arguments failed to persuade FDA, which concluded that the "evidence collected during the inspection showed that KB109 was intended for use as a drug and not as a food."

FDA maintained that "even if there were distinctive nutritional requirements established for COVID-19, Protocols K031-120 and K032-120 went beyond 'dietary management' of COVID-19 to evaluate KB109's ability to treat COVID-19 by measuring the investigational product's effects on fever, oxygen saturation, viral load, and other biomarkers of COVID-19 infection, among other things."

Kaleido's argument that KB109 is a medical food fails on other counts as well, wrote FDA. Since no particular nutritional requirements that might help manage COVID-19 have yet been discovered, KB109 cannot be used in the dietary management of the disease, as would be required for a product classified as a medical food.

Also, the fact that an article has been established to be generally recognized as safe (GRAS) does not preclude its use as a drug. FDA clarified that "an article may be a drug as used in a clinical trial, even if it is considered a food as used in other contexts."

The company has 15 days to file a response.

Daniel Menichella, Kaleido's chief executive officer, told *Focus* in an email that "we are reviewing the letter currently and look forward to providing a response within the given timeframe and continuing our discussions with the FDA."

[Kaleido warning letter](#)

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Advocacy Group Files Lawsuit Seeking FDA Action on Propecia

September 10, 2021

A new lawsuit filed against the FDA this week contends that the male pattern baldness drug Propecia (1 mg) should either be taken off the market or its prescribing information should include warnings about the risk of long-term erectile dysfunction, depression and suicide.

The consumer watchdog group Public Citizen filed the suit Sept. 8 in the U.S. District Court for the District of Columbia on behalf of the Post-Finasteride Syndrome Foundation (PFSF). The complaint asks the FDA to act on a petition the foundation filed in 2017, which has gone unanswered.

The case has more merit than it did when originally filed, Philip Roberts, PFSF's patient manager, told *FDAnews*. "Much more scientific information has come to light since then," he said, including two studies by Harvard investigators confirming the risk of long-term erectile dysfunction, depression, suicidal ideation and suicide; and epidemiologic data showing that the drug alters the expression of more than 3,700 genes in men who take the drug. The drug mimics a male hermaphroditism gene; people who have this are genetic males who sometimes look female and who never go bald.

The 2017 petition asked FDA to either withdraw 1 mg oral Propecia from the market or include a Black Box warning and send out a "Dear Healthcare Provider" letter, alerting clinicians to the risks associated with the medication. The petition included information on about a dozen men whose suicides were considered related to Propecia use and contraindications for patients with a history of sexual dysfunction and depression.

Since then, PFSF has amassed more data, including 17,953 adverse event reports filed in the International Drug Monitoring database. These include reports of erectile and sexual dysfunction (5,234) and 4,313 reports of psychiatric disorders, including anxiety (1,756) and depression (1,386). The database includes 88 cases of completed suicides, 39 attempts and 384 cases of suicidal ideation.

In light of Propecia's enormous global market (almost 9 million users in 2018, according to the website ClinCalc.com), the numbers are very small. Nevertheless, they represent an unnecessary tragedy that needs to be addressed, said Michael Kilpatrick, the Public Citizen attorney who filed the suit.

"It's a small percentage, but the number of prescriptions is so high that it's still a large number of people," he told *FDAnews*. "We think patients should know this. It's a drug for a cosmetic issue. If people knew there was even a slight change you could develop a significant, life-altering syndrome and even think about suicide, they might not want to take it."

The FDA has been sitting on the matter far too long, he said.

"A four-year wait is unreasonable. Our goal is to compel FDA to issue a decision on the original petition. We think it's a strong one and that the decision should be for granting a black box warning or even withdrawing the drug. This delay is

in violation of the Administrative Procedure Act and given the seriousness of the issue and the quality of the petition, it should be resolved.”

Merck didn’t reply to a request for comment. The company has moved all of its Propecia patient and prescribing data to a new company spinoff, Organon, launched on June 3.

Read the PFSF complaint here: www.fdanews.com/09-09-21-Complaint.pdf. — Michele G. Sullivan

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Humanigen’s Lenzilumab for COVID-19 Draws FDA Complete Response Letter

September 10, 2021

The FDA has declined to grant Humanigen an emergency use authorization (EUA) for its antibody lenzilumab for treatment of hospitalized COVID-19 patients.

The agency instead sent the Burlingame, Calif.-based company a Complete Response Letter (CRL), in which it said it was “unable to conclude that the known and potential benefits of lenzilumab outweigh the known and potential risks of its use as a treatment for COVID-19,” Humanigen said.

But the agency also said in the letter that it is committed to working with Humanigen to develop lenzilumab and has invited the company to submit additional data as it becomes available, the company said.

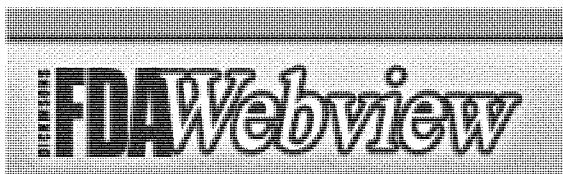
Humanigen CEO Cameron Durrant said the National Institutes of Health’s ACTIV-5/BET-B study, which is enrolling up to 500 patients, is expected to provide further data that may support a new EUA request. The company said it is also committed to completing the regulatory processes it has under way to seek a marketing authorization for lenzilumab to treat hospitalized COVID-19 patients in the UK and other territories.

Humanigen is focused on preventing and treating an immune hyper-response called ‘cytokine storm,’ which has been a major problem for critically ill COVID-19 patients. Lenzilumab neutralizes granulocyte-macrophage colony-stimulating factor (GM-CSF) that plays a role in inflammation that can lead cytokine storm.

The company said its phase 3 LIVE-AIR study “suggests early intervention with lenzilumab may prevent consequences of a full-blown cytokine storm in hospitalized patients with COVID-19.”

Humanigen is also studying the antibody for other inflammatory conditions such as acute graft versus host disease in patients undergoing allogeneic hematopoietic stem cell transplantation, eosinophilic asthma, and rheumatoid arthritis.
— Martin Berman-Gorvine

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Apellis Pharma Plan NDAs for Geographic Atrophy

09/09/2021

Apellis Pharmaceuticals says it plans to file an NDA in the first half of 2022 after reporting top-line results from the Phase 3 DERBY and OAKS studies evaluating intravitreal pegcetacoplan, an investigational targeted C3 therapy, in 1,258 adults with geographic atrophy secondary to age-related macular degeneration. Geographic atrophy is a leading cause of blindness that affects about one million people in the U.S.

In the studies, pegcetacoplan demonstrated a clinically meaningful slowing of disease progression with an even stronger effect in geographic atrophy patients with extrafoveal lesions, the company says. “Monthly and every-other-month treatment with pegcetacoplan met the primary endpoint in OAKS, significantly reducing [geographic atrophy] lesion growth by 22% ($p=0.0003$) and 16% ($p=0.0052$), respectively, compared to pooled sham at 12 months,” it says. “DERBY did not meet the primary endpoint, showing a reduction in GA lesion growth of 12% ($p=0.0528$) and 11% ($p=0.0750$) with monthly and every-other-month treatment, respectively, compared to pooled sham at 12 months.”

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Myfembree sNDA for Endometriosis Pain

09/09/2021

FDA has accepted for review a Myovant Sciences and Pfizer supplemental NDA for Myfembree (relugolix 40 mg, estradiol 1 mg, and norethindrone acetate 0.5 mg) for managing moderate to severe pain associated with endometriosis. The agency set a user fee review action target date of 5/6/2022.

The submission is based on data from the Phase 3 SPIRIT program, which included two multinational, replicate pivotal clinical studies (SPIRIT 1 and SPIRIT 2) in over 1,200 women with pain associated with endometriosis for 24 weeks, and an open-label extension study for eligible women through one year.

Endometriosis is an estrogen-dependent, inflammatory disease in which tissue similar to the uterine lining is found outside the uterine cavity, which results in chronic inflammation and can cause scarring and adhesions.

Myfembree is currently approved for managing heavy menstrual bleeding associated with uterine fibroids in premenopausal women, with a treatment duration of up to 24 months.

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New FDA Jardiance Breakthrough Therapy Designation

09/09/2021

FDA has granted breakthrough therapy designation to Boehringer Ingelheim and Lilly's Jardiance (empagliflozin) for treating heart failure with preserved ejection fraction. An *American Journal of Managed Care* online [post](#) says Jardiance is the first drug in its class to gain the designation. The post says results from the EMPEROR Preserved trial showed that Jardiance, a sodium glucose co-transporter 2 inhibitor reduced cardiovascular death and hospitalization for heart failure by 21%.

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FDA Rejects Lenzilumab EUA for Hospitalized Covid Patients

09/09/2021

FDA has rejected a Humanigen emergency use authorization (EUA) request for the use of its lenzilumab in newly-hospitalized Covid-19 patients. FDA reportedly told the company it was unable to conclude that the known and potential benefits of lenzilumab outweigh the known and potential risks of its use as a treatment for Covid-19.

The company [says](#) the agency committed to working with it in the development of lenzilumab and invited Humanigen to submit additional data as they become available. Humanigen says the National Institutes of Health ACTIV-5/BET-B study is expected to provide further data that may support a new EUA request.

Humanigen describes lenzilumab as a first-in-class antibody that binds to and neutralizes granulocyte-macrophage colony-stimulating factor.

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Congress Must Regulate CBD Since FDA Won't: Steve Mister

09/09/2021

Responding to an FDA objection to a new dietary ingredient notification submitted by Charlotte's Web to demonstrate that its full-spectrum hemp extract is reasonably expected to be safe, Council for Responsible Nutrition CEO **Steve Mister** says Congress must step in and regulate the CBD (cannabidiol) marketplace. Mister appeared with Charlotte's Web CEO **Deanie Elsner** in a "Short Legal Briefs" [video](#) posted by *Natural Products Insider*.

In the video, Elsner said she was disappointed in the way the agency made its decision to object to the notification for two reasons: that CBD products are excluded from the definition of a dietary supplement and that there were concerns about the sufficiency of the safety evidence.

She indicated that her company met multiple times with FDA officials before the notification was submitted to share data. The agency "ignored big parts of our application," she said.

Mister said the FDA denial letter showed that the agency is not going to solve the problem with CBD and thus Congress must act. He said the industry should turn its attention to persuading Congress to intervene. Regulation is needed, he said, because reputable companies that are concerned about the quality of their products are stuck in a "quagmire" with other companies that are not as concerned about quality, and consumers are being hurt.

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FiercePharma

No dice: Court tells AbbVie it can't force Takeda to supply a drug that's in shortage worldwide

by [Eric Sagonowsky](#) | Sep 9, 2021 9:28am

Amid the global shortage of prostate cancer drug Lupron, AbbVie took its partner Takeda to court over alleged failures to live up to supply agreements. Now, a court has denied the company's plea to force Takeda to fill orders.

In an [opinion](#) (PDF) this week, Delaware Chancery Court Vice Chancellor Sam Glasscock III denied AbbVie's request to compel Takeda to fill supply orders from its plant in Hikari, Japan.

Under a deal between the companies, AbbVie sells the drug in the U.S. and Canada. Because Takeda is the sole supplier of the drug worldwide, AbbVie relies on the Japanese drugmaker for its product.

Problem is, the med has been in short supply worldwide after the FDA and Takeda in 2019 found "protocol violations" at the plant, Glasscock wrote in this week's opinion. Thanks to "remediation efforts" including a weeks-long production hold global supply hiccups and shortages have persisted to this day, Glasscock wrote.

AbbVie had asked the court to find that Takeda breached its contract plus issue damages and "injunctive relief," or an order forcing Takeda to supply the drug. In his new opinion, Glasscock said an injunction would be "unworkable" because it'd force the court to oversee Takeda's operations and would "inevitably lead to contempt hearings" over Takeda's ability to fulfill orders.

"In other words, even if I find that Takeda has breached its contract with AbbVie and that, as a result, AbbVie faces irreparable harm, the injunctive relief sought is unavailable," Glasscock wrote.

The Lupron saga started in November 2019, when FDA inspectors traveled to Japan to review production processes at the Hikari site. In a June 2020 warning letter, the FDA slapped Takeda for poor equipment maintenance, faulty documentation and quality shortfalls. The company has been working to resolve those concerns, but the Lupron problem has dragged on throughout this year.

Right now, the FDA [lists](#) most Lupron Depot dose forms as "available" in the U.S., but there's one dose that's not expected to become available until next January.

While Glasscock decided against an injunction forcing Takeda to fill orders, he hasn't made a ruling on whether the company breached its contract. He plans to issue a decision on that issue in "due course," he said, and another trial phase on damages could follow.

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WCLC: Jazz charts new path for small cell lung cancer drug Zepzelca after late-stage flop

by [Angus Liu](#) | Sep 9, 2021 11:00am

Jazz Pharmaceuticals wasn't able to ride a breakthrough FDA nod to a pivotal trial win for conditionally approved small cell lung cancer (SCLC) drug Zepzelca. But after talking with the FDA, the company and partner PharmaMar see a new path forward.

In previously treated SCLC, the combination of Zepzelca and doxorubicin failed to extend patients' lives over physician's choice of doxorubicin-based chemo or Hycamtin, according to data presented at the 2021 World Conference on Lung Cancer virtual meeting.

Jazz and PharmaMar first unveiled the phase 3 ATLANTIS failure in December. Now we know patients who mostly got the Zepzelca regimen as their second-line treatment lived a median 8.6 months, versus 7.6 months in the control arm.

If successful, the trial could have served as the confirmatory trial to convert Zepzelca's accelerated approval earned last June into a full one. In a previous single-arm study, Zepzelca monotherapy shrunk tumors in 35% of relapsed SCLC patients as measured by investigator assessment. The response lasted a median 5.3 months.

Pascal Besman, chief operating officer at PharmaMar, noted that the ATLANTIS study used a 2-mg/m² dose of Zepzelca, which is lower than the FDA-approved dose of 3.2-mg/m². Although the Zepzelca-doxorubicin combo didn't improve survival, "the overall results support the activity and tolerability of Zepzelca monotherapy in second-line therapy," Besman said in an email interview.

As ATLANTIS investigator Luis Paz-Ares, M.D., Ph.D., noted in a prepared presentation, the research team ran a so-called exposure-response analysis to gauge Zepzelca's contribution on patient survival. The model predicted that single-agent Zepzelca, at the 3.2-mg/m² dose, would have had a statistically significant advantage over the control arm at prolonging the lives of patients in the ATLANTIS study.

Jazz stands "firmly behind the favorable benefit/risk profile of monotherapy Zepzelca" in second-line SCLC as shown in its phase 2 trial, the company's chief medical officer, Rob Iannone, M.D., said in a separate email interview.

For now, Zepzelca will remain on the market. Jazz and PharmaMar have agreed "in principle" with the FDA to run another phase 3 as the confirmatory trial, Besman said.

The three-arm trial will test Zepzelca monotherapy at 3.2 mg/m², and a second experimental arm will combine the drug at 2 mg/m² with Pfizer's Camptosar (irinotecan).

Second-line patients in the control arm will get physician's choice or either Camptosar or Hycamtin. The trial, expected to launch this year, will also support a regulatory filing to the European Medicines Agency, Besman added.

In addition, Jazz is collecting data from an observational phase 4 trial to understand Zepzelca's real-world profile in second-line SCLC, Iannone noted.

Meanwhile, Jazz is also looking to move Zepzelca earlier in the treatment line. Through a partnership with Roche, a phase 3 trial dubbed ImForte will test Zepzelca with immunotherapy as a first-line maintenance therapy for patients with extensive-stage SCLC after induction chemotherapy. If successful, the new regimen could pressure AstraZeneca and its Imfinzi, which is currently dominating the front-line SCLC space.

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WCLC: AstraZeneca's detailed Imfinzi, tremelimumab data in metastatic NSCLC are far from a clear-cut win

by [Angus Liu](#) | Sep 9, 2021 11:00am

After two high-profile late-stage trial flops, AstraZeneca has finally scored a victory for Imfinzi and ill-fated tremelimumab in newly diagnosed metastatic non-small cell lung cancer (NSCLC). But in a lucrative field where immuno-

oncology rivals Merck and Bristol Myers Squibb have established a solid presence, a detailed look at the data reveals a win that's perhaps too little, too late.

A cocktail of Imfinzi, investigational CTLA-4 inhibitor tremelimumab and chemotherapy pared down the risk of death by 23% over chemo alone in previously untreated patients, AZ announced Thursday at the 2021 World Conference on Lung Cancer virtual meeting.

The phase 3 POSEIDON trial came after two prior phase 3 flops for Imfinzi in metastatic NSCLC and marked the first positive pivotal readout for treme after many more failures in multiple indications. But the data don't look favorable compared to Merck's Keytruda or Bristol Myers' Opdivo, and efficacy in some patient subgroups showed imbalances that could limit an eventual FDA approval if AZ gets one.

First off, the combination of Imfinzi and chemo failed to significantly outdo chemo alone at extending patients' lives. That pairing only reduced the risk of death by 14% over chemo alone. That already makes Imfinzi look inferior to current standard-of-care Keytruda.

Keytruda's combo with chemo is well known for its impressive 51% death risk reduction over chemo alone in front-line non-squamous NSCLC. Those were the results at an interim analysis in the phase 3 Keynote-189 trial, while the final analysis at 31 months of follow-up showed a 44% risk decrease. As for front-line squamous cell NSCLC, Keytruda and chemo reduced death risk by 36% at interim and 29% at final analysis in the KEYNOTE-407 trial.

Luckily for AZ, treme finally pulled through to help Imfinzi. The CTLA-4 agent has a bad track record with Imfinzi and has been marred with failures in NSCLC as well as small cell lung, head and neck and bladder cancers. In front-line NSCLC alone, the dual immunotherapy combo of Imfinzi and treme flopped the phase 3 MYSTIC trial in a broad patient group and the NEPTUNE trial in patients with high tumor mutation burden.

Now that the Imfinzi-treme-chemo doesn't appear likely to shake up Keytruda's dominance, what about less competitive Opdivo? Data don't look very promising for AZ.

For one thing, while the chemo-free regimen of Imfinzi and treme failed, Opdivo's own CTLA-4 combo with Yervoy is approved for front-line NSCLC patients whose tumors express PD-L1.

For a better apple-to-apple comparison, the Opdivo-Yervoy-chemo combo won an FDA nod last year for front-line NSCLC patients regardless of PD-L1 status. In the CheckMate 9LA trial, Opdivo, Yervoy and chemo were able to show a survival benefit early, having cut patients' risk of death by 31% over chemo after a minimum follow-up of 8.1 months. After a longer follow-up of at least 24.4 months, the rate stood at 28%, as patients on the Opdivo-Yervoy-chemo regimen lived a median 15.8 months versus 11 months for the solo chemo group.

Imfinzi-treme-chemo's 23% death reduction looks slightly weaker than Opdivo-Yervoy-chemo's 28%, but cross-trial comparisons have their problems. In POSEIDON, patients on the AZ therapy lived a median 14 months, versus 11.7 months for those who took chemo alone.

To further complicate the front-line NSCLC landscape, Roche's PD-L1 inhibitor Tecentriq is allowed as a monotherapy for PD-L1-high patients thanks to data from the IMpower110 trial. And its combo with Avastin and chemo has an approval for non-squamous disease after showing it reduced the risk of death by 22% over Avastin and chemo.

Sanofi and Regeneron's Libtayo also bears a monotherapy nod in front-line PD-L1-high NSCLC. The companies recently found that adding Libtayo on top of chemo could further prolong the lives of first-line patients in the phase 3 EMPOWER-lung 3 trial.

Eli Lilly and Innovent Biologics have a front-line non-squamous NSCLC filing for their China-approved PD-1 inhibitor Tyvyt under review at the FDA. The application includes data from a China trial that showed a combination of Tyvyt, Lilly's Alimta and platinum chemo could stave off tumor progression or death over chemotherapy alone.

For AZ, non-squamous NSCLC might be just what it could eventually reach for with the Imfinzi-treme-chemo pairing.

As POSEIDON lead investigator Melissa Johnson, M.D., noted during a prepared presentation Thursday, among patients with the squamous histology, progression-free survival (PFS) "and overall survival were poor across all treatment arms, with little separation of the curves for PFS or overall survival."

In the squamous subgroup, Imfinzi, treme and chemo only managed to reduce the risk of death by 12% over chemo.

What's more, the AZ therapy also showed some worrisome signs in other patient subgroups. Notably, women and Asian patients didn't seem to experience any additional survival benefits from Imfinzi and treme.

The one upside? The efficacy of Imfinzi, treme and chemo didn't seem to be bound by a patient's PD-L1 expression level.

Still, with the mixed bag of data and a poor track record, AZ could face a limited FDA label and a hard time winning over market share in front-line NSCLC. Imfinzi is currently approved to treat stage 3 NSCLC and extensive-stage small cell lung cancer. In the first half of 2022, AZ plans to read out data from PEARL, an IMpower110 counterpart trial that evaluates Imfinzi monotherapy against chemo as front-line treatment for PD-L1-high patients.

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Biogen CEO blames Aduhelm's rocky launch on 'too much confusion, misinformation and controversy'

by [Eric Sagonowsky](#) | Sep 9, 2021 1:44pm

Before Biogen scored an FDA approval for Alzheimer's drug Aduhelm, the company probably hoped its launch would go much smoother than the last three months have been. It turns out Biogen's CEO Michel Vounatsos is willing to say so publicly.

Speaking at a Morgan Stanley healthcare [event](#) Thursday, Vounatsos said there's "clearly too much confusion, misinformation and controversy surrounding our data and the approval process." The launch has been "even more gradual than we had expected," he added.

The company is facing "near-term challenges," Vounatsos told the virtual audience, noting that "everybody can see that." Now, months after the drug's approval, he said it's "imperative" that "society moves its attention [from] the clinical data to the patients in need."

After Aduhelm's accelerated approval in June, a firestorm quickly engulfed the new Alzheimer's drug. Lawmakers and medical experts have questioned the data Biogen used to support an approval plus the FDA's procedures that led to the accelerated nod.

The criticism grew so intense that the FDA and Biogen, in an unusual move, **walked back** Aduhelm's broad label just weeks into the launch. The FDA originally endorsed the drug for all Alzheimer's patients but later restricted the label to those with mild cognitive impairment or mild dementia.

In another big setback for the rollout, the Department of Veteran's Affairs last month decided against covering the drug, citing a "risk of significant adverse drug events" and a "lack of evidence of a positive impact on cognition."

Looking forward, one key question mark for the rollout remains in the Centers for Medicare & Medicaid Services' coverage decision on the drug. That's expected in January and will determine whether federal insurance programs get behind the treatment.

Meanwhile, the company is working to improve the public's understanding of the clinical data and on generating additional data, Vounatsos said. It's also aiming to bring more treatment centers online and negotiate reimbursement deals to "maximize access," he added.

Biogen's drug launched with big sales expectations, but its initial stumbles have dampened some of the enthusiasm on Wall Street. In a note about Vounatsos' Thursday presentation, Jefferies analysts said the Street

is "very negative on the early launch." Analysts still expect the drug to bring in multiple billions of dollars per year once it gets rolling.

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Despite mixed phase 3 showing, Apellis hopes to push eye disorder drug past FDA's finish line

by [Noah Higgins-Dunn](#) | Sep 9, 2021 4:05pm

Apellis Pharmaceuticals' pegcetacoplan posted mixed results in two late-stage trials studying the C3 inhibitor in patients living with geographic atrophy (GA). Despite the mixed showing, CEO Cedric Francois thinks the med's performance was strong enough to warrant an FDA nod.

Apellis studied pegcetacoplan, the active ingredient in rare disease drug Empaveli, in two phase 3 trials, known as Oaks and Derby, as a monthly and every-other-month treatment in 1,258 patients with geographic atrophy (GA) secondary to age-related macular degeneration (AMD). For the studies, investigators weighed efficacy by measuring change in the total area of GA lesions from baseline.

In Oaks, pegcetacoplan met its primary endpoint in both dosing groups, significantly reducing GA lesion growth by 22% in the monthly arm and 16% in the every-other-month group compared with sham injections after 12 months, Apellis said.

The drug failed to do the same in Derby, reducing lesions by just 12% and 11%, respectively. The drug didn't produce any significant safety concerns in both trials, although its use was associated with slightly higher rates of exudation, or emitted fluid.

Despite the conflicting readouts, Apellis is "unbelievably happy" with the results given the totality of pegcetacoplan clinical trial performance, CEO Cedric Francois said in an interview. The company plans to file the med with the FDA in the first half of 2022.

There are no FDA-approved treatments for the condition, an advanced form of AMD that eventually leads to vision loss and blindness in older people.

"This is a drug that, in our opinion, has absolutely no doubt around its efficacy and comes out with a safety profile that I think exceeds our own and other people's expectations," Francois said.

Meanwhile, the drug posted another late-stage upside, Francois argued. In a prespecified analysis of the combined trials, pegcetacoplan was more effective in patients with extrafoveal lesions at the start of the trials, decreasing GA lesion growth in that group by as much as 26% in the monthly arm and 23% in the every-other-month group.

GA patients typically present first with extrafoveal lesions, which then progress toward the fovea where central vision is impacted, Apellis says. That's important because the disease is like a forest fire in the retina, continuously destroying photoreceptor cells until someone's blind with no way to turn it around, Francois said.

There are roughly 1 million Americans living with the condition and 5 million people globally. If Apellis is able to do what others have failed, it could be looking at \$3 billion in peak sales, Jefferies analysts recently **predicted**.

Pharma has tried and failed to push GA treatments over the FDA's finish line in recent years, including an effort by Roche on its complement-inhibiting drug lampalizumab. That drug, however, **crashed** out of two phase 3 trials in 2017.

This has already been a busy year for Apellis, which **scored** its first-ever FDA nod in May for pegcetacoplan in patients with the rare blood disorder paroxysmal nocturnal hemoglobinuria (PNH). That has teed-up a showdown between Apellis' C3 inhibitor and Alexion's heavyweight C5 inhibitors Soliris and its follow-up Ultomiris, which target a different enzyme.

“If this drug gets approved, it will be the first time that complements control would get approved in a large speciality, rather than in a rare disease like PNH,” Francois said. “I think it opens up a new era for what a complement can do in many of these conditions across multiple therapeutic areas.”

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Room Available In US Pharma Industry For OTC Switch Leader: Has Perrigo Made A Reservation?

09 Sep 2021

ANALYSIS

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Executive Summary

Acquiring HRA Pharma, Perrigo could take OTC switch lead, likely starting with HRA's daily oral contraceptive before moving to numerous other potential candidates. Pharmas previously busy in switch have other priorities for their consumer health businesses.

The US pharma industry has a vacancy for an OTC switch leader as longtime sponsors of applications to make additional ingredients available nonprescription prioritize reorganizing their consumer health businesses over expanding their product portfolios.

By acquiring HRA Pharma, [Perrigo Company PLC](#) could fill the space in the pharma industry. Its switch proposals likely will start with HRA's daily oral contraceptive before moving to numerous other potential candidates.

“There aren't a lot of switch powerhouses out there. I think there's an opportunity for a new player to emerge as a switch powerhouse, given the changing market dynamics,” says pharma industry consultant Susan Levy .

“I think this alignment, HRA with Perrigo's scale, is just what the industry needs,” said switch consultant Susan Lavine Coleman, president of NCI Consulting in Moorestown, NJ.

Levy and Coleman spoke with HBW Insight following Perrigo's 8 September announcement of an agreement to acquire Paris-based HRA, listed in France as Héra SAS, for €1.8bn (\$2.1bn) in a cash deal.

Perrigo's predominant revenue stream has been private label/store brand OTC drugs, although it has added brands with its 2014 move into Europe's consumer health market and by acquiring the Prevacid 24 HR (lansoprazole/15 mg) proton pump inhibitor in 2019 and licensing the Burt's Bees brand in May to make and market a line of organic baby formulas and supplements for babies and children.

"I think there's an opportunity for a new player to emerge as a switch powerhouse, given the changing market dynamics." – Susan Levy

But while it consistently is first to launch generic equivalents for brand switches, its name hasn't been linked to submitting proposals for making additional drug ingredients available OTC.

Bringing HRA, including management, under its roof changes the switch outlook for Perrigo.

“These are pros. They know what they're doing,” Perrigo president and CEO Murray Kessler said about HRA’s management during a briefing about the deal. “They've all agreed to stay on with Perrigo.”

Slow Time For Switch NDAs

The HRA acquisition isn’t the only reason Perrigo could become a top OTC switch sponsor in the US. Other pharmas are leaving the top spot open.

Looking over the current lineup of major OTC marketers in the US, Levy, founder and principal of Susan B. Levy Consulting LLC in Cranford, NJ, doesn’t see any interested in sponsoring new drug applications for OTC switches.

“If you look at who has historically been a driver for OTC switches, you've got GSK, Novartis and Pfizer, but they're all one [consumer health] company now, and it's being spun out. It's kind of unclear to what extent they are positioned right now to invest in expensive growth programs,” she said.

GlaxoSmithKline plc is the majority owner of a joint venture with Pfizer Inc. marketing the two firms’ consumer health product lines. GSK, with products including brands it acquired after previously operating a consumer health JV with Novartis AG, plans to spin out its current consumer health business. (Also see "McNamara To Lead GSK’s Standalone Consumer Healthcare Business" - HBW Insight, 23 Jul, 2021.)

“Then you've got Sanofi,” Levy added.

The French firm is known to be considering proposing switches for flu treatment Tamiflu (oseltamivir) and erectile dysfunction drug Cialis (tadalafil), but it too looks to separate its consumer health business. (Also see "Sanofi To Divest 60% Of Brands In Consumer Healthcare Overhaul" - HBW Insight, 10 Feb, 2021.)

“It's unclear where they want to put their bets right now as a result of being spun out independently,” she said.

Bayer AG earlier in 2021 received Food and Drug Administration approval for OTC sales of Astepro Allergy (azelastine hydrochloride nasal spray, 205.5 mcg per spray). Bayer plans to launch the first-in-class switch, the first ingredient available OTC that is a first-generation H1 (histamine receptor inhibitor) antihistamine, in 2022. (Also see "Market Exclusivity Decision Pending For Bayer’s First-In-Class OTC Switch Astepro Allergy" - HBW Insight, 24 Jun, 2021.)

Levy pointed out the previous OTC switch for a product the German firm markets in the US was Oxytrol for Women overactive bladder treatment, a first-in-class switch when it was approved in 2013 when US firm Merck & Co., Inc. owned the brand. After acquiring Merck & Co.’s consumer business in 2014, Bayer relinquished OTC rights to the 3.9mg oxybutynin transdermal patch to Allergan PLC less than a year later.

J&J Large In Consumer, Not In Switch

Johnson & Johnson may be synonymous with OTC drugs due to its market-leading brands, but like Perrigo it hasn’t been a switch driver.

“Who could be the powerhouses? ... J&J has not historically been a switch player. They don't have an experienced team of switch professionals,” Levy said.

“It speaks to the fact that there is a gap in the marketplace because these switch programs are very expensive for an OTC company. They are somewhat risky, and they're much longer-term than typical OTC innovation. ... If you're heading up a consumer health care company and thinking about where you're going to put your R&D bets, much more incentives are there to do some of the shorter-term, easier innovation.”

The FDA is encouraging firms to study making available nonprescription drug ingredients indicated for chronic conditions; in 2012 it opened a docket for recommendations on changing its OTC switch NDA rules to allow for “novel switches” targeting chronic conditions, indications more complicated than most currently available OTC drugs have.

The agency launched the initiative expecting labeling for novel switches to be subject to the same limits as other OTCs, with all necessary information available on a product’s Drug Facts label. However, it has since indicated

in a 2018 guidance that switch sponsors could tap into digital media and other extra-label tools to help demonstrate that consumers can assess whether an OTC drug is appropriate for them and they can use the product safely. (Also see "[Before Asking FDA For Novel OTC Switch, Be Sure DFL Alone 'Can't Get There'](#)" - HBW Insight, 23 May, 2019.)

In a recent FDA workshop conducted online, industry experts and other stakeholders said some problems with OTC DFLs are worse than others and some likely can be corrected with minor changes to the format, but other changes, notably for supporting OTC labeling for drugs that currently are available Rx-only and are indicated for chronic conditions aren't minor and won't happen within the limits of one-dimensional text media printed on nonprescription drug containers, packages and inserts. (Also see "[Progress On Adding OTC Drugs To US Market? Room To Move But Label Format Limits Options](#)" - HBW Insight, 18 Jun, 2021.)

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Sept. 9 Quick Takes: Obsidian readies for IPO with \$115M crossover round

PLUS: VENTURE ROUNDS FOR LEXEO, EVOMMUNE, ALAMAR, AND SETBACKS FOR HUMANIGEN, SANOFI AND MORE

BY BIOCENTURY STAFF

SEP 9, 2021 | 9:44 PM EDT

Cell and gene therapy play Obsidian Therapeutics Inc. looks set to seek a public listing after raising \$115 million in a series B round led by The Column Group Crossover Fund. Other new investors included RA Capital, Surveyor Capital, Cowen Healthcare Investments, Deep Track Capital, Logos Capital, Pivotal BioVenture Partners, [Samsara BioCapital](#) and Soleus Capital. Existing investors Atlas Venture, Vertex Ventures HC, Amgen Ventures, Bristol Myers Squibb Co. (NYSE:BMJ) and Vertex Pharmaceuticals Inc. (NASDAQ:VRTX) also participated. Obsidian's lead tumor infiltrating lymphocyte (TIL) program, cytoTIL15, is expected to enter the clinic next year.

\$100M SERIES B TO SUPPORT LEXEO'S GENE THERAPIES

Adding to an \$85 million January [series A](#), [Lexeo Therapeutics](#) raised \$100 million in a series B led by D1 Capital Partners and Eventide Asset Management. Also backing the round were CAM Capital, Verition Fund Management, Laurion Capital Management, Gray's Creek Capital Partners, and existing investors Longitude Capital, Omega Funds, Lundbeckfonden Ventures, PBM Capital, Janus Henderson Investors, Woodline Partners LP, Invus Capital and Alexandria Venture Investments. The company has three lead AAV-mediated gene therapy programs: LX2006, which is in preclinical testing for cardiomyopathy associated with Friedreich's ataxia, and LX1004 for CLN2 Batten disease and LX1001 for APOE4-associated Alzheimer's disease, both of which are in Phase I/II testing. The company is also developing recently acquired cardiac gene therapies from Stelios Therapeutics Inc. and additional preclinical programs targeting Alzheimer's disease.

EVOMMUNE RAISES \$83M IN SERIES A

[Evommune Inc.](#), which was founded in 2020 by Dermira Inc. alums, raised \$83 million in series A round with participation from Andara Partners, LSP, global syndicate of investors and Pivotal BioVenture Partners, the seed investor, to enable development of therapies to treat inflammatory diseases through industry and academic collaborations. Evommune has exclusive rights to three of the four candidates in its pipeline, from Dermira, now a Eli Lilly and Co. (NYSE:LLY) company.

SHERPA LEADS ALAMAR'S \$80M SERIES B

An \$80 million series B round led by Sherpa Healthcare Partners will enable Bay Area-based proteomics company Alamar Biosciences Inc. to accelerate the development of its NULISA and Attobody technology platforms to deliver single-digit attomolar detection sensitivity. NULISA (NUcleic acid-Linked Immuno-Sandwich Assay) seeks to provide "ultra-high sensitivity

and massively parallel scale” to proteomic analysis, while Attobody, the company says, yields highly specific picomolar affinity antibodies. Other firms participating in the round included new investors Morningside Ventures and Samsara Biocapital and existing investors Qiming Venture Partners, which lead the series A, and Illumina Ventures. The company has now raised \$110 million.

LILLY TAKES EQUITY STAKE IN LYCIA AS PART OF \$70M SERIES B

New Lycia Therapeutics, Inc. partner Eli Lilly and Co. (NYSE:LLY) has invested in the South San Francisco company’s \$70 million series B, which Redmile Group led. Founding investor Versant Ventures and new investors Cowen Healthcare Investments, Invus, RTW Investments and Alexandria Venture Investments also participated. The financing comes two weeks after Lycia picked Lilly as its collaborator to develop LYTACs against targets implicated in the pharma’s areas of focus.

FIRST APPROVAL FOR PFIZER’S ORAL ATOPIC DERMATITIS THERAPY COMES IN U.K.

The U.K.’s MHRA approved Cibirgo abrocitinib from Pfizer Inc. (NYSE:PFE) to treat atopic dermatitis, marking the first global approval for the JAK1 inhibitor. FDA pushed back the therapy’s PDUFA date due to a class-wide safety review. Last month, the oral therapy demonstrated superiority to leading atopic dermatitis therapy Dupixent dupilumab in the Phase III JADE DARE trial.

CENTESSA REPORTS FIRST POSITIVE DATA READOUT SINCE FORMATION

Asset-centric play Centessa Pharmaceuticals plc (NASDAQ:CNTA) had its first positive data readout since the company was formed this year by Medicxi. Centessa subsidiary ApcinteX Ltd. said that in the Phase IIa portion of a Phase I/IIa trial, the high dose of SerpinPC over six months led to an 88% reduction in annualized bleeding rate compared with a pre-exposure observation period in patients with either hemophilia A or hemophilia B. The PROC inhibitor was well tolerated with no cases of sustained elevation of D-dimer, a marker of excess thrombin generation. Data from an open-label extension of the study are expected next year. Centessa rose \$1.21 to \$22.26

HUMANIGEN SINKS AFTER FDA DECLINES TO ISSUE EUA

Shares of Humanigen Inc. (NASDAQ:HGEN) shed nearly half their value Thursday after the biotech said FDA declined to grant an emergency use authorization for lenzilumab, its mAb targeting GM-CSF, to treat hospitalized patients with COVID-19. Humanigen said FDA could not yet conclude that lenzilumab’s benefits outweigh its risks, but the biotech believes the NIH-funded ACTIV-5/BET-B study may generate data sufficient to support the mAb’s emergency authorization. Humanigen lost \$7.14 (47%) to \$7.97 on Thursday.

ACQUIRED IN PRINCIPIA DEAL, SANOFI’S BTK MISSES IN PEMPHIGUS

Sanofi (Euronext:SAN; NASDAQ:SNY) said Btk inhibitor rilzabrutinib failed to significantly improve remission rates in the Phase III PEGASUS trial to treat pemphigus, missing the study’s primary endpoint. The pharma gained the molecule via its \$3.4 billion buyout of Principia Biopharma Inc. (NASDAQ:PRNB) last year. Rilzabrutinib is also in a Phase III study to treat chronic immune thrombocytopenia.

AZ-AVILLION COMBO MEETS MARK IN ASTHMA READOUTS

AstraZeneca plc (LSE:AZN; NYSE:AZN) and Avillion LLP said PT027, the partners’ fixed-dose combination of albuterol and budesonide, met the primary endpoints in the Phase III DENALI and MANDALA trials to treat asthma. The treatment significantly improved lung function compared with each of its components in DENALI, and significantly reduced exacerbations compared with albuterol alone as a rescue medicine in MANDALA. Blackstone Life Sciences, Abingworth and Royalty Pharma plc (NASDAQ:RPRX) are backing the PT027 development program in a risk-sharing arrangement.

POSITIVE READOUT FOR A SUBCUTANEOUS GENMAB BISPECIFIC

Epcoritamab, a subcutaneously administered CD3 x CD20 bispecific antibody from Genmab A/S (CSE:GMAB; NASDAQ:GMAB), led to an 88% ORR in relapsed or refractory diffuse B cell lymphoma patients, providing support that the formulation may compete with more burdensome IV infusions on efficacy. Among eight patients who received the recommended Phase II dose of 48 mg, 38% achieved a complete response. ORR was 90% and CR was 50% in the 10 follicular lymphoma patients treated across dose ranges in the dose-escalation study. There was no Grade 3 or higher cytokine release syndrome.

Odronextamab (REGN1979), a CD3 x CD20 bispecific from Regeneron Pharmaceuticals Inc. (NASDAQ:REGN), led to an ORR of 92.9% in follicular lymphoma patients in a Phase I readout last year, though a partial clinical hold was placed on the program in December due to incidence of Grade 3 and higher CRS during step-up dosing.

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Cancer leads way to routine use of accelerated approval: Data Byte

AN ANALYSIS FROM BIOCENTURY'S 2021 BACK TO SCHOOL PACKAGE

BY SELINA KOCH, EXECUTIVE EDITOR

SEP 9, 2021 | 7:08 PM EDT

Accelerated approval is becoming the *de facto* route for approvals of new molecular entities in cancer.

In three of the past five years, the number of new cancer drugs granted accelerated approval by FDA has outnumbered those given full approval. Halfway through this year, the count stood at eight accelerated to four standard approvals.

BioCentury's annual Back to School project explored cancer as a case study of the promise and problems of accelerated approval, and argued for changes that could usher in routine use of the pathway for all serious diseases with unmet needs.

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COVID-19

Pfizer Says Its Antiviral Pill Reduces Hospitalizations, Deaths Among High-Risk Patients With COVID-19 By 89%. [[HYPERLINK](#)

"<https://www.bloomberg.com/news/articles/2021-11-05/pfizer-to-look-for-covid-pill-after-strong-results?srnd=premium&sref=3IOEzKok>"] (11/5, Langreth, 3.57M) reported Pfizer on Friday "said its Covid-19 pill [Paxlovid] reduced hospitalizations and deaths in high-risk patients by 89%, a result that has the potential to upend how the disease caused by the coronavirus is treated and alter the course of the pandemic." The drugmaker "said...it was no longer taking new patients in a clinical trial of the treatment 'due to the overwhelming efficacy' and planned to submit the findings to U.S. regulatory authorities for emergency authorization as soon as possible." [[HYPERLINK](#) "<https://www.reuters.com/business/healthcare-pharmaceuticals/pfizer-says-antiviral-pill-cuts-risk-severe-covid-19-by-89-2021-11-05/>"] (11/5, Beasley) said the findings "suggest that Pfizer's drug surpasses Merck & Co Inc's pill, molnupiravir, which was shown last month to halve the chance of dying or being hospitalized for COVID-19 patients at high risk of serious illness." The [[HYPERLINK](#) "<https://www.nytimes.com/2021/11/05/health/pfizer-covid-pill.html>"] (11/5, A1, Robbins, 20.6M) reported Merck's drug "was about 50 percent effective when given within five days of the onset of symptoms, though the different designs and timing of the Pfizer and Merck trials make such comparisons imprecise."

According to the [[HYPERLINK](#) "<https://www.wsj.com/articles/pfizer-says-covid-19-pill-is-89-effective-in-preliminary-assessment-11636109100>"] (11/5, A1, Hopkins, Subscription Publication, 8.41M), Pfizer will ask the FDA this month to authorize the drug's use. The [[HYPERLINK](#) "<https://apnews.com/article/coronavirus-pandemic-science-business-health-medication-eeee3c6a5f6428479e35bc1bcd7aec52>"] (11/5, Perrone) reported that "once Pfizer applies, the FDA could make a decision within weeks or months," and the [[HYPERLINK](#) "<https://www.washingtonpost.com/health/2021/11/05/pfizer-covid-pill/>"] (11/5, A1, Johnson, 10.52M) said if the FDA finds the therapy is "safe and effective," they "are expected to become available right away." [[HYPERLINK](#) "<https://www.usatoday.com/story/news/health/2021/11/05/pfizer-antiviral-therapy-prevents-covid-deaths-drug-study/6284097001/>"] (11/5, Weintraub, 12.7M) said, "An FDA advisory committee is scheduled to meet later this month to discuss the companies' request for an emergency use authorization for molnupiravir."

On [[HYPERLINK](#) "<https://playvideo.bulletinintelligence.com/c9f76b755f9d44d49d40989a700b08cf?pubid=fda>"] (11/5, story 2, 2:06, Muir, 5.81M), Whit Johnson hailed Pfizer's pill as "a potential new weapon in the fight against COVID." Johnson added, "Unlike monoclonal antibody treatments, which are given through an IV at a hospital or clinic, the antiviral pills could be taken at home over five days soon after a person tests positive for COVID. Merck's drug is on track for potential authorization by the end of the year, Pfizer's by early 2022." Gabe Gutierrez reported on [[HYPERLINK](#) "<https://playvideo.bulletinintelligence.com/e72001040e814c7b8c540e7b267b237d?pubid=fda>"] (11/5, story 3, 1:44, Holt,

4.81M) that patients would take Pfizer's pill within three days of symptoms. [[HYPERLINK "https://www.cnbc.com/2021/11/05/pfizer-says-its-covid-pill-with-hiv-drug-cuts-the-risk-of-hospitalization-or-death-by-89percent.html"](https://www.cnbc.com/2021/11/05/pfizer-says-its-covid-pill-with-hiv-drug-cuts-the-risk-of-hospitalization-or-death-by-89percent.html)] (11/5, Lovelace, 7.34M) reported on its website that Pfizer's pill, "scientifically known as PF-07321332, is part of a class of medicines called protease inhibitors and works by inhibiting an enzyme the virus needs to replicate in human cells."

According to [[HYPERLINK "https://abcnews.go.com/Health/pfizers-covid-19-pill-reduces-risk-hospitalized-dying/story?id=80979366"](https://abcnews.go.com/Health/pfizers-covid-19-pill-reduces-risk-hospitalized-dying/story?id=80979366)] (11/5, Salzman, 2.44M), Pfizer's findings come "from one of three clinical trials that the company is running. The results from the other two trials are expected by the end of the year. Pfizer then plans to submit all the data and seek authorization at that time, meaning the new medication may be available in early 2022." [[HYPERLINK "https://www.cnbc.com/2021/11/05/pfizer-says-its-covid-pill-with-hiv-drug-cuts-the-risk-of-hospitalization-or-death-by-89percent.html"](https://www.cnbc.com/2021/11/05/pfizer-says-its-covid-pill-with-hiv-drug-cuts-the-risk-of-hospitalization-or-death-by-89percent.html)] (11/5, Lovelace, 7.34M) reported, "Pfizer said there were six hospitalizations and zero deaths out of the 607 trial participants who received the pill in combination" with an antiviral drug called ritonavir. That contrasts "with 41 hospitalizations and 10 deaths out of the 612 people who received a placebo." [[HYPERLINK "https://www.cnn.com/2021/11/05/health/pfizer-covid-antiviral-pill/index.html"](https://www.cnn.com/2021/11/05/health/pfizer-covid-antiviral-pill/index.html)] (11/5, Fox, Sealy, 89.21M) reported Pfizer "said 19% of patients given the treatment suffered adverse events, compared to 21% who got placebo, but declined to disclose what those adverse events were."

However, [[HYPERLINK "https://www.axios.com/pfizer-pill-cuts-covid-death-hospitalization-risk-e79e0254-7f41-4b51-a963-fe6db2da4e45.html"](https://www.axios.com/pfizer-pill-cuts-covid-death-hospitalization-risk-e79e0254-7f41-4b51-a963-fe6db2da4e45.html)] (11/5, Falconer, 1.26M) says the announcement comes with "some caveats. Pfizer's findings came through a corporate press release, not a peer-reviewed journal, and federal regulators haven't had a chance to scrutinize the drug's clinical trial data yet."

According to [[HYPERLINK "https://thehill.com/policy/healthcare/580215-pfizer-antiviral-pill-cuts-risk-of-coronavirus-death-hospitalization-by-89"](https://thehill.com/policy/healthcare/580215-pfizer-antiviral-pill-cuts-risk-of-coronavirus-death-hospitalization-by-89)] (11/5, Vakil, 5.69M), President Biden's "chief medical adviser Anthony Fauci has previously warned that the Merck drug should not be considered an alternative to the COVID-19 vaccine, it could help alleviate the possibility of escalating COVID-19 cases." Meanwhile, [[HYPERLINK "https://www.bloomberg.com/news/articles/2021-11-05/pfizer-to-submit-covid-pill-for-authorization-by-thanksgiving?smd=premium"](https://www.bloomberg.com/news/articles/2021-11-05/pfizer-to-submit-covid-pill-for-authorization-by-thanksgiving?smd=premium)] (11/5, Griffin, 3.57M) reported Pfizer CEO Albert Bourla on Friday "said Pfizer hasn't yet disclosed how it will price the antiviral pill but said it would cost less than Covid-19 antibody treatments."

Additional outlets reporting included: [[HYPERLINK "https://playvideo.bulletinintelligence.com/1d4622677c794ee3"](https://playvideo.bulletinintelligence.com/1d4622677c794ee3)

<a110a239d482b94e?pubid=fda>] (11/5, story 4, 0:34, O'Donnell, 3.62M), [[HYPERLINK "https://www.nbcnews.com/health/health-news/pfizers-covid-antiviral-pill-may-cut-severe-illness-89-percent-rcna4593"](https://www.nbcnews.com/health/health-news/pfizers-covid-antiviral-pill-may-cut-severe-illness-89-percent-rcna4593)] (11/5, Edwards, 4.91M), [[HYPERLINK "https://www.npr.org/sections/coronavirus-live-updates/2021/11/05/1052679112/pfizer-covid-pill-treatment"](https://www.npr.org/sections/coronavirus-live-updates/2021/11/05/1052679112/pfizer-covid-pill-treatment)] (11/5, Chappell, 3.69M), [[HYPERLINK "https://news.bloomberglaw.com/pharma-and-life-sciences/pfizer-to-submit-covid-pill-for-authorization-by-thanksgiving"](https://news.bloomberglaw.com/pharma-and-life-sciences/pfizer-to-submit-covid-pill-for-authorization-by-thanksgiving)] (11/5, Subscription Publication, 4K), the [[HYPERLINK "https://www.bostonherald.com/2021/11/05/pfizer-coronavirus-pill-cuts-risk-of-hospitalization-or-death-by-89-studies-show/"](https://www.bostonherald.com/2021/11/05/pfizer-coronavirus-pill-cuts-risk-of-hospitalization-or-death-by-89-studies-show/)] (11/5, Cohan, 327K), [[HYPERLINK "https://www.forbes.com/sites/alexknapp/2021/11/05/pfizer-says-its-covid-pill-reduced-risk-of-hospitalization-and-death-by-89-in-clinical-study/"](https://www.forbes.com/sites/alexknapp/2021/11/05/pfizer-says-its-covid-pill-reduced-risk-of-hospitalization-and-death-by-89-in-clinical-study/)] (11/5, Knapp, 10.33M), [[HYPERLINK "https://www.newsweek.com/pfizer-covid-pill-prevents-89-percent-hospitalizations-show-it-compares-mercks-1646331"](https://www.newsweek.com/pfizer-covid-pill-prevents-89-percent-hospitalizations-show-it-compares-mercks-1646331)] (11/5, 2.67M), the [[HYPERLINK "https://www.nydailynews.com/coronavirus/ny-covid-pfizer-pill-cuts-hospitalization-death-20211105-as7ff524evg2hwmwpotjlo3ejie-story.html"](https://www.nydailynews.com/coronavirus/ny-covid-pfizer-pill-cuts-hospitalization-death-20211105-as7ff524evg2hwmwpotjlo3ejie-story.html)] (11/5, Schladebeck, 2.51M), the [[HYPERLINK "https://r.bulletinintelligence.com/56fd83efe4074ba0aaec8a9e0381e7ad"](https://r.bulletinintelligence.com/56fd83efe4074ba0aaec8a9e0381e7ad)] (11/5, Feuer, 7.45M), [[HYPERLINK "https://www.statnews.com/2021/11/05/experimental-pfizer-pill-prevents-covid-hospitalizations-and-deaths/"](https://www.statnews.com/2021/11/05/experimental-pfizer-pill-prevents-covid-hospitalizations-and-deaths/)] (11/5, Herper, 262K), [[HYPERLINK "https://www.usnews.com/news/health-news/articles/2021-11-05/pfizer-covid-19-antiviral-pill-reduces-hospitalization-death-by-nearly-90"](https://www.usnews.com/news/health-news/articles/2021-11-05/pfizer-covid-19-antiviral-pill-reduces-hospitalization-death-by-nearly-90)] (11/5, 1.91M), [[HYPERLINK "https://www.science.org/content/article/pfizer-antiviral-slashes-covid-19-hospitalizations"](https://www.science.org/content/article/pfizer-antiviral-slashes-covid-19-hospitalizations)] (11/5, Couzin-Frankel, 484K), [[HYPERLINK "https://www.fiercebiotech.com/biotech/pfizer-s-oral-covid-19-antiviral-cuts-hospitalization-death-by-85-sending-team-barreling-to"](https://www.fiercebiotech.com/biotech/pfizer-s-oral-covid-19-antiviral-cuts-hospitalization-death-by-85-sending-team-barreling-to)] (11/5, Taylor, 4K), and the [[HYPERLINK "https://www.ft.com/content/848cce54-0d47-4488-b829-5539abddcf6e?FTCamp=engage/CAP/webapp/Channel_Bulletin/B2B"](https://www.ft.com/content/848cce54-0d47-4488-b829-5539abddcf6e?FTCamp=engage/CAP/webapp/Channel_Bulletin/B2B)] (11/5, Kuchler, Subscription Publication, 1.48M).

Administration To Defend OSHA COVID-19 Vaccine Requirement For Business. [[HYPERLINK](#)

["https://playvideo.bulletinintelligence.com/d9818bac82d142bb825eb646b2e073b2?pubid=fda"](https://playvideo.bulletinintelligence.com/d9818bac82d142bb825eb646b2e073b2?pubid=fda)] (11/7, 6:37 p.m. EST, story 2, 2:00, Johnson, 3.92M) reported, "Tonight, the Administration firing back after an appeals court blocked the requirement, questioning its constitutionality. ABC's Phil Lipof reports." ABC (Phil Lipof) added, "And tonight, the White

House is not backing down as the Justice Department vowing to defend the Biden Administration's vaccine mandate for all businesses with 100 employees or more, after a federal appeals court blocked it this weekend, questioning its constitutionality." Dr. Vivek Murthy, US Surgeon General, said, "It's good for people's health, it's good for the economy. That's why these requirements make so much sense."

The [HYPERLINK "https://www.nytimes.com/2021/11/07/health/biden-oshavaccine-mandate.html"] (11/7, Imbler, 20.6M) reports that Surgeon General Murthy said that the Administration will defend the rules. Appearing on ABC's "This Week," Dr. Murthy said, "The president and the administration wouldn't have put these requirements in place if they didn't think that they were appropriate and necessary." The Times also reports that White House chief of staff Ron Klain "said he was 'quite confident' the mandate would be upheld in an interview on NBC's 'Meet the Press.'"

[HYPERLINK "https://thehill.com/homenews/sunday-talk-shows/580443-white-house-chief-of-staff-confident-vaccine-or-test-mandate-will"] (11/7, Vakil, 5.69M) reports on Sunday that Klain, appearing on NBC's "Meet The Press," said, "I'm quite confident that when this finally gets fully adjudicated, not just a temporary order, the validity of this requirement will be upheld. It's common sense...if OSHA can tell people to wear a hard hat on the job, to be careful around chemicals, it can put in place these simple measures to keep our workers safe."

[HYPERLINK "https://www.reuters.com/world/us/federal-appeals-court-issues-stay-bidens-vaccine-rule-us-companies-2021-11-06/"] (11/6, Scarcella) reports Solicitor of Labor Seema Nanda "said the Labor Department was 'confident in its legal authority' to issue the rule," as does the [HYPERLINK "https://apnews.com/article/appeals-court-stays-vaccine-mandate-coronavirus-biden-b235b6fa858bd23b02ab5f9c009428db"] (11/6), which also quotes Justice Department spokesman Anthony Coley as saying, "The OSHA emergency temporary standard is a critical tool to keep America's workplaces safe as we fight our way out of this pandemic. The Justice Department will vigorously defend this rule in court."

In addition, [HYPERLINK "https://www.foxbusiness.com/politics/federal-court-of-appeals-issues-temporary-halt-to-biden-vaccine-mandate"] (11/6, 3.06M) reports on its website that White House Deputy Press Secretary Karine Jean-Pierre "responded to a question at a Friday press briefing about whether or not she is confident the mandate will stand up to legal challenges." She said, "We are very confident that it can." She continued, "As for the legal side of this, let me be crystal clear to avoid what appears to be possible misinformation or disinformation around the emergency temporary standard being a vaccine mandate. That would be on its face incorrect as has been

explicit for months. It is a standard for safe workplace to either comply with weekly testing or to be vaccinated."

The [HYPERLINK "https://nypost.com/2021/11/05/white-house-calling-vaccine-policy-mandate-is-misinformation/"] (11/5, Nelson, 7.45M) reported that "Jean-Pierre on Friday said that it's 'misinformation' to use the term 'vaccine mandate' to describe a requirement that most private-sector workers get vaccinated, submit to regular testing or lose their jobs." Instead, she called it a "vaccination requirement."

[HYPERLINK "https://www.axios.com/appeals-court-issues-stay-vaccine-rule-us-companies-6cd05a8a-7a5e-4124-8cd7-8794e1d26389.html"] (11/6, 1.26M) and the [HYPERLINK

"https://www.washingtontimes.com/news/2021/nov/7/white-house-says-ruling-against-vaccine-mandate-st/"] (11/7, 626K) also covered the story.

Fifth Circuit Issues Stay Of OSHA COVID-19 Vaccine Requirement For Businesses. The [HYPERLINK "https://www.washingtonpost.com/business/2021/11/06/court-suspends-biden-vaccine-mandate/"] (11/6, Rosenberg, 10.52M) reported "a panel of three judges appointed by Republican presidents" of the US Court of Appeals for the Fifth Circuit on Saturday "suspended the Biden administration's new vaccine requirement for private companies, delivering a major blow for one of the White House's signature attempts to increase the number of vaccinations to corral the pandemic."

The [HYPERLINK "https://www.nytimes.com/live/2021/11/06/world/covid-booster-vaccine"] (11/6, Hirsch, Paz, 20.6M) called the order "a sign that the Biden administration may face an uphill battle in its biggest effort yet to combat the virus among the American work force." The Times says the stay "doesn't have an immediate impact" since "the first major deadline in the new rule is Dec. 5, when companies with at least 100 employees must require unvaccinated employees to wear masks indoors," and "businesses have until Jan. 4 to mandate Covid vaccinations or start weekly testing of their workers," but it "provided momentum for a wide coalition of opponents of the rule, who have argued that it is unconstitutional."

[HYPERLINK "https://thehill.com/policy/healthcare/580403-appeals-court-stays-biden-vaccine-mandate-for-businesses"] (11/6, Lonas, 5.69M) reported the ruling came "after Texas Attorney General Ken Paxton (R) filed a challenge to the mandate requiring employers with over 100 or more employees to get vaccinated or undergo frequent testing directly with the court."

[HYPERLINK "https://www.bloomberg.com/news/articles/2021-11-06/appeals-court-halts-roll-out-of-biden-s-big-company-vaccine-rule?srd=politics-vp"] (11/6, Brubaker Calkins,

3.57M) reported the request for the stay "came late Friday from more than two dozen private companies suing the U.S. Occupational Safety and Health Administration and Labor Department, as part of a court challenge led by Texas, Louisiana, Utah and South Carolina."

[[HYPERLINK "https://www.cnn.com/2021/11/06/us-federal-appeals-court-freezes-bidens-vaccine-mandate-for-companies.html"](https://www.cnn.com/2021/11/06/us-federal-appeals-court-freezes-bidens-vaccine-mandate-for-companies.html)] (11/6, Kimball, 7.34M) noted the judges "who issued the order – Stuart Kyle Duncan, Kurt Damian Englehardt and Edith Hollan Jones – were appointed by former Presidents Donald Trump and Ronald Reagan."

[[HYPERLINK "https://thehill.com/homenews/administration/580340-white-house-facing-onslaught-of-challenges-to-coronavirus-vaccine"](https://thehill.com/homenews/administration/580340-white-house-facing-onslaught-of-challenges-to-coronavirus-vaccine)] (11/7, Gangitano, Weixel, 5.69M) reports, "Administration officials insist they are on solid legal footing, but it's not stopping business groups, religious organizations and GOP state attorneys general from filing a barrage of lawsuits or Republican lawmakers from threatening legislative actions" after "the Labor Department on Thursday released the sweeping vaccine-or-test mandate, which President Biden had previously announced was coming." The Hill adds, "A coalition of 11 states led by Missouri Attorney General Eric Schmitt (R), who is running for Senate, argued the Occupational Safety and Health Administration (OSHA) doesn't have the authority to put in place such sweeping federal public health regulations and that the rule unconstitutionally infringes on states' powers." Meanwhile "a separate group led by West Virginia Attorney General Patrick Morrisey (R) filed a similar lawsuit in the 6th Circuit." On Friday, White House deputy press secretary Karine Jean-Pierre "said the administration is 'very confident' the mandate can withstand legal challenges."

[[HYPERLINK "https://www.politico.com/news/2021/11/06/biden-vaccine-order-blocked-federal-court-519908"](https://www.politico.com/news/2021/11/06/biden-vaccine-order-blocked-federal-court-519908)] (11/6, Rainey, 6.73M) reported "more than two dozen states have filed multiple legal challenges in federal court against the Biden administration's vaccinate-or-test mandate for private businesses, arguing that the Occupational Safety and Health Administration doesn't have the authority to issue the requirements." Politico adds the four lawsuits "were filed by groups of 26 states in the 8th Circuit, 11th Circuit, 6th Circuit and 5th Circuit over the past few days." Politico says the lawsuits "broadly...argue that the Department of Labor lacks the authority to issue a rule and that it did not follow the proper procedure in issuing the emergency temporary standard."

The [[HYPERLINK "https://helenair.com/news/state-and-regional/govt-and-politics/montana-ag-challenges-vax-mandate-for-large-businesses/article_e04fb654-746e-5438-b95c-70ae13ce5ffe.html"](https://helenair.com/news/state-and-regional/govt-and-politics/montana-ag-challenges-vax-mandate-for-large-businesses/article_e04fb654-746e-5438-b95c-70ae13ce5ffe.html)] (11/5, Wilson, 97K) reported that Montana Attorney General Austin Knudsen "joined a lawsuit filed by 11 states to block the Biden administration's new rule

requiring businesses with 100 or more employees to mandate COVID-19 vaccinations in the workplace or conduct weekly coronavirus testing."

[[HYPERLINK "https://www.bloomberg.com/news/articles/2021-11-07/biden-adviser-says-big-company-vaccine-mandate-on-legal-ground?srnd=premium"](https://www.bloomberg.com/news/articles/2021-11-07/biden-adviser-says-big-company-vaccine-mandate-on-legal-ground?srnd=premium)] (11/7, 3.57M) reports, "President Joe Biden's requirement for employees at companies with more than 100 workers to get Covid-19 vaccinations or regular testing is on solid legal ground, presidential adviser Cedric Richmond said" in an appearance on CBS's "Face the Nation" on Sunday.

However, [[HYPERLINK "https://www.cnn.com/2021/11/06/politics/court-blocks-biden-administration-vaccine-rule-larger-employers/index.html"](https://www.cnn.com/2021/11/06/politics/court-blocks-biden-administration-vaccine-rule-larger-employers/index.html)] (11/6, Vogue, Janfaza, 89.21M) reported that Sean Marotta, a lawyer at Hogan Lovells, "said he was surprised that the Fifth Circuit Court of Appeals acted to block the mandate on a Saturday, particularly because the mandate does not require employers or employees to act for months."

The [[HYPERLINK "https://www.wsj.com/articles/federal-appeals-court-temporarily-blocks-biden-administration-vaccine-rules-for-private-employers-11636226904"](https://www.wsj.com/articles/federal-appeals-court-temporarily-blocks-biden-administration-vaccine-rules-for-private-employers-11636226904)] (11/6, Kendall, Siddiqui, Subscription Publication, 8.41M) provided similar coverage as did [[HYPERLINK "https://www.foxbusiness.com/politics/federal-court-of-appeals-issues-temporary-halt-to-biden-vaccine-mandate#&_intcmp=hp1bt1_1_hp1bt"](https://www.foxbusiness.com/politics/federal-court-of-appeals-issues-temporary-halt-to-biden-vaccine-mandate#&_intcmp=hp1bt1_1_hp1bt)] (11/6, 3.06M) and the [[HYPERLINK "https://www.sfchronicle.com/us-world/article/Court-freezes-Biden-s-nationwide-vaccination-16598190.php"](https://www.sfchronicle.com/us-world/article/Court-freezes-Biden-s-nationwide-vaccination-16598190.php)] (11/7, Said, 2.44M).

Employees Not Allowed To Self-Administer And Self-Report Tests. The [[HYPERLINK "https://www.wsj.com/articles/new-biden-vaccine-rules-dont-allow-self-administered-self-reported-covid-19-tests-11636147556"](https://www.wsj.com/articles/new-biden-vaccine-rules-dont-allow-self-administered-self-reported-covid-19-tests-11636147556)] (11/5, Calfas, Cutter, Subscription Publication, 8.41M) reported that under the rule, employees will be not be able to self-administer and self-report their COVID-19 tests. Instead they must be observed by the employer or another authorized person.

OSHA Seeking Comment On Whether To Extend COVID-19 Vaccine And Testing Requirement To Businesses With Fewer Than 100 Employees. [[HYPERLINK "https://www.foxbusiness.com/politics/biden-admin-considering-vaccine-mandate-small-businesses-fewer-than-100-employees"](https://www.foxbusiness.com/politics/biden-admin-considering-vaccine-mandate-small-businesses-fewer-than-100-employees)] (11/5, Olson, 3.06M) reported, "The Occupational Safety and Health Administration (OSHA) says it is 'seeking comment' about whether businesses with fewer than 100 employees should be subject to the COVID-19 vaccine and testing mandate it is implementing on big businesses." The current rule "will force businesses with more than 100 employees to mandate vaccines or else their

employees will need to wear masks and be tested for COVID-19 weekly."

OSHA Report Says People With Prior COVID-19 Case Less Protected Than Those With Vaccine. [

HYPERLINK "<https://www.foxbusiness.com/politics/osha-vaccine-mandate-covid-grave-danger>"] (11/6, Aitken, 3.06M) reported, "The Occupational Safety and Health Administration (OSHA) has issued a report in which it explains that even if a person had COVID-19, they face significant danger from the virus." OSHA stated in the report, "A considerable number of individuals who were previously infected with SARS-CoV-2 do not appear to have acquired effective immunity to the virus."

Republicans In Congress To Use Congressional Review Act To Vote Against COVID-19 Vaccine Rule. [

HYPERLINK "<https://www.foxnews.com/politics/house-republicans-move-stall-biden-vaccine-mandate>"] (11/5, McFall, 23.99M) reported Rep. Fred Keller (R-PA), ranking member of the Education and Labor Subcommittee on Workforce Protections, "will take steps next week to block President Biden's 'unconstitutional' coronavirus vaccine mandate." Keller said that in cooperation with Sen. Mike Braun (R-IN), they "will attempt to use the Congressional Review Act to nullify the executive order." Braun had previously announced that he had the backing of "at least 42 other Senate Republicans in challenging the president's executive order."

Kansas Governor Opposes OSHA COVID-19 Vaccine Requirement. The [HYPERLINK

"<https://apnews.com/article/coronavirus-pandemic-joe-biden-business-health-kansas-86d570bbf1eb4376a5e7b519c4cb4903>"] (11/5, Field) reported that Kansas Gov. Laura Kelly (D) "grew more forceful Friday in opposing President Joe Biden's COVID-19 vaccine mandates, saying it's 'too late' in the coronavirus pandemic to impose them after Kansas and other states tailored responses to their needs." Kelly said, "While I appreciate the intention to keep people safe, a goal I share, I don't believe this directive is the correct, or the most effective, solution for Kansas." Also on Friday, Kansas Attorney General Derek Schmidt (R), "who hopes to unseat Kelly next year, announced that Kansas is among seven states that filed a federal lawsuit with the 6th U.S. Circuit Court of Appeals in Cincinnati against a Biden mandate that applies to companies with more than 100 workers."

[HYPERLINK "<https://www.foxnews.com/politics/democratic-governor-kelly-kansas-biden-vaccine-mandate>"] (11/7, Colton, 23.99M) reports that Gov. Kelly said in a statement, "States have been leading the fight against COVID-19 from the start of the pandemic. It is too late to impose a federal standard now that we have already developed systems and strategies that are tailored for our specific needs."

[HYPERLINK "<https://thehill.com/policy/healthcare/580256-democratic-governor-comes-out-against-bidens-vaccine-mandate>"]

(11/5, Coleman, 5.69M) reported that Kelly's "disapproval of the administration's move comes as many Republican governors and attorneys general have voiced their opposition to the vaccine requirements for businesses."

[HYPERLINK "<https://www.newsweek.com/kansas-democratic-governor-calls-bidens-vaccine-mandate-too-late-1646437>"] (11/5, Fung, 2.67M) reported that "Kelly questioned how effective President's Biden's vaccine mandate would be for residents of her state and said it was 'too late' for the federal government to be implementing such efforts this late into the coronavirus pandemic."

US To Lift Travel Ban On 33 Nations For People Fully Vaccinated Against COVID-19. The

[HYPERLINK "<https://playvideo.bulletinintelligence.com/d68bbc0f4bca4f74931b7fdc93d0836f?pubid=fda>"] (11/7, 6:04 p.m. EST, story 2, 2:10, Duncan, 9.45M) reported, "The United States reopens its borders to international visitors" on Monday, "as long as travelers are fully vaccinated" against COVID-19. It is "the first time since COVID restrictions closed crossings nearly 20 months ago." CBS (Carter Evans) reported, "US airports are facing and preparing for big crowds. [Monday] alone, United Airlines is anticipating there will be more than 30,000 inbound international travelers and it's not just by air. Our land borders are expected to be crowded as well. Excitement and relief for thousands of people on both sides of the US-Canada border. Starting Monday, after being shut down for nearly 600...days, the world's longest land border will be open to the vaccinated."

The [HYPERLINK

"https://www.washingtonpost.com/world/europe/europe-travel-coronavirus-ban/2021/11/06/db3324ee-3d67-11ec-8ee9-4f14a26749d1_story.html"] (11/6, Stein, 10.52M) reported, "On Monday, the United States will lift the travel ban on 33 nations – including many in Europe and several other places including China – for people who are fully vaccinated" against COVID-19 "or show a negative coronavirus test before boarding a flight." The travel ban lift "gives more families a chance to reunite and offers a boost to the world's sagging tourism industry." The United States has been criticized by European businesses, lawmakers, and residents for months "for failing to allow vaccinated EU citizens into the country – particularly as the vaccination rates in many European countries began to eclipse rates in the United States."

The [HYPERLINK

"<https://www.nytimes.com/2021/11/07/travel/us-travel-ban-reunions.html>"] (11/7, Murphy, 20.6M) reports that under the

new rules, travelers fully vaccinated against COVID-19 "will be allowed to enter the United States if they can show proof of vaccination and a negative coronavirus test taken within three calendar days of travel." Those Americans not vaccinated against COVID-19 as well as "children under the age of 18 are exempt from the requirement, but must take a coronavirus test within 24 hours of travel."

The [HYPERLINK "https://apnews.com/article/coronavirus-pandemic-lifestyle-health-travel-relationships-528d66c448c9b6fc7dd50ac5ddef03fc"] (11/6, Loller) reported, "Loved ones have missed holidays, birthdays and funerals while nonessential air travel was barred from a long list of countries that includes most of Europe, Brazil and South Africa." Closed land border crossings "with Mexico and Canada have devastated the border towns where traveling back and forth, sometimes daily, is a way of life."

Biden Says US Has Secured Millions Of Doses Of Experimental COVID-19 Pill. [HYPERLINK

"https://www.reuters.com/article/us-health-coronavirus-pfizer-biden/biden-says-u-s-has-secured-doses-of-new-pfizer-covid-pill-idUSKBN2HQ1PP"] (11/5, Alper, Hunnicutt) reported President Biden on Friday said the US has secured millions of doses of Pfizer's investigational antiviral oral COVID-19 therapy should it receive FDA authorization.

[HYPERLINK "https://thehill.com/policy/healthcare/580271-biden-us-accelerating-our-path-out-of-this-pandemic"] (11/5, Samuels, 5.69M) reported Biden said: "If authorized by the FDA, we may soon have pills and may treat the virus of those who become infected. We have already secured millions of doses, and the therapy would be another tool in our toolbox to protect people from the worst outcomes of Covid."

[HYPERLINK "https://www.forbes.com/sites/lisakim/2021/11/05/us-has-secured-millions-of-pfizer-antiviral-pills-president-biden-says"] (11/5, Kim, 10.33M) provided additional coverage.

First Lady Launches Nationwide Effort To Encourage COVID-19 Vaccine For Children 5 To 11. [HYPERLINK

"https://thehill.com/policy/healthcare/580218-jill-biden-to-kick-off-effort-to-encourage-vaccines-among-children-5-to-11"] (11/5, Chalfant, 5.69M) reported the White House on Friday announced First Lady Dr. Jill Biden on Monday will "begin a nationwide effort by the Biden administration to encourage COVID-19 vaccinations among children ages 5 to 11 next week, after the Pfizer vaccine was approved for use in this age group." The Hill says Dr. Biden will "visit a pediatric coronavirus vaccine clinic at Franklin Sherman Elementary School. The elementary school has historical significance

because it was the first to administer the polio vaccine more than half a century ago, which the White House noted." According to The Hill, the Administration "has undertaken similar campaigns to encourage vaccines among those ages 15 and up, seeking to answer questions that unsure Americans may have and to combat misinformation about the shots."

[HYPERLINK "https://www.cnn.com/2021/11/05/politics/jill-biden-child-vaccinations/index.html"] (11/5, Klein, 89.21M) reported that Dr. Biden, "who is also a community college professor, has previously traveled the country to encourage vaccinations and has also visited schools, highlighting the enormous deficit Covid-19 has caused for many families and teachers and school systems. Biden's new efforts will target the nation's youngest, most newly eligible vaccine candidates."

[HYPERLINK "https://www.forbes.com/sites/masonbissada/2021/11/05/first-lady-jill-biden-will-launch-campaign-to-encourage-covid-vaccinations-for-children-ages-5-11/"] (11/5, Bissada, 10.33M) also covered the First Lady's vaccination campaign.

Murthy On ABC's "This Week" Says Administration Will Defend COVID-19 Vaccine Requirement. [HYPERLINK

"https://playvideo.bulletinintelligence.com/338899c7122848fc9f3223b1a89e7a33?pubid=fda"] (11/7, 2.23M) hosted US Surgeon General Dr. Vivek Murthy and asked him about the decision by the 5th Circuit Court of Appeals decision to issue a stay of the OSHA COVID-19 vaccine requirement. Dr. Murthy said, "The President and the Administration wouldn't have put these requirements in place if they didn't think they were appropriate and necessary. The administration is certainly prepared to defend them." Asked about the suits filed by 27 states in opposition to the rule, Murthy said, "One of the reasons why you would see thousands of companies actually moving forward with requirements even before the federal deadline is because they've realized how important and helpful it is for the health of their workers and for the health of their businesses to have everyone vaccinated."

[HYPERLINK "https://abcnews.go.com/Politics/dr-vivek-murthy-defends-bidens-vaccine-mandate/story?id=81009953"] (11/7, Demissie, 2.44M) reports that Dr. Murthy "defended the actions of the Biden administration in a Sunday interview on ABC's 'This Week' after it announced a sweeping COVID-19 vaccine mandate for companies with 100 employees or more." ABC's Martha Raddatz "pressed Murthy on the impact that this mandate could have on businesses and the economy," and Murthy responded, "I hear time and time again, from small businesses, large businesses and workers is that what's really hurting the economy is actually COVID itself."

[<https://www.foxnews.com/health/surgeon-general-murthy-covid-19-is-whats-really-hurting-the-economy>] (11/7, Aitken, 23.99M) reports that Dr. Murthy “defended the Biden administration’s workplace COVID-19 vaccine mandate by insisting that it is the virus that is hurting the economy over any other factor.”

[<https://www.forbes.com/sites/teakvetenadze/2021/11/07/surgeon-general-says-nothing-is-off-the-table-when-it-comes-to-vaccine-mandates-for-smaller-companies/>] (11/7, Kvetenadze, 10.33M) reports, “As the White House vowed on Sunday morning to defend its federal vaccine mandates against court challenges, Surgeon General Dr. Vivek Murthy didn’t rule out the possibility of future mandates for smaller companies.” On “This Week,” “Murthy hinted that the administration would consider extending the decree to include those companies with fewer than 100 employees, telling” ABC’s Martha Raddatz “certainly nothing is off the table.”

[<https://thehill.com/policy/healthcare/public-global-health/580438-surgeon-general-says-administration-prepared-to-defend>] (11/7, Mastrangelo, 5.69M) reports that “Murthy said President Biden’s administration is ready to fight for the implementation of a vaccine requirement for large businesses.”

[<https://www.politico.com/news/2021/11/07/murthy-vaccine-mandate-biden-520015>] (11/7, Cohen, 6.73M) reports “Murthy on Sunday defended the Biden administration’s workplace rules on vaccine mandates” by “citing historical precedents dating back to George Washington during the American Revolution,” and “said President Joe Biden had faith in both the legality of the mandate and the effectiveness of such requirements.”

[<https://www.axios.com/murthy-biden-defend-vaccine-mandate-cbd87e3d-6d49-4f3d-b49d-dd647d4bcae6.html>] (11/7, Saric, 1.26M), [https://www.huffpost.com/entry/surgeon-general-defends-vaccine-rule_n_6187fb08e4b0ad6f588482a0] (11/7, 363K), the [<https://nypost.com/2021/11/07/surgeon-general-suggests-vax-mandate-could-expand-to-small-biz/>] (11/7, 7.45M), and the [<https://www.nydailynews.com/news/politics/us-elections-government/ny-covid-vaccine-large-business-vaccine-mandate-vivek-murthy-20211107-t4r5awl3rvfyljxumbsb7fnvy-story.html>] (11/7, Shahrighian, 2.51M) also provide coverage.

Schools Across US Preparing To Offer COVID-19 Vaccine To Younger Children.

The [[https://apnews.com/article/coronavirus-](https://apnews.com/article/coronavirus-pandemic-health-education-970897279539c6e004eaab4040e879b5)

[pandemic-health-education-970897279539c6e004eaab4040e879b5](https://apnews.com/article/coronavirus-pandemic-health-education-970897279539c6e004eaab4040e879b5)] (11/7, Eaton-Robb) reports that following the approval of the Pfizer-BioNTech COVID-19 vaccine “for younger children, many elementary schools around the US are preparing to offer the shots, which educators see as key to keeping students learning in person and making the classroom experience closer to what it once was.” However, “many school systems are choosing not to offer elementary schools as hosts for vaccination sites after some middle and high schools that offered shots received pushback.”

The [<https://www.washingtonpost.com/dc-md-va/2021/11/06/covid-vaccines-dc-kids/>] (11/6, Svruga, 10.52M) reported “the go-ahead Tuesday night from federal officials allowing the Pfizer-BioNTech shots to be given to millions of 5-to-11-year-olds had many parents in Washington dropping everything to stand in line. ... It marked a turning point for many families: a welcome sense of relief, and anticipation.”

[<https://www.bloomberg.com/news/articles/2021-11-06/child-vaccine-demand-underscores-deep-divisions-in-u-s?srnd=premium>] (11/6, Edney, 3.57M) and [<https://www.cbsnews.com/news/covid-19-vaccine-new-york-city-100-dollars-kids/>] (11/5, Gibson, 5.39M) also covered the story.

Cities, States Incentivizing Younger Kids To Receive COVID-19 Vaccine.

[<https://www.usatoday.com/story/news/health/2021/11/05/incentives-kids-get-covid-vaccine-include-money-gift-cards/6300733001/>] (11/5, Miranda, 12.7M) reported, “Children age[d] 5 to 11 are now eligible for the Pfizer-BioNTech COVID-19 vaccine and cities like New York City and Chicago are incentivizing kids to get vaccinated.” Those “in New York City can either gain \$100 if they receive their first dose of the vaccine within the city or tickets to city attractions.” Furthermore, “Louisiana is offering \$100 to children who get vaccinated, and in Texas, San Antonio officials announced parents can claim a \$100 gift card for H-E-B grocery stores.”

Big Bird Tweeted He Got COVID-19 Vaccine.

[<https://www.nbcnews.com/news/us-news/big-bird-vaccine-announcement-sparks-backlash-conservatives-gop-n1283425>] (11/7, Kessler, 4.91M) reports, “Big Bird’s seemingly innocuous – and obviously fictional – announcement Saturday that he has been vaccinated against Covid-19 caused a stir online, as Republicans like Sen. Ted Cruz of Texas accused the yellow anthropomorphic bird of tweeting ‘government propaganda.’” Big Bird tweeted, “I got the COVID-19 vaccine today! My wing is feeling a little sore, but it’ll give my body an extra protective boost that keeps me

and others healthy." NBC also reports, "Little research has been done about birds' getting Covid-19, but the Centers for Disease Control and Prevention says 'chickens and ducks do not seem to become infected or spread the infection based on results from studies.'"

[<https://thehill.com/homenews/senate/580426-ted-cruz-labels-big-birds-tweet-about-vaccination-propaganda>] (11/7, Schnell, 5.69M), [<https://www.newsweek.com/ted-cruz-slams-government-propaganda-after-sesame-streets-big-bird-gets-vaccine-1646733>] (11/7, Rahman, 2.67M), [<https://www.foxnews.com/media/twitter-calls-out-cnn-sesame-street-town-hall-for-covid-vaccines>] (11/7, Kornick, 23.99M), [<https://www.forbes.com/sites/danidiplacido/2021/11/07/pro-vaccine-tweets-from-big-bird-and-elmo-spark-a-big-conservative-backlash/>] (11/7, Placido, 10.33M), and the [<https://r.bulletinintelligence.com/48e3e03bd1c84b10b7749f45dfdcd6d2>] (11/7, Feldman, 2.51M) also provide coverage

Murthy Discusses COVID-19 Vaccine With Sesame Street's Granny Bird. [<https://playvideo.bulletinintelligence.com/92ba0bd0ec864d968229a5be94dddb7?pubid=fda>] (11/6, 384K) carried video of US Surgeon General Dr. Vivek Murthy appearing in a segment with Sesame Street's Granny Bird character to discuss the COVID-19 vaccines, their safety and efficacy. Murthy said, "this vaccine works to protect our kids against COVID. But it is also very safe. The only side effects that they're really seeing have popped up are soreness, in the arm where you get the shot, and some people have had headaches and felt a little tired afterwards."

Global COVID-19 Cases Reach 250M. [<https://www.reuters.com/business/healthcare-pharmaceuticals/global-covid-19-cases-near-250-million-delta-surge-eases-2021-11-07/>] (11/7, Abraham, Biswas) reports, "Global COVID-19 cases surpassed 250 million on Monday as some countries in eastern Europe experience record outbreaks, even as the Delta variant surge eases and many countries resume trade and tourism." The average of daily cases "has fallen by 36% over the past three months, according to a Reuters analysis, but the virus is still infecting 50 million people every 90 days due to the highly transmissible Delta variant."

CDC Data Show Cruises Reported Over 1,350 COVID-19 Cases Since June. The [<https://www.washingtonpost.com/travel/2021/11/05/covid-cruise-cdc-positive-us/>] (11/5, 10.52M) reported, "A report from the Centers for Disease Control and Prevention offers the first publicly available look at how many coronavirus

cases were discovered on cruises in the United States following an industry-wide shutdown." In spite of "cruise lines' efforts to impose strict public health rules, covid-19 has still found a way on board." According to the data from the CDC, "cruise operators confirmed 1,359 cases between June 26 and Oct. 21, a roughly four-month period that represents a rebound for the industry." Many of the cases "involved breakthrough infections of people who were fully vaccinated." Furthermore, "during that time, operators reported 49 hospitalizations and 38 medical evacuations for covid-19 or covid-like illnesses. At least one person died after testing positive during a cruise."

Arizona Reports More Than 3K COVID-19 Cases For Fourth Consecutive Day. The [<https://apnews.com/article/coronavirus-pandemic-health-arizona-9eec66493de408f324e9dbd5cc47f6f5>] (11/7) reports, "Health officials in Arizona on Sunday reported over 3,000 confirmed COVID-19 cases for the fourth consecutive day." The state's "coronavirus dashboard reported 3,231 additional cases but just one death." These newest numbers "increased Arizona's pandemic totals to 1,189,708 cases and 21,409 known deaths."

Over 10K Patients Diagnosed With COVID-19 In US Hospital After Being Admitted For Something Else, Analysis Suggests. [<https://www.cnn.com/2021/11/07/health/covid-19-hospital-died-khn-partner/index.html>] (11/7, Jewett) reports "more than 10,000 patients were diagnosed with" COVID-19 "in a U.S. hospital last year after they were admitted for something else, according to federal and state records analyzed exclusively for KHN." The analysis also revealed that nearly "21% of the patients who contracted covid in the hospital from April to September last year died," while "nearly 8% of other Medicare patients died in the hospital at the time."

California COVID-19 Case Rate Now Higher Than Texas, Florida. The [<https://www.mercurynews.com/2021/11/06/californias-covid-19-case-rate-now-twice-floridas-whats-up-with-that/>] (11/6, Woolfolk, 432K) reported, "COVID-19 cases aren't falling in California anymore. They have climbed back up to the CDC's blood-red 'high' level of virus transmission as the highly contagious Delta variant continues to wreak havoc." The case rate in the state "is now well above Texas' and double Florida's, which along with the rest of the Gulf Coast are down to the CDC's orange 'substantial' transmission level."

Las Vegas Nursing Facility Claims It Is Not Liable In COVID-19 Death Lawsuits. The [[HYPERLINK](#)

"<https://www.reviewjournal.com/investigations/summerlin-nursing-home-says-its-not-liable-in-covid-death-lawsuits-2473881/>"] (11/7, Erickson, 372K) reports, "A Las Vegas nursing home where 30 residents died from COVID-19 is facing several negligence lawsuits, but the facility asserts it is protected from the claims because it followed federal safety directives." The facility "is arguing that a longstanding law guarantees it the same liability protections as companies like COVID-19 vaccine manufacturers, according to court documents." The home "is invoking the Public Readiness and Emergency Preparedness Act (PREP Act), a longstanding federal law that is meant to shield companies fighting public health emergencies." This January, "the U.S. Department of Health and Human Services clarified that nursing homes and other facilities are immune from liability, except for cases where virus mitigation measures were totally disregarded."

Opinion: Lower Vaccination Rates Lead To Losing Ground Against Diseases We Have Already Defeated. In an opinion piece for the [[HYPERLINK](#)

"<https://www.washingtonpost.com/opinions/2021/11/07/were-losing-ground-against-diseases-weve-already-defeated/>"] (11/7, Bhadelia, 10.52M), infectious disease physician Nahid Bhadelia writes, "As we race to build global immunity against covid-19, we're losing ground against diseases we've already defeated. There are two reasons for this: growing vaccine hesitancy as a result of the politicization of coronavirus vaccines, and the disruption of routine childhood inoculation." Bhadelia says, "Health officials – and the public at large – must shore up our defenses against all diseases and keep routine childhood vaccination requirements from becoming enmeshed in our growing ideological divide." She discusses further the reasons behind growing anti-vaccination sentiment, and concludes with suggestions to combat lowering vaccination rates: "First, we must support routine childhood vaccination regardless of our political affiliations." Additionally, "clinicians must be more vigilant about detecting vaccine-preventable diseases that might appear in our clinics with greater frequency."

Opinion: COVID-19 Presents Opportunity For Tennessee To Expand Medicaid. In an opinion piece for the [[HYPERLINK](#)

"<https://www.tennessean.com/story/opinion/2021/11/07/covid-19-makes-expanding-medicaid-even-more-vital/6304638001/>"] (11/7, 645K), Kevin Gibas, an infectious diseases physician at Vanderbilt University Medical Center, says "the COVID-19 pandemic has had a devastating impact

on the health of communities across the southeastern United States, including here in Tennessee." The impact "has been exacerbated by high numbers of residents lacking insurance in Tennessee and throughout the region." Gibas asserts that "a critical component of any response to COVID-19 is addressing the health insurance coverage gap – a gap that is largely the result of Tennessee's failure to expand Medicaid." According to Gibas, "Medicaid expansion is Tennessee's best hope of closing the state's health care coverage gap, and COVID-19 presents a unique opportunity to act."

Judge Orders California Officials To Provide Distance Learning For Students With Disabilities. The [[HYPERLINK](#)

"<https://www.latimes.com/california/story/2021-11-05/court-order-reinstates-distance-learning-for-disabled-students>"] (11/5, Blume, 3.37M) reported California "officials must act immediately to provide distance learning that is comparable to last year for students with disabilities and also adequate to their overall needs, a judge has ordered." The court ruling "will provide immediate relief for 15 students...but there are broad implications for students across California." The policies "at issue arise from Assembly Bill 130, which put in place rules meant to ensure that school districts provided and prioritized in-person instruction for all students this fall." The alleged fallout "in this case was that families that wanted remote learning for students with disabilities...faced delays and roadblocks that kept them out of school and denied them needed services." The judge wrote, "The Court finds that plaintiffs have demonstrated irreparable harm."

Biden Administration's COVID-19 Vaccine Rules Will Apply To Many Workers Who Support People With Developmental Disabilities. [[HYPERLINK](#)

"<https://r.bulletinintelligence.com/8ba94e487be04757b176027912b3c27b>"] (11/8, Diamant) reports "new Biden administration rules requiring millions of American workers to receive COVID-19 vaccines will apply to many of those who support people with developmental disabilities." Under a rule proposed by CMS, "workers at health care facilities that participate in the Medicare and Medicaid programs will need to be fully vaccinated by Jan. 4." Along with "hospitals and long-term care facilities, the rule will apply to staff at intermediate care facilities for individuals with intellectual disabilities, psychiatric residential treatment facilities, home health agencies and other providers." Meanwhile, "providers of home and community-based services could be subject to Biden's other rule, a mandate...that workers at any business employing 100 or more people be vaccinated or be tested weekly for COVID-19."

Lockdown In Auckland, New Zealand Likely To Be Over By Month's End, Prime Minister Says.

The [[HYPERLINK "https://apnews.com/article/coronavirus-pandemic-business-health-new-zealand-auckland-1c8b69ccf90bea1039b2c90d22e53661"](https://apnews.com/article/coronavirus-pandemic-business-health-new-zealand-auckland-1c8b69ccf90bea1039b2c90d22e53661)] (11/8) reports "the lockdown of New Zealand's largest city is likely to end later this month, with some coronavirus restrictions eased from Tuesday, Prime Minister Jacinda Ardern said." The city of "Auckland has been in lockdown for almost three months after an outbreak of the delta variant." The prime minister "said Monday the city's improving vaccination rate among people age 12 and older meant it could continue cutting back on restrictions."

[[HYPERLINK "https://www.reuters.com/business/healthcare-pharmaceuticals/virus-curbs-ease-auckland-new-zealand-hits-vaccination-targets-2021-11-08/"](https://www.reuters.com/business/healthcare-pharmaceuticals/virus-curbs-ease-auckland-new-zealand-hits-vaccination-targets-2021-11-08/)] (11/8, Menon) reports "a pick-up in vaccination rates and mounting pressure from critics calling for more freedoms have prompted Ardern to end the curbs."

Columnist Calls Florida's Surgeon General Awaiting Confirmation The "Anti-Fauci."

[[HYPERLINK "https://nypost.com/2021/11/07/meet-the-anti-fauci-floridas-surgeon-general-joseph-ladapo/"](https://nypost.com/2021/11/07/meet-the-anti-fauci-floridas-surgeon-general-joseph-ladapo/)] (11/7, 7.45M) columnist Karol Markowicz says "America has had 20 long months with Dr. Anthony Fauci as the spokesman for the government's COVID response, poorly explaining to us both the pandemic and the concurrent illogical restrictions." Markowicz writes, "Perhaps it's time for some new voices." The media call "Joseph Ladapo, Florida's surgeon general awaiting confirmation, a 'firebrand' who's 'controversial.'" Ladapo "opposes mask and vaccine mandates – and so is erroneously called 'anti-vax' by his foes." However, Markowicz says "Ladapo came off as none of these things when I spoke to him for an exclusive interview last week." She asserts that "in so many ways, the Nigerian-born Ladapo is the anti-Fauci."

Residents In Major US Cities More Willing To Receive COVID-19 Vaccine Than Rest Of Country, Survey Suggests.

The [[HYPERLINK "https://nypost.com/2021/11/07/new-yorkers-urbanites-more-accepting-of-covid-vaccine-cuny-poll/"](https://nypost.com/2021/11/07/new-yorkers-urbanites-more-accepting-of-covid-vaccine-cuny-poll/)] (11/7, Campanile, 7.45M) reports "residents of the Big Apple metro area and other major US cities are more willing to get vaccinated against COVID-19 than the rest of the US, a new survey suggests." The [[HYPERLINK "https://www.nature.com/articles/s41598-021-00794-6"](https://www.nature.com/articles/s41598-021-00794-6)], which was "conducted by the CUNY School of Public Health and just published in nature.com, surveyed 6,037 Americans in mid-April with a particular focus on four large metro areas

in different regions of the country: New York City, Chicago, Los Angeles and Dallas." According to the Post, "the number of people unwilling to get vaccinated in each city was: 10.1 percent in New York City; 11.2 percent in Chicago; 11.5 percent in LA and 19.7 percent in Dallas, according to the survey." However, "the resistance rate for the entire country was more than 21 percent."

Protest Against COVID-19 Safety Measures Brings Rallyists, Counter-Protestors To Boston Common.

[[HYPERLINK "https://thehill.com/homenews/state-watch/580487-protesters-clash-in-boston-over-mask-and-vaccine-mandates"](https://thehill.com/homenews/state-watch/580487-protesters-clash-in-boston-over-mask-and-vaccine-mandates)] (11/7, Beals, 5.69M) reports "a rally against COVID-19 public safety measures like mask and vaccine mandates brought dozens of protesters and even more counter-protestors to Boston Common on Sunday, where the participants breached police barricades and repeatedly clashed, according to The Boston Globe." Police officers "said two people were arrested, the Globe reported." The Globe "estimated that 'scores' of right-wing protesters turned out, while hundreds of counter-demonstrators showed up – along with hundreds of police trying to keep them apart."

Pfizer CEO Says Vaccines, Boosters Will Remain Key In Fight Against COVID-19.

[[HYPERLINK "https://www.cnbc.com/2021/11/05/pfizer-ceo-says-covid-vaccines-boosters-still-needed-even-with-pills.html"](https://www.cnbc.com/2021/11/05/pfizer-ceo-says-covid-vaccines-boosters-still-needed-even-with-pills.html)] (11/5, Mendez, 7.34M) reported Pfizer CEO Albert Bourla said in an interview with CNBC on Friday that while the company's investigational antiviral oral COVID-19 pill appears promising, vaccines and vaccine boosters remain key in combatting SARS-CoV-2. Bourla said, "The fact that we have a treatment is not at all a reason not to take the vaccine, in fact we should take the vaccine."

[[HYPERLINK "https://www.foxbusiness.com/healthcare/pfizer-ceo-says-covid-pill-a-game-changer"](https://www.foxbusiness.com/healthcare/pfizer-ceo-says-covid-pill-a-game-changer)] (11/5, Kaplan, 3.06M) reported Bourla added, "People should get vaccinated and they should be able to protect themselves and their families by doing that because otherwise they can spread the disease."

UK To Begin Distributing COVID-19 Antiviral Pill Molnupiravir.

[[HYPERLINK "https://www.reuters.com/business/healthcare-pharmaceuticals/uk-roll-out-covid-19-antiviral-drug-trial-this-month-health-security-agency-2021-11-07/"](https://www.reuters.com/business/healthcare-pharmaceuticals/uk-roll-out-covid-19-antiviral-drug-trial-this-month-health-security-agency-2021-11-07/)] (11/7, MacLellan) reports the UK will begin rolling out Merck & Co.'s "molnupiravir COVID-19 antiviral pill through a drug trial later this month, Susan Hopkins, Chief Medical Adviser at the UK Health Security Agency said on Sunday." The UK last week

"became the first country in the world to approve the potentially game-changing COVID-19 antiviral pill."

FDA Approves Two More Batches Of J&J COVID-19 Vaccine Manufactured At Emergent.

[[HYPERLINK "https://www.reuters.com/world/us/us-authorizes-more-batches-jj-covid-19-vaccine-made-emergent-2021-11-05/"](https://www.reuters.com/world/us/us-authorizes-more-batches-jj-covid-19-vaccine-made-emergent-2021-11-05/)] (11/5, Roy) reported the Food and Drug Administration "said on Friday it had authorized the use of two more batches of Johnson & Johnson's one-dose COVID-19 vaccine manufactured at the problem-plagued Baltimore factory of Emergent BioSolutions Inc," bringing the total to 11 approved batches. The "authorization was based on a thorough review of facility records and quality testing by the manufacturer, and considering the current COVID-19 public health emergency, the health agency said in a statement."

Moderna Facing COVID-19 Vaccine Vial-Filling Slowdowns.

[[HYPERLINK "https://www.bloomberg.com/news/articles/2021-11-06/moderna-finds-that-inventing-a-covid-vaccine-was-the-easy-part?srnd=premium"](https://www.bloomberg.com/news/articles/2021-11-06/moderna-finds-that-inventing-a-covid-vaccine-was-the-easy-part?srnd=premium)] (11/6, Langreth, LaVito, 3.57M) reported that "more than 10 months after U.S. regulators first authorized its potent shot" against SARS-CoV-2, "Moderna is finding that inventing a Covid-19 vaccine was actually a relatively straightforward task compared with what would come next: ramping up vial-filling operations on multiple continents and distributing the vaccine to countries around the world in the middle of a devastating pandemic." Moderna on Thursday "announced it would be able to supply only 700 million to 800 million vaccine doses this year, down from its previous projection of 800 million to 1 billion doses."

In an analysis of Moderna's partnership with the National Institutes of Health, [[HYPERLINK "https://www.politico.com/news/2021/11/05/trump-deal-moderna-global-vaccine-effort-519771"](https://www.politico.com/news/2021/11/05/trump-deal-moderna-global-vaccine-effort-519771)] (11/5, Cancryn, Oermohle, Banco, 6.73M) said the agreement the federal government "negotiated with Moderna has become a key stumbling block in the global fight against Covid-19. Armed with the generous language in its contract, the company has refused to grant access to its technology to vaccine manufacturers serving the developing world and at multiple points resisted pleas to increase aid to the neediest countries."

Researchers Explore Heart-Related Risks Tied To COVID-19 Vaccines.

The [[HYPERLINK "https://www.wsj.com/articles/researchers-probe-link-between-covid-19-vaccines-and-myocarditis-11636290002"](https://www.wsj.com/articles/researchers-probe-link-between-covid-19-vaccines-and-myocarditis-11636290002)] (11/7, Loftus, Subscription Publication, 8.41M) reports that as health authorities are expanding the use of COVID-19

vaccines in the US, researchers exploring heart-related risks tied to the shots, such as myocarditis or pericarditis, are following several emerging theories. The Journal says the FDA has held back on authorizing Moderna's coronavirus vaccine in adolescents while the agency investigates the risk.

Novavax To Complete Submission Of COVID-19 Vaccine Data To FDA By Year End.

On its website, [[HYPERLINK "https://www.cnn.com/2021/11/05/health/novavax-covid-19-vaccine-submit-data-plans-wellness/index.html"](https://www.cnn.com/2021/11/05/health/novavax-covid-19-vaccine-submit-data-plans-wellness/index.html)] (11/5, Howard, 89.21M) reported Novavax CEO Stanley Erck in a Friday interview revealed the company "plans to submit complete data to the US Food and Drug Administration soon for possible emergency use authorization of its coronavirus vaccine." Erck said, "We'll have a file in the US hopefully before the end of the year." The vaccine, "called NVX-CoV2373, is made using somewhat more conventional methods than the vaccines already authorized for use in the US."

Use Of Monoclonal Antibodies In Massachusetts Has Surged In Recent Months.

The [[HYPERLINK "https://www.bostonherald.com/2021/11/06/massachusetts-hospitals-see-sharp-increase-in-demand-for-monoclonal-antibody-treatment/"](https://www.bostonherald.com/2021/11/06/massachusetts-hospitals-see-sharp-increase-in-demand-for-monoclonal-antibody-treatment/)] (11/6, Cohan, 327K) reported, "Demand for monoclonal antibodies to treat coronavirus has surged at Massachusetts hospitals in recent months as awareness about the once-little-known drug and its incredible effects is increasing, bringing patients in from a dozen different states." While the FDA authorized the therapy's use in December 2020, its use in state hospitals plateaued across the spring and summer months before increasing "dramatically" this fall.

FDA May Soon Authorize Use Of Monoclonal Antibodies As PrEP Against SARS-CoV-2.

[[HYPERLINK "https://www.nbcnews.com/health/health-news/covid-antibody-drugs-protect-people-weak-immune-systems-rcna4597"](https://www.nbcnews.com/health/health-news/covid-antibody-drugs-protect-people-weak-immune-systems-rcna4597)] (11/7, Ryan, 4.91M) reports, "The Food and Drug Administration could soon grant emergency authorization to monoclonal antibodies from drugmaker Regeneron for what is known as pre-exposure prophylaxis, or PrEP, against Covid-19." If authorized, the therapy could provide the "ticket back to some semblance of normalcy" for many immunocompromised patients. Still, "questions remain about how accessible such routine preventive therapy would be to any of the estimated 3 percent of Americans who have compromised immune systems."

Ocugen Submits Emergency Use Authorization To FDA For Use Of Investigational COVID-19 Vaccine BBV152 In Pediatric Patients.

The [

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"<https://www.bizjournals.com/philadelphia/news/2021/11/05/ocugen-malvern-covid-19-vaccine-covaxin-children.html>"]

(11/5, George, Subscription Publication, 875K) reported Ocugen "has submitted an emergency use authorization application to the Food and Drug Administration for use of the company's Covid-19 vaccine candidate BBV152, known as Covaxin outside the United States, in children ages 2 to 18." Ocugen is working with India-based Covaxin developer Bharat Biotech "to bring the Covid-19 vaccine to the United States under a partnership formed in February."

WPost: Manufacturers Should Focus On Sending COVID-19 Pills To Developing Nations.

A [HYPERLINK

"<https://www.washingtonpost.com/opinions/2021/11/06/pfizer-oral-antiviral-pill-new-promise-covid-19/>"] (11/6, 10.52M) editorial was hopeful Pfizer's new COVID-19 pill could fill the gaps in protection vaccines leave such as challenges with accessibility and distribution. It could also lessen the burden on hospitals. The Post concludes that drug manufacturers should prioritize sending pills to developing nations after wealthy nations received initial shipments of vaccines.

Increased Binge-Drinking Among Some Americans Could Increase Risk From COVID-19 Breakthrough Infections, NIDA Study Finds.

In an article highlighting alcohol use trends in the US, [HYPERLINK

"<https://www.usatoday.com/in-depth/graphics/2021/11/04/trends-in-alcohol-use-during-the-covid-19-pandemic-in-graphics/6239987001/>"]

(11/4, Loehrke, 12.7M) reported that while "some research shows many Americans are drinking less, some are binge-drinking more," an issue that could weaken immune systems putting heavy drinkers "at risk for breakthrough infections of COVID-19." For example, one "[HYPERLINK

"<https://www.drugabuse.gov/news-events/news-releases/2021/10/people-with-substance-use-disorders-may-be-at-higher-risk-for-sars-cov-2-breakthrough-infections>"] by the National Institute on Drug Abuse found that people who binge-drink had a higher rate of breakthrough infections of COVID-19 compared with those with no substance disorders. Binge-drinking is defined as men consuming five drinks or more and women consuming four drinks or more in a two-hour period."

Murthy Defends COVID-19 Vaccine Requirement, Vaccines For Children.

[

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"<https://www.npr.org/2021/11/05/1052650965/as-u-s-cases-drop-surgeon-general-warns-covid-has-been-underestimated-before>"] (11/5, 3.69M) featured Steve Inskeep talking to US Surgeon General Vivek Murthy "about President Biden's latest vaccine rules for 100 million workers." Murthy said, "I certainly understand the frustrations people may have. But what we have to keep in mind here is that the real burden that we've been facing in workplaces has been from COVID itself. ... So if we really want to safeguard our workers, if we want to create safer workplaces, getting people vaccinated is actually the best pathway to go down." Asked, "is there a risk of another wave in the United States like they're experiencing in Europe right now?" Murthy said, "A number of times in the past 20 months, we have underestimated this virus. ... we still have 60 million-plus people who are not vaccinated. They remain vulnerable to COVID-19." Asked about COVID-19 vaccines for children, Murthy said, "No. 1 – the risk of COVID to children is not zero. It's not negligible. ... No. 2 – recognize that the vaccine is actually very effective, more than 90% effective in reducing the risk of children getting symptomatic COVID. ... And third is safety. ... There were no serious adverse effects seen."

US Secretary of Education Dr. Miguel A. Cardona, and US Surgeon General Dr. Vivek H. Murthy wrote for [HYPERLINK

"<https://www.usatoday.com/story/opinion/2021/11/05/covid-kids-mental-health-education-secretary-surgeon-general/6285131001/>"] (11/5, 12.7M), "Between the two of us, we have kids in pre-K, elementary, middle school and high school." And "so far, we've been heartened by what we see in schools. Recent data from the Centers for Disease Control and Prevention shows that 98% of schools are fully open this fall, up from about 70% at the end of the 2020-21 school year." They added, "we're fortunate not to have to choose between reducing our kids' COVID risk and keeping them connected to their support systems in school. But we do need to support and implement the strategies backed by scientists, mental health professionals and more than eighteen months of experience."

Reviewing Applications For Exemptions To COVID-19 Vaccine Requirement Among Federal Employees May Take Months.

The [HYPERLINK

"https://www.washingtonpost.com/politics/federal-workers-vaccines-exemptions/2021/11/07/761eb9d8-3da3-11ec-8ee9-4f14a26749d1_story.html"] (11/7, Rein, Duncan, Horton, 10.52M) reports, "With a Monday deadline looming, high percentages of federal workers are reporting they have been vaccinated against the coronavirus," yet, "tens of thousands of holdouts have requested exemptions on religious grounds,

complicating President Biden's sweeping mandate." In addition, "A far smaller number of employees has asked for exemptions on medical grounds." The Post adds that consideration of requests for exemptions "could take months for officials designated at each agency." The Post also reports that "Jeffrey Zients, the White House's covid-19 response coordinator, said this week that religious exemptions have existed for past vaccines and pledged that federal agencies 'are following rigorous processes to evaluate religious and medical exemptions.'"

The [[HYPERLINK](https://apnews.com/article/coronavirus-pandemic-joe-biden-health-lawsuits-1054028d7cbf0ef46f7ddadbdc4ae167) "https://apnews.com/article/coronavirus-pandemic-joe-biden-health-lawsuits-1054028d7cbf0ef46f7ddadbdc4ae167"] (11/7, Long, Sisak) reports, "About 4 million federal workers are to be vaccinated by Nov. 22 under the president's executive order." That "deadline is the first test of Biden's push." The AP adds that "officials at Health and Human Services, U.S. Food and Drug Administration and Housing and Urban Development said they were working on getting their employees vaccinated but had no figures yet."

Defense Industry Concerned By "Ambiguities" In COVID-19 Vaccine Requirement For Federal Contractors.

[[HYPERLINK](https://www.rollicall.com/2021/11/05/federal-contractors-see-risks-in-unclear-vaccine-mandate/) "https://www.rollicall.com/2021/11/05/federal-contractors-see-risks-in-unclear-vaccine-mandate/"] (11/5, Donnelly, 130K) reported, "Defense industry executives are concerned that ambiguities in President Joe Biden's vaccine mandate for federal contractors will create financial and legal risks for some 20 percent of America's businesses." According to Roll Call, business is concerned about "how ambiguities in the order have created other forms of uncertainty for their businesses." One attorney summarized their concerns as "the loss of key employees, the lack of clarity on whether and how the contractors will be able to recover costs associated with the disruptions to their business because of the mandate, and their ability to meet delivery requirements of the customers up the federal supply chain."

Evictions Increasing, Though They Remain Below Pre-Pandemic Levels.

The [[HYPERLINK](https://www.nytimes.com/2021/11/07/us/evictions-crisis-us.html) "https://www.nytimes.com/2021/11/07/us/evictions-crisis-us.html"] (11/7, Kasakove, 20.6M) reports that instead of a "sudden surge of evictions that tenants and advocates feared after the Supreme Court ruled in August that President Biden's extension of the eviction moratorium was unconstitutional," what is being seen "is a more gradual eviction crisis that is increasingly hitting communities across the country, especially those where the distribution of federal rental assistance has been slow, and where tenants have few protections." The Times adds, "the number of eviction filings

remained at nearly half of prepandemic averages during the first two weeks of October," but "filings are also increasing."

COVID-19 Vaccines For Children May Be Required By Schools Soon.

[[HYPERLINK](https://www.theatlantic.com/health/archive/2021/11/kids-school-covid-vaccine-mandate/620622/) "https://www.theatlantic.com/health/archive/2021/11/kids-school-covid-vaccine-mandate/620622/"] (11/5, Gutman, 3.53M) reported, that while "school mandates for new vaccines tend to lag behind CDC recommendations by about half a decade, but COVID-19 shots appear to be in the express lane." The Atlantic adds, "The kid-size COVID vaccine fights a disease that is about as dangerous as others for which schools regularly require vaccination, and, at least for now, it appears to be about as effective as these other vaccines."

Commentary: Despite Progress During Pandemic, Government Has Much Work To Do To Improve Data Handling.

Ali S. Khan, dean of the College of Public Health at the University of Nebraska Medical Center, and William J. Kassler, Palantir's chief medical officer working with the U.S. government, write in an op-ed for the [[HYPERLINK](https://www.latimes.com/opinion/story/2021-11-07/coronavirus-data-u-s-government-cdc) "https://www.latimes.com/opinion/story/2021-11-07/coronavirus-data-u-s-government-cdc"] (11/7, 3.37M), that at the beginning of the COVID-19 pandemic, "the speed, scope and scale of the data overwhelmed the government's outdated infrastructure until it finally created new systems for functions such as disease tracking and race and ethnicity reporting." As a result, "our nation relied on academic, media and other volunteers...rather than on our nation's public health agency, the Centers for Disease Control and Prevention." Now, "the HHS Protect hub – built for the Department of Health and Human Services with technology from the company Palantir...makes available near-real-time data," but, they add, "data are still largely locked into the government's outdated legacy tech systems that can't communicate with one another." They call for more improvements in the collection and handling of data by the government.

US To Reopen To Foreign Travelers As Long As Their COVID-19 Vaccinations Are Authorized By FDA.

The [[HYPERLINK](https://apnews.com/article/coronavirus-pandemic-lifestyle-business-travel-health-e1b651734559f0dfdbeb4301f44ed636) "https://apnews.com/article/coronavirus-pandemic-lifestyle-business-travel-health-e1b651734559f0dfdbeb4301f44ed636"] (11/7, Spike) reports, "Starting Monday, the United States plans to reopen to foreign travelers who are fully vaccinated against the coronavirus." It comes with a condition: "non-immigrant adults need to have received vaccines authorized by the Food and Drug Administration or which received an emergency use

listing from the World Health Organization." The decision "leaves many hopeful travelers across the globe who [have] taken full courses of vaccines widely used in other parts of the world – Sputnik V and the China-produced CanSino jab, in particular – scrambling to get reinoculated with shots approved by US authorities."

Canada, US Opening Border, Though Canadian COVID-19 Rules Likely To Deter Many. The [[HYPERLINK](#)

"<https://www.washingtonpost.com/world/2021/11/07/united-states-canada-border-reopen/>"] (11/7, 10.52M) reports, "On Monday, for the first time in more than 19 months, fully vaccinated Canadians will be allowed to cross the US land border for such nonessential purposes as tourism or family visits." Though "the reopening is being cheered in the tightknit communities that straddle the 5,500-mile border – and by the Canadian snowbirds who prefer to drive South to warmer climes – few are expecting an immediate flood of tourists." One reason is "because those entering Canada – including Canadians returning from even the briefest of visits on the American side – must present a negative coronavirus molecular test result within 72 hours of arrival."

Europe Again Becomes COVID-19 Epicenter. [[HYPERLINK](#)

"<https://www.bloomberg.com/news/articles/2021-11-06/europe-relapses-as-covid-hot-spot-in-warning-sign-for-recovery?srnd=premium>"] (11/6, Loh, 3.57M) reported, "Europe has again become an epicenter for the coronavirus, calling into question the region's efforts to recover from the pandemic."

Russia Hits Another Single-Day High For New COVID-19 Cases. The [[HYPERLINK](#)

"<https://apnews.com/article/coronavirus-pandemic-europe-health-russia-vladimir-putin-e2dc21ece794eb65297ddedf9e0111ee>"] (11/6) reported, "Russia's COVID-19 cases hit another one-day record as the country struggles to contain a wave of infections and deaths that has persisted for more than a month." Russia's "national coronavirus task force on Saturday reported 41,335 new cases since the previous day, exceeding the previous daily record of 40,993 from Oct. 31." It also "said 1,188 people with COVID-19 died, just seven fewer than the daily death record reported Thursday."

Sydney To Further Ease Social Distancing COVID-19 Curbs, Officials Say. [[HYPERLINK](#)

"<https://www.reuters.com/world/asia-pacific/sydney-further-ease-covid-19-curbs-monday-vaccinations-pick-up-2021-11-07/>"] (11/7, Kelly) reports, "Australia's largest city of Sydney will further ease social distancing curbs on Monday, a month

after emerging from a coronavirus lockdown that lasted nearly 100 days, as close to 90% of people have got both doses of vaccine, officials said." Though only available for those who are fully vaccinated against COVID-19, "the relaxation in the state of New South Wales, home to Sydney, lifts limits on house guests or outdoor gatherings, among other measures." Hundreds of "people held an anti-vaccine protest on Sunday in the state's capital of Sydney, but such opposition is slight in Australia, where polls show vaccination opponents number in the single digits."

Austrian Government Bans Those Unvaccinated Against COVID-19 From Restaurants, Hotels As Coronavirus Cases Rise. The [[HYPERLINK](#)

"<https://apnews.com/article/coronavirus-pandemic-lifestyle-europe-health-austria-b10bc4b4c1e049e45dc0afb875de835d>"] (11/6) reported, "Unvaccinated people in Austria who also haven't had COVID-19 will no longer be allowed to enter restaurants, hotels and hair salons or attend public events larger than 25 people under new rules that take effect Monday, the government said." Alexander Schallenberg, Austria's chancellor, "outlined the rules Friday night after a meeting with state-level leaders to discuss the country's response to rapidly rising coronavirus cases." Formerly, "people could enter restaurants, hotels and other areas if they were vaccinated, had recovered from the virus or could show results from a negative test."

[[HYPERLINK](#)]
"<https://www.reuters.com/world/europe/austrian-chancellor-expects-tighter-covid-rules-remain-past-christmas-newspaper-2021-11-07/>"] (11/7, Revill) reports, "Austria's latest measures to tackle the spread of coronavirus will likely stay in force over Christmas and New Year, Chancellor Alexander Schallenberg was quoted as saying on Sunday, as the country posted a record number of infections." Austria "reported 9,943 new infections within a 24-hour period on Saturday, surpassing the previous worst day of Nov. 13 2020 when 9,586 cases were recorded."

Costa Rica Issues COVID-19 Vaccine Mandate For Children Aged Five And Older. [[HYPERLINK](#)

"<https://www.reuters.com/world/americas/costa-rica-issues-covid-19-vaccine-requirement-children-2021-11-06/>"] (11/6, Murillo) reported, "Costa Rican children aged five and up must get COVID-19 vaccinations, according to a new health ministry mandate, making the Central American country one of the first to adopt such a requirement for kids." The mandate "would add COVID-19 to a list of other infectious diseases in which vaccines for children have for years been required, including for polio and smallpox." According to

official data, "coronavirus infection rates in Costa Rica have been trending down recently, with confirmed COVID-19 deaths totaling more than 7,000...out of a population of around 5.1 million."

People Not Vaccinated Against COVID-19 In Greece Face New Restrictions As Coronavirus Cases Soar. [[HYPERLINK](#)

"<https://www.reuters.com/world/europe/unvaccinated-greece-face-new-restrictions-covid-cases-soar-2021-11-06/>"] (11/6, Fronista) reported, "Queues formed outside shops in Athens on Saturday on the first day of new restrictions to curb soaring coronavirus infections which require the unvaccinated to have negative COVID-19 tests." Coronavirus "infections in Greece hit a new daily high almost every day in November, prompting authorities to announce new measures on Tuesday, which also restrict access to cafes and restaurants, state services and banks to those who are either vaccinated or have a negative test." People "vaccinated against COVID-19 also have to present their vaccination certificates, triggering long lines outside shops in the capital's busiest shopping street, Ermou."

China Has Given 1.072 Billion People Complete COVID-19 Vaccine Doses, Spokesman Says. [[HYPERLINK](#)

"<https://www.reuters.com/world/china/china-gives-107-blm-people-complete-covid-19-vaccine-doses-by-nov-5-official-2021-11-06/>"] (11/6, Liu, Stanway) reported, "China had given 1.072 billion people complete COVID-19 vaccine doses by Nov. 5, Mi Feng, spokesman at the National Health Commission, told a briefing on Saturday." Commission official Wu Liangyou said at the briefing that "a total of 37.97 million people in China had received a booster shot as of Friday." In a bulletin, the commission said "that China had administered 2.312 billion doses of COVID-19 vaccines by the end of Nov. 5, an increase of about 8.9 million from the previous day."

[<https://www.bloomberg.com/news/articles/2021-11-06/china-says-it-will-continue-covid-curbs-amid-overseas-outbreaks?srnd=premium>] (11/6, 3.57M) reported, "China said it will continue with stringent curbs to disrupt the coronavirus's domestic transmission, suggesting the nation won't break away from its COVID-Zero strategy anytime soon."

Australia's 80-Percent Vaccination Rate Against COVID-19 A "Magnificent Milestone," Prime Minister Says. [[HYPERLINK](#)

"<https://www.reuters.com/world/asia-pacific/australia-hits-magnificent-milestone-with-80-rate-vaccinations-2021-11-06/>"] (11/6, Kelly) reported, "Australia reached on Saturday a full

inoculation rate of 80% of those aged 16 and older, which Prime Minister Scott Morrison called a 'magnificent milestone' on the path to becoming one of the world's most vaccinated countries against COVID-19." An early advocate "of a COVID-zero strategy to manage the pandemic, the country of 25 million has moved towards living with the virus through extensive vaccinations, as the Delta variant has proven too infectious to suppress." While inoculations against COVID-19 "remain voluntary on the federal level, Australia's states and territories introduced mandatory measures for many occupations and workers."

[<https://www.bloomberg.com/news/articles/2021-11-05/australia-hits-80-full-covid-vaccine-rate-for-people-16-and-up?srnd=premium>] (11/5, Leigh, 3.57M) reported, "Australia hit its target of having 80% of people aged 16 and older fully vaccinated against COVID-19, Prime Minister Scott Morrison said, as the country shifts from a strict containment policy toward living with the virus as endemic."

Britain's Health Ministry To Open Up Bookings For COVID-19 Booster Shots. [[HYPERLINK](#)

"<https://www.reuters.com/world/uk/britain-allows-early-booking-booster-shots-speed-up-rollout-2021-11-06/>"] (11/6, Smout) reported, "Britain's health ministry on Saturday said it would open up bookings for booster shots" against COVID-19 "a month before people were eligible to receive the shots to help speed up the rollout ahead of the challenging winter months." Boris Johnson, Britain's prime minister, "is relying on booster doses as a major plank of his plan to avoid lockdown this winter through COVID-19 vaccines rather than social distancing rules or mask mandates, but has been criticized for a slow start to the program."

UK's Health Secretary Urges Eligible Residents To Get COVID-19 Booster Shots. The [[HYPERLINK](#)

"<https://www.nytimes.com/2021/11/07/world/europe/uk-virus-surge-booster-shots.html>"] (11/7, Kwai, 20.6M) reports, "Britain's health secretary is urging eligible residents to get booster shots of the coronavirus vaccine, aiming to reduce pressure on the country's health system as winter approaches." Ten million British people, most of whom are over age 50, "have received booster shots since the government began offering them, and millions more will be invited in the coming weeks to book appointments."

[<https://www.politico.com/www.politico.eu/article/uk-health-minister-sajid-javid-booster-coronavirus-vaccine-christmas-restrictions/>] (11/7, Haeck, 15K) reports that UK Health Secretary Sajid "Javid 'strongly urged' everyone to get a third dose of the coronavirus vaccine – saying 'immunity begins to

wane after six months,' which means a booster shot is needed to keep older people and those at risk safe over the winter."

Fire In COVID-19 ICU Ward In India Kills 11. The [[HYPERLINK "https://www.nytimes.com/2021/11/06/world/asia/india-fire-covid-icu.html"](https://www.nytimes.com/2021/11/06/world/asia/india-fire-covid-icu.html)] (11/6, Kumar, Schmall, 20.6M) reported, "Eleven people died after a fire broke out in a coronavirus intensive care unit in the western state of Maharashtra, the latest in a series of fatal disasters in COVID-19 wards in India." Shankar Misal, the fire chief in the Ahmednagar district, said "hospital staff tried to douse the fire that started Saturday morning with fire extinguishers, but the flames spread quickly in the airtight room, cutting the power out and forcing people to flee to safety." Misal said, "Most of the 11 patients who died suffocated from smoke."

[[HYPERLINK "https://thehill.com/policy/international/india/580374-11-dead-after-fire-breaks-out-in-covid-19-ward-of-indian-hospital"](https://thehill.com/policy/international/india/580374-11-dead-after-fire-breaks-out-in-covid-19-ward-of-indian-hospital)] (11/6, Lonas, 5.69M) reported, "Police Inspector Jyoti Karkade said 17 patients were staying in the ward at the hospital in the state of Maharashtra, with 11 of them dying, The Associated Press reported." The six survivors "are in stable condition." The cause of the fire is unknown.

Fears Grow That Diwali Gatherings In India Could Start Another COVID-19 Case Spike. The [[HYPERLINK "https://www.nytimes.com/2021/11/05/world/asia/india-diwali-covid.html"](https://www.nytimes.com/2021/11/05/world/asia/india-diwali-covid.html)] (11/5, Yasir, 20.6M) reported, "When a devastating second wave of coronavirus infections hit India in the spring, hospitals were overwhelmed with sick patients and crematories struggled to bury the dead as the bodies piled up." India is now "celebrating one [of] its most important holidays – Diwali, the festival of lights – and many fear another wave of infections as millions gather for the celebrations." Diwali "began on Thursday, and there is no data yet on what impact the festivities might be having on the country's battle to curb the spread of the virus."

Germany Post-Merkel Will Not Reimpose Strict Pandemic Lockdowns, Coalition Partner Pledges. [[HYPERLINK "https://www.politico.com/www.politico.eu/article/germany-lockdown-coronavirus-fdp-christian-lindner/"](https://www.politico.com/www.politico.eu/article/germany-lockdown-coronavirus-fdp-christian-lindner/)] (11/6, Bennett, 15K) reported, "Germany's expected incoming government will not reimpose strict pandemic lockdowns, a coalition partner pledged Saturday amid a record spike in COVID-19 cases." The promise "came from Christian Lindner, head of the pro-business Free Democrats (FDP), one of three parties working to form a coalition to replace outgoing Chancellor

Angela Merkel's government." While the Free Democrats "is the smallest of the three parties, Lindner is seen as the front-runner to become Germany's next finance minister."

Op-Ed Criticizes Inequities In Worldwide Distribution Of COVID-19 Vaccine Doses. Thomas J. Bollyky, director of the Global Health Program at the Council on Foreign Relations, wrote in a [[HYPERLINK "https://www.wsj.com/articles/getting-vaccines-to-the-world-next-time-11636118973"](https://www.wsj.com/articles/getting-vaccines-to-the-world-next-time-11636118973)] (11/5, Subscription Publication, 8.41M) op-ed that more than 94% of COVID-19 vaccine doses administered worldwide to date went to just 10 nations. Canada, Japan, and the US will likely have small children vaccinated before Ethiopia and Nigeria will inoculate their healthcare workers and high-risk elderly. NIH was mentioned.

Desperate Officials In Ukraine Enact Ad Campaign To Scare People Into Receiving COVID-19 Vaccine. The [[HYPERLINK "https://www.washingtonpost.com/world/europe/coronavirus-ukraine-vaccine-russia/2021/11/05/f63e23f6-3ca4-11ec-bd6f-da376f47304e_story.html"](https://www.washingtonpost.com/world/europe/coronavirus-ukraine-vaccine-russia/2021/11/05/f63e23f6-3ca4-11ec-bd6f-da376f47304e_story.html)] (11/5, Stern, 10.52M) reported, "Ukraine's pandemic figures are scary: skyrocketing infections, record deaths and among the lowest vaccination rates" against COVID-19 in Europe. In response, the country's "officials decided to become scary themselves with an advertising campaign aimed at young people: If you're unvaccinated, you may die." The campaign is "a noticeable escalation in the government's struggle to get the upper hand on the pandemic."

LGBTQ People More Likely To Lose Income During Pandemic, US Census Bureau Survey Suggests. [[HYPERLINK "https://www.bloomberg.com/news/articles/2021-11-05/lgbtq-people-were-more-likely-to-lose-income-during-the-pandemic?srnd=premium"](https://www.bloomberg.com/news/articles/2021-11-05/lgbtq-people-were-more-likely-to-lose-income-during-the-pandemic?srnd=premium)] (11/5, 3.57M) reported, "In a first-of-its-kind survey from the US Census Bureau, nearly a quarter of LGBTQ people reported losing income during the COVID-19 pandemic, a higher share than non-LGBTQ respondents." Unemployment and income statistics in the United States "don't specifically measure the LGBTQ experience." For the first time this past July, the US Census Bureau "collected information about sexual orientation and gender identity of respondents to its Household Pulse Survey."

With Bigger US Companies Relaxing COVID-19 Mask Guidelines, Employees At Other Places Wonder If They Can Do The Same. The [[HYPERLINK "https://www.wsj.com/articles/the-rules-on-face-masks-at-the-office-are-changing-11636281002"](https://www.wsj.com/articles/the-rules-on-face-masks-at-the-office-are-changing-11636281002)] (11/7,

Smith, Subscription Publication, 8.41M) reports that watching larger American employers such as Apple, Amazon, and JPMorgan Chase & Co. eliminate mask requirements as COVID-19 vaccination rates rise and case numbers fall has workers at other companies wondering if they can do the same.

COVID-19 Vaccine Mandate Divides Small Washington State Hospital.

The [[HYPERLINK "https://www.washingtonpost.com/nation/2021/11/06/vaccine-mandate-hospital-washington-state/"](https://www.washingtonpost.com/nation/2021/11/06/vaccine-mandate-hospital-washington-state/)] (11/6, A1, Saslow, 10.52M) reported on its front page that Washington "state's vaccination deadline for health-care workers was less than a week away when Shane McGuire," the CEO of Dayton General Hospital in southeast Washington, "called his HR department for another daily update." The CEO "liked to refer to his small staff as a family, and many in fact were family, but it had been splitting in two since the beginning of the year, when exactly 50 percent of the hospital's few hundred employees chose to be vaccinated and 50 percent refused." The prime example of the division due to the COVID-19 vaccination mandate was where McGuire's "medical director told the staff that mass vaccination was 'safe, wildly effective and absolutely necessary,'" while his director of nursing "wrote that it was 'government overreach and medical tyranny.'"

Commentary Questions If Debate Over SARS-CoV-2's Origin Is Worth Having.

In a [[HYPERLINK "https://www.statnews.com/2021/11/05/is-debate-about-origin-of-covid-19-still-worth-having/"](https://www.statnews.com/2021/11/05/is-debate-about-origin-of-covid-19-still-worth-having/)] (11/5, 262K) op-ed, John P. Moore, professor of microbiology and immunology at Weill Cornell Medicine in New York City, wrote, "The American public is understandably interested in how a pandemic that has killed nearly 750,000 people in this country and almost 5 million worldwide – with few signs of slowing down – emerged." Moore wrote, "But the US intelligence community has now concluded that the precise sequence of events by which SARS-CoV-2, the virus that causes COVID-19, entered the human population may never be known." Moore questioned if the hand-wringing is worth it, concluding, "Is there any point to continuing an increasingly fractious debate on the origin of COVID-19, or should we now focus on applying the lessons already learned, and think about the future?" NIAID Director Dr. Anthony Fauci and NIH were mentioned in this story.

Healthcare Professionals Urge Facebook To Disclose Its Data On COVID-19 Disinformation.

[[HYPERLINK "https://www.usatoday.com/story/tech/2021/11/05/facebook-covid-misinformation-doctors-letter/6275730001/"](https://www.usatoday.com/story/tech/2021/11/05/facebook-covid-misinformation-doctors-letter/6275730001/)] (11/5,

Collins, 12.7M) reported that Dr. Kavita "Patel is among the more than 500 US public health care professionals battling the ongoing spread of COVID who signed a letter demanding Facebook disclose its data regarding disinformation about the virus and the vaccine." The document "comes a week after Facebook CEO Mark Zuckerberg changed the parent company's name to Meta as a pivot toward the metaverse." The letter by the physicians, which was in the works for two months, "further gained momentum following leaked documents provided by whistleblower Frances Haugen to the Securities and Exchange Commission and Congress about the harms caused on Facebook and Instagram, and its parent company's failure to take action."

[[HYPERLINK "https://thehill.com/policy/technology/580242-doctors-urge-facebook-to-disclose-data-on-covid-19-misinformation"](https://thehill.com/policy/technology/580242-doctors-urge-facebook-to-disclose-data-on-covid-19-misinformation)] (11/5, Klar, 5.69M) reported, "Hundreds of health care professionals are urging Facebook to disclose data on the breadth of COVID-19 disinformation on the platform, piling onto the criticism the social media giant has faced in recent weeks after a whistleblower leaked internal company documents." The professionals "underscored the importance of combating false claims after the vaccine" against COVID-19 "became available to younger children." A letter written by the Doctors For America to Facebook on Thursday "was signed by more than 500 health care professionals, including medical professors at New York University, Columbia University and Johns Hopkins Medicine."

Op-Ed: Diversion Of Resources During COVID-19 Pandemic Has Allowed Tuberculosis Death Rates To Increase For First Time In 15 Years.

In a [[HYPERLINK "https://www.forbes.com/sites/joshuacohen/2021/11/05/impact-of-covid-19-pandemic-extends-to-tuberculosis-and-neglected-tropical-diseases/"](https://www.forbes.com/sites/joshuacohen/2021/11/05/impact-of-covid-19-pandemic-extends-to-tuberculosis-and-neglected-tropical-diseases/)] (11/5, 10.33M) op-ed, Joshua Cohen, an independent healthcare analyst, wrote, "Last month, the World Health Organization reported that for the first time in 15 years the number of people who have died from tuberculosis has increased." Last year, worldwide, "more than 1.5 million deaths were attributed to tuberculosis; the first year-on-year increase since 2005." Cohen wrote that multiple reasons were mentioned, including "diversion of resources due to the COVID-19 pandemic." Worse, Cohen wrote, "tuberculosis is not the only disease that has been impacted, with disproportionately severe health burdens on the world's poorest populations."

Despite Signals That Things Are Improving, Americans Seem Stuck In "A Pandemic Hangover Of Pessimism."

The [[HYPERLINK "https://www.nytimes.com/2021/11/05/us/pandemic-funk-](https://www.nytimes.com/2021/11/05/us/pandemic-funk-)

america.html"] (11/5, A1, Healy, Burch, Mazzei, 20.6M) reported on its front page, "Despite many signals that things are improving – the stock market is hitting record highs, hiring is accelerating sharply with 531,000 jobs added in October, workers are earning more, and COVID hospitalizations and deaths are dropping from their autumn peaks – many Americans seem stuck in a pandemic hangover of pessimism." In opinion surveys, "more than 60 percent of voters...say that the country is heading in the wrong direction – a national funk that has pummeled" President Joe "Biden's approval ratings and fueled a backlash against Democrats that could cost them control of Congress in next year's midterm elections."

WPost: China's Prosecution Of Woman Who Told Truth About Wuhan And Is Now Near Death In Prison Is An "Act Of Barbarity." The [[HYPERLINK](https://www.washingtonpost.com/opinions/2021/11/07/zhang-zhan-prisoner-wuhan-china-covid-origin/)

"https://www.washingtonpost.com/opinions/2021/11/07/zhang-zhan-prisoner-wuhan-china-covid-origin/"] (11/7, 10.52M) editorializes, "China committed one act of barbarity when it prosecuted the citizen journalist Zhang Zhan for her revealing look at Wuhan in the first stages of what became a global pandemic." The woman "was sentenced in December to four years in prison on the specious charge of 'picking quarrels and provoking trouble,' which China uses to suffocate free speech." The editorial board writes, "Now her health has deteriorated, and relatives say she is near death." The board concludes that "China will compound the barbarity unless it sets her free and saves her life."

Woman Who Died From Long-COVID Sparks Debate Of Whether Organs Are Safe, Healthy Enough To Be Transplanted. The [[HYPERLINK](https://www.nytimes.com/2021/11/07/health/covid-organ-transplants.html)

"https://www.nytimes.com/2021/11/07/health/covid-organ-transplants.html"] (11/7, Rabin, 20.6M) reports that a case of a 50-year-old woman ravaged by long-COVID who died "highlights an urgent debate among medical professionals about whether the organs of people who survived COVID, and even of those who died with the illness, are really safe and healthy enough to be transplanted." As a general rule, "the organs are considered safe for transplantation if the test is negative, even if the donor has recovered from COVID." There is also "no universally accepted set of recommendations regarding when organs can be safely recovered from virus-positive bodies and transplanted to patients in need." Creating more complications "is the fact that people with long COVID, whose debilitating symptoms may persist for months, mostly do not test positive for the infection."

Cruz Pushes Legislation To Block COVID-19 Vaccine Mandate For Children. The [[HYPERLINK](https://www.houstonchronicle.com/politics/texas/article/Sen-Ted-Cruz-files-bill-to-block-COVID-vaccine-16596097.php) "https://www.houstonchronicle.com/politics/texas/article/Sen-Ted-Cruz-files-bill-to-block-COVID-vaccine-16596097.php"] (11/5, Wermund, 982K) reported, "US Sen. Ted Cruz is pushing legislation that would block schools from requiring students to get" vaccinated against COVID-19. Cruz "filed a bill this week that would prohibit the federal government or any entity at the federal, state or local level that receives federal funding, including school districts, from requiring COVID-19 vaccines for minors." The bill "comes as the Food and Drug Administration has authorized the emergency use of the Pfizer vaccine for children 5 through 11 years of age."

Face Masks No Longer Required In Buildings In Miami-Dade County, Mayor Says. The [[HYPERLINK](https://www.miamiherald.com/news/coronavirus/article255590771.html)

"https://www.miamiherald.com/news/coronavirus/article255590771.html"] (11/5, Cetoute, 647K) reported, "Masks will no longer be required in Miami-Dade County buildings, Miami-Dade Mayor Daniella Levine Cava said Friday." The mayor tweeted that the reversal "is due to 'enormous progress we have made against the Delta variant, and in consultation with our Chief Medical Officer.'" She "had reinstated the mask mandate in July and urged businesses to do the same due to a spike in COVID cases and deaths, which was later recognized as Florida's third wave."

Schools In Minnesota Encouraging, But Not Requiring, Children To Get Vaccinated Against COVID-19. The [[HYPERLINK](https://www.startribune.com/minnesota-schools-encourage-covid-19-vaccines-for-students-but-so-far-don-t-plan-to-require-them/600113778/)

"https://www.startribune.com/minnesota-schools-encourage-covid-19-vaccines-for-students-but-so-far-don-t-plan-to-require-them/600113778/"] (11/6, Lonetree, Klecker, 855K) reported, "Schools across Minnesota have encouraged children to get vaccinated against COVID-19 – even hosting vaccine clinics in school buildings – but so far district leaders have stopped short of requiring the immunizations." Though "all school-age children are eligible for the vaccine, state education and health officials say it's unlikely districts will move to require students to get the shots." COVID-19 vaccine requirement decisions "are now left to individual school districts, and many school boards have already opted out of other COVID-related precautions, including requiring students to wear face masks." The FDA was mentioned in this story.

Chicago Schools Will Close Friday To Boost COVID-19 Vaccinations. The [[HYPERLINK](https://apnews.com/article/coronavirus-pandemic-health-education-chicago-public-health-df7841d7a3d2ed1ea16b9a1aa086cd96)

"https://apnews.com/article/coronavirus-pandemic-health-education-chicago-public-health-df7841d7a3d2ed1ea16b9a1aa086cd96"] (11/5) reported Chicago Public Schools officials sent a letter to parents and

families announcing "schools will be closed Nov. 12 for 'Vaccination Awareness Day.'" District CEO Pedro Martinez "said...Friday's canceled classes will provide 'an opportunity for parents and guardians to take their children five years of age and older to get vaccinated.'"

The [[HYPERLINK "https://www.chicagotribune.com/coronavirus/ct-chicago-coronavirus-vaccines-kids-schools-20211105-fxa5d4c5gba5djateisu2lurpu-story.html#ed=rss_www.chicagotribune.com/arcio/rss/category/news/breaking/"](https://www.chicagotribune.com/coronavirus/ct-chicago-coronavirus-vaccines-kids-schools-20211105-fxa5d4c5gba5djateisu2lurpu-story.html#ed=rss_www.chicagotribune.com/arcio/rss/category/news/breaking/)] (11/5, Pratt, Casanova, Yin, 2.03M) reported Chicago city employees will also "be let off work two hours early that day to get a vaccine shot or booster, an effort city officials said is aimed at increasing shots ahead of the Thanksgiving holiday."

Lawsuits Against Illinois Over Lack Of COVID-19 Vaccine Religious Exemption Accumulating.

The [[HYPERLINK "https://apnews.com/article/coronavirus-pandemic-us-supreme-court-business-illinois-lawsuits-72ecbfa1c21272c73bd8aa0d76b536e"](https://apnews.com/article/coronavirus-pandemic-us-supreme-court-business-illinois-lawsuits-72ecbfa1c21272c73bd8aa0d76b536e)] (11/7, O'Connor) reports, "Lawsuits against Illinois over required COVID-19 vaccination were accumulating and the reason was clear: the legal claims leverage of the state's Health Care Right of Conscience Act in stating religious objections to the shot and in appealing sanctions, including losing a job, that come with refusal." The Health Care Right of Conscience Act "has long been considered protection from repercussions against physicians who refuse on moral grounds to perform abortions." However, "Democrats' clarification, which has yet to be sent to [Gov.] Pritzker for his OK, would ensure that the law's prohibition on penalties for religious objections doesn't apply to COVID-19 vaccine refusal."

Los Angeles To Require Proof Of COVID-19 Vaccination To Enter Many Businesses.

The [[HYPERLINK "https://apnews.com/article/coronavirus-pandemic-entertainment-lifestyle-business-yoga-8a1373530c5bc46b396e6af9559911bb"](https://apnews.com/article/coronavirus-pandemic-entertainment-lifestyle-business-yoga-8a1373530c5bc46b396e6af9559911bb)] (11/7, Weber, Dazio) reports, "Los Angeles is among a growing number of cities...requiring people show proof of [COVID-19] vaccination to enter various types of businesses and venues." However, "rules in the nation's second-most-populous city, called SafePassLA, apply to more types of businesses and other indoor locations including museums and convention centers." The order takes effect Monday, but "city officials say they won't start enforcing it until Nov. 29 to give businesses time to adjust."

The [[HYPERLINK "https://www.latimes.com/food/story/2021-11-07/restaurants-vaccine-mandate-los-angeles"](https://www.latimes.com/food/story/2021-11-07/restaurants-vaccine-mandate-los-angeles)] (11/7, Harris, 3.37M) reports that "if a business doesn't comply with the ordinance, the first

response is a warning," and the "second violation could result in a \$1,000 fine." Subsequent "fines can escalate up to \$5,000."

Oklahoma City School District Fires Six Teachers For Refusing To Wear Masks.

The [[HYPERLINK "https://apnews.com/article/coronavirus-pandemic-health-oklahoma-oklahoma-city-392cf2a690d4c086758eb0151d8aa7c1"](https://apnews.com/article/coronavirus-pandemic-health-oklahoma-oklahoma-city-392cf2a690d4c086758eb0151d8aa7c1)] (11/7) reports, "The Oklahoma City school district violated state law by firing six teachers who refused to wear masks, according to Oklahoma Gov. Kevin Stitt." The governor said, "This is about a school district not following state law, this isn't a debate about masks."

Maryland Could Lose Federal Funding For Rent Because Of Its Failure To Distribute Aid.

The [[HYPERLINK "https://apnews.com/article/coronavirus-pandemic-larry-hogan-health-baltimore-chris-van-hollen-21f72c3fdf126eb3ec49db36557a6d16"](https://apnews.com/article/coronavirus-pandemic-larry-hogan-health-baltimore-chris-van-hollen-21f72c3fdf126eb3ec49db36557a6d16)] (11/6) reported, "Maryland failed to reach a federal benchmark early this fall for distributing COVID-19 relief funds it received for rent to tenants in danger of eviction, according to the US Treasury Department." The Department claims Maryland is "among the states that could lose some funding because it failed to spend 30% of the money by Sept. 30." US Sen. Chris Van Hollen (D-MD) and Sen. Ben Cardin (D-MD) wrote to "Housing & Community Development Secretary Kenneth Holt on Friday urging more disbursements." The letter indicated that "the state needs to act quickly 'to avoid having these funds revoked by the Treasury Department beginning on November 15th.'"

Kansas City Rescinds Mask Order.

The [[HYPERLINK "https://apnews.com/article/coronavirus-pandemic-health-kansas-city-missouri-efc22eef6245108cc5e723a764d7d273"](https://apnews.com/article/coronavirus-pandemic-health-kansas-city-missouri-efc22eef6245108cc5e723a764d7d273)] (11/6) reported, "Business operators in Kansas City, Missouri, are hopeful that the end of the city's mask mandate will mean a return of more customers." On Thursday, city council voted "to rescind the mask order, though it kept the mandate for children and adults in school."

Florida Judge Tosses School Districts' Lawsuit Over Mask Mandate Ban.

[[HYPERLINK "https://thehill.com/policy/healthcare/580363-judge-tosses-florida-school-districts-lawsuit-over-mask-mandate-ban"](https://thehill.com/policy/healthcare/580363-judge-tosses-florida-school-districts-lawsuit-over-mask-mandate-ban)] (11/5, Williams, 5.69M) reported, "A Florida judge tossed a challenge from several of the state's school districts over the Sunshine State's ban on school mask mandates." On Friday, "an administrative law judge ruled that officials from six counties failed to prove that the ban was 'an invalid exercise of delegated legislative authority.'"

Demand For COVID-19 Booster Shots Slower Than Expected In California.

The [[HYPERLINK "https://www.latimes.com/california/story/2021-11-05/far-fewer-californians-getting-covid-19-booster-shots-than-expected-bringing-new-alarms"](https://www.latimes.com/california/story/2021-11-05/far-fewer-californians-getting-covid-19-booster-shots-than-expected-bringing-new-alarms)] (11/5, Lin, Money, Smith, 3.37M) reported, "Demand for COVID-19 booster shots in California has been slower than some health officials anticipated...sparking new concerns of at-risk populations losing immunity as the risky holiday season approaches." As of late October, health officials reported that only 20% of eligible seniors received a booster in San Francisco, and just 27% of fully vaccinated seniors in Los Angeles County have done so.

West Virginia Grandparents Who Are Primary Caregivers For Grandchildren Could Receive School Voucher If Vaccinated Against COVID-19.

The [[HYPERLINK "https://apnews.com/article/coronavirus-pandemic-lifestyle-health-education-wv-state-wire-bfb2f403cc62ec5cfc3d725af7da3e51"](https://apnews.com/article/coronavirus-pandemic-lifestyle-health-education-wv-state-wire-bfb2f403cc62ec5cfc3d725af7da3e51)] (11/5) reported that on Friday, West Virginia Gov. Jim Justice (R) announced "grandparents who are the primary caregivers for their grandchildren age[d] 5 to 11 can receive a \$150 school voucher if they are vaccinated against COVID-19." To receive a voucher, each "family must be enrolled in West Virginia State University's Healthy Grandfamilies program."

Florida Governor Bobblehead Will Help Raise Money For Fight Against COVID-19.

The [[HYPERLINK "https://www.orlandosentinel.com/politics/os-neron-desantis-bobblehead-20211105-q2d3rtzogr4nkhys6ietzoam-story.html"](https://www.orlandosentinel.com/politics/os-neron-desantis-bobblehead-20211105-q2d3rtzogr4nkhys6ietzoam-story.html)] (11/5, Hylton, 599K) reported that on Friday, the National Bobblehead Hall of Fame and Museum announced its newest bobblehead – Florida Gov. Ron DeSantis (R). A \$5 donation will be made to the Protect the Heroes fund for each bobblehead sold. The organization helps "raise funds for hospitals in their fight against COVID-19."

Alabama Governor Signs Protections For Workers Unvaccinated Against COVID-19 Because Of Religious, Health Reasons.

The [[HYPERLINK "https://apnews.com/article/coronavirus-pandemic-legislature-health-kay-ivey-alabama-392af450ac7e5f7b7968afc55cf9f030"](https://apnews.com/article/coronavirus-pandemic-legislature-health-kay-ivey-alabama-392af450ac7e5f7b7968afc55cf9f030)] (11/5, Chandler) reported that on Friday, Alabama Gov. Kay Ivey (R) "signed employment protections for workers who claim a religious or health reason for not getting vaccinated against COVID-19." Under the new law, "state employers can't fire workers for being unvaccinated against COVID-19 if the employee

returns a new standardized state form to claim a religious, medical exemption."

Court Ruling Accelerates End Of Palm Beach County Schools' Mask Mandate.

The [[HYPERLINK "https://www.palmbeachpost.com/story/news/education/2021/11/06/palm-beach-county-school-mask-mandate-ends-monday-thanks-court-ruling/6321596001/"](https://www.palmbeachpost.com/story/news/education/2021/11/06/palm-beach-county-school-mask-mandate-ends-monday-thanks-court-ruling/6321596001/)] (11/6, Isger, 223K) reported, "Pressed by a court ruling Friday, the mask mandate in Palm Beach County schools will fall Monday, one week earlier than an already accelerated timeline and only days after vaccines became available to the district's youngest students."

Nearly All Philadelphia Nursing Homes Satisfy Mandate For Staff To Be Vaccinated Against COVID-19.

The [[HYPERLINK "https://www.inquirer.com/business/health/philadelphia-covid-19-vaccine-mandate-nursing-homes-report-20211106.html"](https://www.inquirer.com/business/health/philadelphia-covid-19-vaccine-mandate-nursing-homes-report-20211106.html)] (11/6, Brubaker) reported, "All but one of Philadelphia's 47 nursing homes have satisfied the city mandate to have all staff vaccinated against COVID-19 unless the workers have approved exemptions, the Philadelphia Department of Public Health said Friday." A survey sent to these 47 nursing homes found "that 8,460 of 9,313 nursing home workers, or 90.8%, were fully vaccinated."

Less Than Half Of San Francisco's COVID-19 Booster Shots Administered To Seniors.

The [[HYPERLINK "https://www.sfchronicle.com/health/article/Less-than-half-of-San-Francisco-s-COVID-19-16597161.php"](https://www.sfchronicle.com/health/article/Less-than-half-of-San-Francisco-s-COVID-19-16597161.php)] (11/6, Neilson, 2.44M) reported San Francisco Department of Health data show that as of November 1, "just under 23,000 of the city's approximately 118,000 seniors eligible to receive COVID-19 booster shots – about 1 in 5 – had received them." Additionally, the data indicate that less the half of the 52,000 booster shots administered by the city "have been given to people 65 and older."

Mississippi Behind On Spending Federal COVID-19 Relief.

The [[HYPERLINK "https://apnews.com/article/coronavirus-pandemic-business-jackson-state-governments-congress-646e85afb5e23bc6bca39bace316b12b"](https://apnews.com/article/coronavirus-pandemic-business-jackson-state-governments-congress-646e85afb5e23bc6bca39bace316b12b)] (11/6, Pettus) reported, "Mississippi is running behind many other states in deciding how to spend billions of federal dollars for pandemic relief." According to Lt. Gov. Delbert Hosemann (R), it is "imperative that the money go to 'transformational projects.'" Meanwhile, Northern District Public Service Commissioner Brandon Presley (D) "wrote in a recent newspaper column

that Mississippi needs to invest pandemic relief money in projects that could boost people's quality of life, including expansion of broadband access and improvement of water systems."

COVID-19 Vaccine Mandates Stirring Controversy At NIH.

The [[HYPERLINK "https://www.wsj.com/articles/vaccine-mandate-debate-makes-it-to-top-federal-research-agency-11636286400"](https://www.wsj.com/articles/vaccine-mandate-debate-makes-it-to-top-federal-research-agency-11636286400)] (11/7, Strasburg, Subscription Publication, 8.41M) reports COVID-19 vaccine mandates are causing controversy at the NIH. On December 1, the NIH's main bioethics department will host a roundtable session over the ethics of mandates.

NIH Launches Tool Recommending When Americans Should Get Tested For COVID-19.

The [[HYPERLINK "https://www.dailymail.co.uk/health/article-10170603/NIH-launches-new-tool-tell-Americans-not-tested-COVID-19.html?ito=1490"](https://www.dailymail.co.uk/health/article-10170603/NIH-launches-new-tool-tell-Americans-not-tested-COVID-19.html?ito=1490)] (11/5, Kekatos, 4.11M) reported, "The National Institutes of Health (NIH) has launched a new tool for Americans to use to determine whether or not they should get tested for COVID-19." The When To Test Calculator for Individuals "uses factors such as vaccination status, protective measures taken by the user and rates of spread where a person lives to recommend testing." The calculator "spits out results based on whether or not it determines someone is at low or high risk of contracting COVID-19 or spreading it to others."

NIH To Study Impacts Of COVID-19 On Pregnant Women.

[[HYPERLINK "https://www.foxnews.com/health/nih-study-covid-long-term-impact-pregnancy"](https://www.foxnews.com/health/nih-study-covid-long-term-impact-pregnancy)] (11/6, Musto, 23.99M) reported the NIH announced "it will study the long-term impact of COVID-19 on pregnant women." The four-year follow-up "study will enroll some participants from an earlier study by the Maternal-Fetal Medicine Units (MFMU) Network, which was supported by NIH's Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD)." Researchers "will also follow the offspring of those women to see whether or not there have been long-term health effects on them." The team "aims to understand what proportion of patients with COVID-19 in pregnancy are at risk for Long COVID, whether the severity of COVID-19 infection in pregnancy influences the likelihood of developing Long COVID and how the proportion of patients who develop Long COVID after COVID-19 in pregnancy compares to that of non-pregnant women who develop it."

Opinion: COVID-19 Exposes How Science Vs. Faith Disclosure A False Dichotomy.

In an opinion piece for the [[HYPERLINK "https://www.nytimes.com/2021/11/07/opinion/faith-science-covid.html"](https://www.nytimes.com/2021/11/07/opinion/faith-science-covid.html)]

(11/7, 20.6M), Anglican Church priest Tish Harrison Warren writes, "I have never had much interest in faith versus science debates," but "these past two years have exposed how the science vs. faith discourse isn't an abstract ideological debate but a false dichotomy that has disastrous real-world consequences." Warren interviewed BioLogos President Deborah Haarsma "to better understand this cultural division." NIH Director Francis Collins is the founder of BioLogos, "an organization that explores the relationship between faith and science." In fact, "some questioned whether Collins's religious faith should disqualify him from" his position at NIH. Haarsma concluded by saying "these two communities share a goal: seeking truth."

NIAID Funding Research To Assess Antibody Response To Additional COVID-19 Vaccine Dose In People With Autoimmune Diseases.

The [[HYPERLINK "https://www.bizjournals.com/triad/news/2021/11/07/triangle-organization-running-covid-vaccine-study.html?ana=RSS&s=article_search"](https://www.bizjournals.com/triad/news/2021/11/07/triangle-organization-running-covid-vaccine-study.html?ana=RSS&s=article_search)] (11/7, Ezzone, Subscription Publication, 845K) reports the NIAID is funding a trial "which will assess the antibody response to an additional COVID-19 vaccine dose in people with an autoimmune disease who did not respond to an initial vaccine regimen." The study "will also investigate whether pausing immunosuppressive therapy improves the antibody response to an additional COVID-19 vaccine dose." Rho, a long-term partner of NIH, will serve "as the clinical coordinating center."

NIH Director Says Long COVID Research Could "Improve Our Understanding Of Other Chronic Post-Viral Syndromes."

The [[HYPERLINK "https://www.washingtonpost.com/health/2021/11/07/long-covid-fatigue-research/"](https://www.washingtonpost.com/health/2021/11/07/long-covid-fatigue-research/)] (11/7, Sellers, 10.52M) reports, "The National Institutes of Health earlier this year launched a \$1.15 billion initiative to study long covid, and researchers and patients hope it will provide answers." However, "NIH Director Francis S. Collins said the \$1.15 billion investment in research would go beyond long covid to 'improve our understanding of other chronic post-viral syndromes and autoimmune diseases, as well as other diseases with similar symptoms.'"

FDA IN THE NEWS

New FDA Commissioner Must Restore Trust To Agency, Former FDA Officials Say.

[[HYPERLINK "https://news.bloomberglaw.com/pharma-and-life-sciences/mending-trust-on-tap-for-next-fda-head-as-](https://news.bloomberglaw.com/pharma-and-life-sciences/mending-trust-on-tap-for-next-fda-head-as)

clock-ticks-on-nominee"] (11/5, Castronuovo, Subscription Publication, 4K) reported "a new permanent commissioner at the" FDA "will need to bring more clarity to the government's pandemic response and restore trust to an agency that remains without a Senate-confirmed leader nearly a year into the Biden administration, policy analysts and former FDA officials say." President "Biden has just weeks left to name a replacement for acting Commissioner Janet Woodcock, who was ruled out for the post due to opposition on Capitol Hill." Former FDA Deputy Chief of Staff Kalah Auchincloss said, "It's hard to have a vision and goals that you want to achieve as an acting commissioner; you're more just keeping the ship steered in the right direction." Former FDA Commissioner Robert Califf "is being reported as the top contender to lead the agency once again." Former FDA Commissioner Scott Gottlieb and Howard Sklamberg, who worked as the FDA's deputy commissioner for global regulatory operations, were mentioned.

US House Committee's Opioid Crisis Probe Focuses On Consulting Firm.

The [[HYPERLINK "https://www.nytimes.com/2021/11/05/business/congress-mckinsey-opioid-crisis.html"](https://www.nytimes.com/2021/11/05/business/congress-mckinsey-opioid-crisis.html)] (11/5, Bogdanich, Forsythe, 20.6M) reported the US House Committee on Oversight and Reform is conducting an opioid crisis-related investigation that is focused on the consulting firm McKinsey. The committee has asked the company to provide it with records that relate to this matter. On Friday, an unnamed McKinsey spokesperson said the company "will engage directly with the committee" as it handles that request. The article mentions the FDA.

[[HYPERLINK "https://r.bulletinintelligence.com/7b36d43170c54545b382767b77fa219f"](https://r.bulletinintelligence.com/7b36d43170c54545b382767b77fa219f)] (11/5, Brennan) reported Sen. Maggie Hassan (D-NH) "directly questioned FDA acting commissioner Janet Woodcock on Thursday about how the FDA didn't know until recently about its conflicting work with McKinsey, which was also consulting for opioids manufacturers while working on the FDA's track-and-trace system. But Woodcock said that she did know, and she said she didn't think there was anything improper going on." Woodcock said: "I was aware, at that time that McKinsey was only doing consulting work of an administrative nature for the FDA. ... Nothing to do with any product or standard or anything like that."

Endo Pharmaceuticals Discontinues Cheaper Version Of Treatment For Central Precocious Puberty.

[[HYPERLINK "https://www.npr.org/sections/health-shots/2021/11/06/1051878026/drugmaker-drops-cheaper-version-of-drug-leaving-patients-stuck-with-pricier-one"](https://www.npr.org/sections/health-shots/2021/11/06/1051878026/drugmaker-drops-cheaper-version-of-drug-leaving-patients-stuck-with-pricier-one)] (11/6, Lupkin, 3.69M) reported Endo Pharmaceuticals is

discontinuing its medication for central precocious puberty histrelin acetate (Vantas), forcing patients to turn to the more expensive version of the drug, Supprelin LA. The company "told NPR there was a manufacturing problem." NPR added, "It's hard to know exactly how many children will be affected by the Vantas discontinuation because data about off-label use is hard to come by." FDA spokesman Jeremy Kahn wrote in an email: "The FDA expects that manufacturers will notify the agency before a meaningful disruption in their own supply occurs."

Gottlieb Says COVID-19 Pandemic In US Could Be Over By The Time Biden's Workplace Vaccine Mandates Take Effect.

[[HYPERLINK "https://www.cnbc.com/2021/11/05/pfizer-board-member-gottlieb-says-the-covid-pandemic-could-be-over-in-the-us-by-january-.html"](https://www.cnbc.com/2021/11/05/pfizer-board-member-gottlieb-says-the-covid-pandemic-could-be-over-in-the-us-by-january-.html)] (11/5, Towey, 7.34M) reported, "The COVID-19 pandemic could be over in the US by the time President Joe Biden's workplace vaccine mandates take effect in early January, Pfizer board member Dr. Scott Gottlieb told CNBC's 'Squawk Box' on Friday." OSHA's COVID-19 vaccine mandates "begin on Jan. 4 for any company with at least 100 employees."

[[HYPERLINK "https://thehill.com/policy/healthcare/580298-gottlieb-says-pandemic-in-us-could-be-over-by-january"](https://thehill.com/policy/healthcare/580298-gottlieb-says-pandemic-in-us-could-be-over-by-january)] (11/5, Sullivan, 5.69M) reported, "Former Food and Drug Administration Commissioner Scott Gottlieb said Friday that he thinks the COVID-19 pandemic in the United States could be over by January, transitioning to a lower-level 'endemic' presence." COVID-19 "is not expected to disappear entirely, but it could settle into a lower-level, constant presence that does not cause the disruption and harm of the past year and a half."

[[HYPERLINK "https://www.newsweek.com/gottlieb-safe-partially-vaxxed-kids-close-grandparents-thanksgiving-1646800"](https://www.newsweek.com/gottlieb-safe-partially-vaxxed-kids-close-grandparents-thanksgiving-1646800)] (11/7, Zhao, 2.67M) reports Gottlieb "said Sunday that families can safely gather for the Thanksgiving holiday, with partially vaccinated children in close proximity around their vaccinated grandparents, depending on local case rates in their community."

[[HYPERLINK "https://www.axios.com/gottlieb-pandemic-end-close-56a7ef11-6030-4404-9c26-5ff0b892e6d2.html"](https://www.axios.com/gottlieb-pandemic-end-close-56a7ef11-6030-4404-9c26-5ff0b892e6d2.html)] (11/7, 1.26M) also covers the story.

Gottlieb Expects "Broad Immunity" Among Younger Children As More Get Vaccinated Against COVID-19.

[[HYPERLINK "https://www.cbsnews.com/news/scott-gottlieb-broad-immunity-children-covid-19-vaccine-face-the-nation/"](https://www.cbsnews.com/news/scott-gottlieb-broad-immunity-children-covid-19-vaccine-face-the-nation/)] (11/7, Hayes, 5.39M) reports, "Dr. Scott Gottlieb, the former commissioner of the Food and Drug Administration who now serves on Pfizer's board of directors, said Sunday that he

expects 'broad immunity' against COVID-19 among younger children as more get vaccinated." The CDC "recommended the pediatric version of Pfizer's COVID-19 vaccine for children 5 to 11 years old" last week. Throughout the country, "vaccine clinics and pediatricians' offices...have already received shipments of the smaller vaccine and started putting shots in arms."

[[HYPERLINK "https://thehill.com/homenews/sunday-talk-shows/580447-gottlieb-not-expecting-travel-from-outside-us-to-feed-a-lot-of"](https://thehill.com/homenews/sunday-talk-shows/580447-gottlieb-not-expecting-travel-from-outside-us-to-feed-a-lot-of)] (11/7, Schnell, 5.69M) reports, "Gottlieb on Sunday said he is not expecting travel from outside the country to 'feed a lot of additional infections,' one day before the US is set to lift border restrictions for fully vaccinated international travelers."

MEDICAL PRODUCT SAFETY

Study Suggests MDMA May Improve Outcomes Among Individuals With Severe PTSD.

[[HYPERLINK "https://www.today.com/health/mdma-ptsd-study-veteran-shares-experience-rcna4495"](https://www.today.com/health/mdma-ptsd-study-veteran-shares-experience-rcna4495)] (11/5, Carroll, 2.24M) reported that 67% of individuals with severe post-traumatic stress disorder (PTSD) who received MDMA "no longer qualified for a diagnosis of PTSD two months after the treatment," according to [[HYPERLINK "https://www.nature.com/articles/s41591-021-01336-3"](https://www.nature.com/articles/s41591-021-01336-3)] published in Nature Medicine. Those findings may "help get PTSD therapy with MDMA approved by the Food and Drug Administration." TODAY added, "According to the National Institute on Drug Abuse, MDMA is also in clinical trials as a possible treatment aid for anxiety in terminally ill patients and for social anxiety in autistic adults. The FDA recently classified MDMA-assisted psychotherapy for PTSD as a 'breakthrough therapy.'"

FDA Grants Breakthrough Device Designation To ReWalk For ReBoot Product.

[[HYPERLINK "https://www.fiercebiotech.com/medtech/rewalk-lands-fda-breakthrough-label-for-at-home-exoskeleton-device-to-restore-post-stroke"](https://www.fiercebiotech.com/medtech/rewalk-lands-fda-breakthrough-label-for-at-home-exoskeleton-device-to-restore-post-stroke)] (11/5, Park, 4K) reported, "ReWalk Robotics is picking up the pace on its trek down the FDA's review pathway." The FDA "has granted ReWalk's latest offering a breakthrough device designation" for Reboot, "a soft exoskeleton that's designed to be worn by patients undergoing rehabilitation for lower limb disabilities caused by stroke and other neurological injuries." This designation gives "the company access to an expedited evaluation." Additionally, "once ReWalk has submitted its regulatory application for the ReBoot system, not only will the FDA prioritize the submission, but experts from the agency will also keep in touch with ReWalk throughout the review so any

issues can be addressed as quickly and efficiently as possible."

FDA Panel Rejects Nasal Spray For Treating PWS.

[[HYPERLINK "https://www.medpagetoday.com/endocrinology/generalendocrinology/95477"](https://www.medpagetoday.com/endocrinology/generalendocrinology/95477)] (11/5, Grant, 183K) reported, "An investigational carbetocin nasal spray for treating extreme hunger and other symptoms of Prader-Willi syndrome (PWS), a rare genetic disorder, received a near-unanimous thumbs-down from FDA advisors on Thursday." For some advisors, "the decision to vote 'no' came down to how FDA framed the question at hand – in short, whether there was 'substantial evidence' of efficacy with the product." NHLBI affiliate James Troendle, PhD, "emphasized the importance of the question's wording before explaining his 'no' vote." He said, "I don't even think they even met a low bar for evidence, let alone 'substantial' evidence – it's far from that."

Novel Drug For Patients With Anemia From CKD Succeeds In Two Phase 3 Trials.

[[HYPERLINK "https://www.fiercepharma.com/pharma/glaxosmithkline-s-novel-anemia-drug-succeeds-where-astrazeneca-fibrogen-akebia-failed"](https://www.fiercepharma.com/pharma/glaxosmithkline-s-novel-anemia-drug-succeeds-where-astrazeneca-fibrogen-akebia-failed)] (11/5, Liu, 12K) reported, "After the FDA's resounding rejection for AstraZeneca and FibroGen's roxadustat, attention on novel oral anemia therapies has shifted to later players," and "now, third-in-line GlaxoSmithKline has notched up a first-in-class success." For "dialysis-dependent and nondialysis patients with anemia from chronic kidney disease, GSK's daprodustat matched up to traditional erythropoiesis-stimulating agents (ESAs) in terms of heart safety, as measured by a composite marker of time to first major adverse cardiovascular event." The data, "from two phase 3 trials, ASCEND-D and ASCEND-ND, were unveiled at American Society of Nephrology Kidney Week 2021."

Opinion: Drug Pricing Legislation Should Preserve Savings From Generics, Biosimilars.

Association for Accessible Medicines President and CEO Dan Leonard wrote in an opinion for [[HYPERLINK "https://www.statnews.com/2021/11/06/hurried-bills-congress-shouldnt-undermine-vast-savings-generic-biosimilar-drugs/"](https://www.statnews.com/2021/11/06/hurried-bills-congress-shouldnt-undermine-vast-savings-generic-biosimilar-drugs/)] (11/6, 262K), "Americans rightfully expect Congress to address the ever-increasing prices of brand-name prescription drugs." However, Leonard cautioned that "the wholesale policy changes now under rushed consideration in Congress will undermine the only proven solution to this long-standing problem: competition from Food and Drug Administration-approved generic and biosimilar medicines." He said that while "Congress is right to try to

meet the expectations of a nation concerned about the high prices of brand-name drugs...policies to reform drug pricing should be focused on ensuring that Americans have access to more-affordable medicines by eliminating barriers and improving incentives for competition."

Columnist: Science Should Not Have To Depend On 20th-Century Institutions To Guide 21st-Century Progress.

In his column for [[HYPERLINK](https://www.theatlantic.com/ideas/archive/2021/11/grants-american-scientific-revolution/620609/) "https://www.theatlantic.com/ideas/archive/2021/11/grants-american-scientific-revolution/620609/"] (11/5, 3.53M), Derek Thompson wrote, "Two stories in science are worth cheering right now: the amazing amount of knowledge humanity is gathering about COVID-19 and the quietly revolutionary ways we're accelerating the pace of discovery." He added that last year, George Mason University economist Tyler Cowen and Stripe CEO Patrick Collison "co-founded a new program for quickly funding scientific research on COVID-19." Dubbed Fast Grants, they "aimed to solve the speed problem in several ways." For example, "Its application process was designed to take half an hour, and many funding decisions were made within a few days." Thompson wrote, "The US relies on a fleet of scientific agencies – the CDC, FDA, NIH, and NSF – that are decades old and that, in many cases, act their age." He concluded, "We shouldn't have to depend on 20th-century institutions to guide 21st-century progress."

FOOD SAFETY

Tastykake Krimpets Recalled Over Possible Metal Pieces.

[[HYPERLINK](https://www.cbsnews.com/news/tastykake-recall-krimpets-cupcakes-metal-pieces-cake/) "https://www.cbsnews.com/news/tastykake-recall-krimpets-cupcakes-metal-pieces-cake/"] (11/5, 5.39M) reported, "Tastykake cake treats sold by retailers across the U.S. are being added to a recall of cupcakes in more than half a dozen states because the baked goods could contain tiny fragments of metal mesh wire, according to a notice posted by the Food and Drug Administration." The cakes "have been added to the list of products being recalled by Thomasville, Georgia-based Flowers Foods, the maker of Tastykakes and one of the biggest U.S. producers of packaged bakery foods."

The [[HYPERLINK](https://www.miamiherald.com/news/recalls/article255620686.html#storylink=mainstage_lead) "https://www.miamiherald.com/news/recalls/article255620686.html#storylink=mainstage_lead"] (11/7, Neal, 647K) also covers the story.

Jumbo Cooked Shrimp Recalled For Possible Listeria.

The [[HYPERLINK](https://www.miamiherald.com/news/recalls/article255598611.html#storylink=mainstage_card) "https://www.miamiherald.com/news/recalls/article255598611.html#storylink=mainstage_card"] (11/6, Cohen, 647K) reported, "Southeastern Grocers, parent company of Winn-

Dixie, Fresco y Más and Harveys Supermarkets, has recalled its Fisherman's Wharf brand Jumbo Cooked Shrimp that is sold frozen in 16-20 count, 16-ounce bags" for possible listeria. The issue "with these affected bags was found after routine testing detected listeria in the 16-20 count, 16-ounce bags of the jumbo cooked shrimp, according to the U.S. Food and Drug Administration's recall alert issued on Nov. 5."

Effects Of Poppy Seed Consumption Highlighted.

According to [[HYPERLINK](https://r.bulletinintelligence.com/9f12210cb6414f2cb052828f891f679f) "https://r.bulletinintelligence.com/9f12210cb6414f2cb052828f891f679f"] (11/6, Wells, 645K), "A sprinkling of poppy seeds in a muffin or over a bagel may sound utterly innocent, but the consumption of these seeds is still regularly flagged by urine tests as opium," and "the National Institute of Drug Abuse suggests skipping a poppy seed snack altogether before a drug test, just to be safe." Sam Houston State University assistant professor of forensic toxicology Madeleine Swortwood said, "What came to our attention a few years ago was that there were companies that were intentionally not washing the seeds, not processing the seeds, so that they could sell seeds that were essentially laced with morphine and codeine." However, she added, "The FDA and DEA have done a really good job of shutting down some of those companies that might be selling the unwashed poppy seeds. As long as you're buying them at grocery stores and major retailers they should be fairly safe."

TOBACCO

Democrats' Spending Bill Imposes Heavy Taxes On E-Cigarettes And Vapes.

[[HYPERLINK](https://www.foxbusiness.com/politics/democrats-spending-bill-nicotine-tax-cripple-vaping-industry) "https://www.foxbusiness.com/politics/democrats-spending-bill-nicotine-tax-cripple-vaping-industry"] (11/4, Hasson, 3.06M) reported, "The latest version of Democrats' Build Back Better Act is poised to cripple the multi-billion dollar American e-cigarette and vaping industry and could drive tobacco-free nicotine pouches from the market altogether, experts warn." The current proposal "leaves tobacco taxes untouched, while imposing heavy taxes on e-cigarettes and vapes, as well as tobacco-free nicotine pouches and lozenges." The provision is "intended to help fund Democrats' massive proposed social spending package by trying to bring taxes on tobacco-free nicotine products in line with the tax rates on tobacco products." However, critics find the method "ill-conceived in its attempt to treat tobacco products and tobacco-free alternatives the same," and say "many tobacco-free products will be at a severe disadvantage to similar tobacco products."

[[HYPERLINK](https://reason.com/2021/11/05/democrats-hike-taxes-on-vaping-but-not-tobacco-in-latest-version-of-build-back-better-bill/) "https://reason.com/2021/11/05/democrats-hike-taxes-on-vaping-but-not-tobacco-in-latest-version-of-build-back-better-bill/"] (11/5, 350K) reported, "The proposed nicotine tax

would raise about \$10 billion at most" and possibly "much less given the Food and Drug Administration's current crackdown on which vaping products are allowed in the market."

FDA Rescinds MDO For Humble Juice E-Liquids.

The [[HYPERLINK "https://tobaccoreporter.com/2021/11/05/fda-rescinds-marketing-denial-for-humble-juice-e-liquids/"](https://tobaccoreporter.com/2021/11/05/fda-rescinds-marketing-denial-for-humble-juice-e-liquids/)] (11/5) reported, "The U.S. Food and Drug Administration has rescinded the marketing denial order (MDO) issued Sept. 15, 2021, for Humble Juice Co.'s flavored e-liquid products, the company announced on Nov. 5." In October, Humble filed a petition "challenging the FDA's decision and seeking to have the MDO vacated." The FDA stated "that upon further review it identified information contained in Humble's PMTA that requires additional evaluation such as 'randomized controlled trials comparing tobacco-flavored ENDS to flavored ENDS as well as several cross-sectional surveys evaluating intentions to use or likelihood of use in current smokers, current ENDS users, former tobacco users, and never users.'"

Vuse Closing Market-Share Gap With Juul.

The [[HYPERLINK "https://journalnow.com/business/local/reynolds-vuse-closing-gap-with-top-selling-e-cigarette-juul/article_9e9de9b4-3bf6-11ec-bd91-cf6bd160ac28.html"](https://journalnow.com/business/local/reynolds-vuse-closing-gap-with-top-selling-e-cigarette-juul/article_9e9de9b4-3bf6-11ec-bd91-cf6bd160ac28.html)] (11/5, Craver, 226K) reported, "The market-share gap tightened slightly again between top-selling electronic cigarette Juul and No. 2 Vuse of R.J. Reynolds Vapor Co. in the latest Nielsen analysis of convenience store data." In its report covering "the four-week period ending Oct. 23," Nielsen found "Vuse had a 34.3% market share, up from 33.3% in the previous report." Juul was "at 40.6%, down from 41% in the previous report." Sales of e-cigarette products "overall have slumped since February 2020, when the Food and Drug Administration implemented its latest round of heightened regulations on the products."

Baby Monkey Forced To Smoke Cigarette For Public Health Campaign, Sparking Backlash.

The [[HYPERLINK "https://www.dailymail.co.uk/news/article-10168635/Baby-monkey-forced-smoke-cigarette-Chinese-zoo.html"](https://www.dailymail.co.uk/news/article-10168635/Baby-monkey-forced-smoke-cigarette-Chinese-zoo.html)] (11/5, Davies, 4.11M) reported a Chinese zoo "has sparked outrage after forcing a baby monkey to smoke a cigarette on camera as part of a supposed public health campaign." In a promotional video, a monkey is seen smoking and "a woman off-screen removes the cigarette and tells off the animal, saying smoking is harmful." The video sparked backlash with many saying "the video was cruel." The FDA has "said animals exposed to cigarette smoke have been found to be at higher risk of illnesses such as pneumonia, eye problems, heart disease, and cancer."

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COVID-19

Pfizer Says Its Antiviral Pill Reduces Hospitalizations, Deaths Among High-Risk Patients With COVID-19 By 89%.

Bloomberg (11/5, Langreth, 3.57M) reported Pfizer on Friday "said its Covid-19 pill [Paxlovid] reduced hospitalizations and deaths in high-risk patients by 89%, a result that has the potential to upend how the disease caused by the coronavirus is treated and alter the course of the pandemic." The drugmaker "said...it was no longer taking new patients in a clinical trial of the treatment 'due to the overwhelming efficacy' and planned to submit the findings to U.S. regulatory authorities for emergency authorization as soon as possible." Reuters (11/5, Beasley) said the findings "suggest that Pfizer's drug surpasses Merck & Co Inc's pill, molnupiravir, which was shown last month to halve the chance of dying or being hospitalized for COVID-19 patients at high risk of serious illness." The New York Times (11/5, A1, Robbins, 20.6M) reported Merck's drug "was about 50 percent effective when given within five days of the onset of symptoms, though the different designs and timing of the Pfizer and Merck trials make such comparisons imprecise."

According to the Wall Street Journal (11/5, A1, Hopkins, Subscription Publication, 8.41M), Pfizer will ask the FDA this month to authorize the drug's use. The AP (11/5, Perrone) reported that "once Pfizer applies, the FDA could make a decision within weeks or months," and the Washington Post (11/5, A1, Johnson, 10.52M) said if the FDA finds the therapy is "safe and effective," they "are expected to become available right away." USA Today (11/5, Weintraub, 12.7M) said, "An FDA advisory committee is scheduled to meet later this month to discuss the companies' request for an emergency use authorization for molnupiravir."

On ABC World News Tonight (11/5, story 2, 2:06, Muir, 5.81M), Whit Johnson hailed Pfizer's pill as "a potential new weapon in the fight against COVID." Johnson added, "Unlike monoclonal antibody treatments, which are given through an IV at a hospital or clinic, the antiviral pills could be taken at home over five days soon after a person tests positive for COVID. Merck's drug is on track for potential authorization by the end of the year, Pfizer's by early 2022." Gabe Gutierrez reported on NBC Nightly News (11/5, story 3, 1:44, Holt, 4.81M) that patients would take Pfizer's pill within three days of symptoms. CNBC (11/5, Lovelace, 7.34M) reported on its website that Pfizer's pill, "scientifically known as PF-07321332, is part of a class of medicines called protease inhibitors and works by inhibiting an enzyme the virus needs to replicate in human cells."

According to ABC News (11/5, Salzman, 2.44M), Pfizer's findings come "from one of three clinical trials that the company is running. The results from the other two trials are expected by the end of the year. Pfizer then plans to submit all the data and seek authorization at that time, meaning the new medication may be available in early 2022." CNBC (11/5, Lovelace, 7.34M) reported, "Pfizer said there were six hospitalizations and zero deaths out of the 607 trial participants who received the pill in combination" with an antiviral drug called ritonavir. That contrasts "with 41 hospitalizations and 10 deaths out of the 612 people who received a placebo." CNN (11/5, Fox, Sealy, 89.21M) reported Pfizer "said 19% of patients given the treatment suffered adverse events, compared to 21% who got placebo, but declined to disclose what those adverse events were."

However, Axios (11/5, Falconer, 1.26M) says the announcement comes with "some caveats. Pfizer's findings came through a corporate press release, not a peer-reviewed journal, and federal regulators haven't had a chance to scrutinize the drug's clinical trial data yet."

According to The Hill (11/5, Vakil, 5.69M), President Biden's "chief medical adviser Anthony Fauci has previously warned that the Merck drug should not be considered an alternative to the COVID-19 vaccine, it could help alleviate the possibility of escalating COVID-19 cases." Meanwhile, Bloomberg (11/5, Griffin, 3.57M) reported Pfizer CEO Albert Bourla on Friday "said Pfizer hasn't yet disclosed how it will price the antiviral pill but said it would cost less than Covid-19 antibody treatments."

Additional outlets reporting included: CBS Evening News (11/5, story 4, 0:34, O'Donnell, 3.62M), NBC News (11/5, Edwards, 4.91M), NPR (11/5, Chappell, 3.69M), Bloomberg Law (11/5, Subscription Publication, 4K), the Boston Herald (11/5, Cohan, 327K), Forbes (11/5, Knapp, 10.33M), Newsweek (11/5, 2.67M), the New York Daily News (11/5, Schladebeck, 2.51M), the New York Post (11/5, Feuer, 7.45M), STAT (11/5, Herper, 262K), US News & World Report (11/5, 1.91M), Science (11/5, Couzin-Frankel, 484K), FierceBiotech (11/5, Taylor, 4K), and the Financial Times (11/5, Kuchler, Subscription Publication, 1.48M).

Administration To Defend OSHA COVID-19 Vaccine Requirement For Business. ABC World News Tonight (11/7, 6:37 p.m. EST, story 2, 2:00, Johnson, 3.92M) reported, "Tonight, the Administration firing back after an appeals court blocked the requirement, questioning its constitutionality. ABC's Phil Lipof reports." ABC (Phil Lipof) added, "And tonight, the White House is not backing down as the Justice Department vowing to defend the Biden Administration's vaccine mandate for all businesses with 100 employees or more, after a federal appeals court blocked it this weekend, questioning its constitutionality." Dr. Vivek Murthy, US Surgeon General, said, "It's good for people's health, it's good for the economy. That's why these requirements make so much sense."

The New York Times (11/7, Imbler, 20.6M) reports that Surgeon General Murthy said that the Administration will defend the rules. Appearing on ABC's "This Week," Dr. Murthy said, "The president and the administration wouldn't have put these requirements in place if they didn't think that they were appropriate and necessary." The Times also reports that White House chief of staff Ron Klain "said he was 'quite confident' the mandate would be upheld in an interview on NBC's 'Meet the Press.'"

The Hill (11/7, Vakil, 5.69M) reports on Sunday that Klain, appearing on NBC's "Meet The Press," said, "I'm quite confident that when this finally gets fully adjudicated, not just a temporary order, the validity of this requirement will be upheld. It's common sense...if OSHA can tell people to wear a hard hat on the job, to be careful around chemicals, it can put in place these simple measures to keep our workers safe."

Reuters (11/6, Scarcella) reports Solicitor of Labor Seema Nanda "said the Labor Department was 'confident in its legal authority' to issue the rule," as does the AP (11/6), which also quotes Justice Department spokesman Anthony Coley as saying, "The OSHA emergency temporary standard is a critical tool to keep America's workplaces safe as we fight our way out of this pandemic. The Justice Department will vigorously defend this rule in court."

In addition, Fox Business (11/6, 3.06M) reports on its website that White House Deputy Press Secretary Karine Jean-Pierre "responded to a question at a Friday press briefing about whether or not she is confident the mandate will stand up to legal challenges." She said, "We are very confident that it can." She continued, "As for the legal side of this, let me be crystal clear to avoid what appears to be possible misinformation or disinformation around the emergency temporary standard being a vaccine mandate. That would be on its face incorrect as has been explicit for months. It is a standard for safe workplace to either comply with weekly testing or to be vaccinated."

The New York Post (11/5, Nelson, 7.45M) reported that "Jean-Pierre on Friday said that it's 'misinformation' to use the term 'vaccine mandate' to describe a requirement that most private-sector workers get vaccinated, submit to regular testing or lose their jobs." Instead, she called it a "vaccination requirement."

Axios (11/6, 1.26M) and the Washington Times (11/7, 626K) also covered the story.

Fifth Circuit Issues Stay Of OSHA COVID-19 Vaccine Requirement For Businesses. The Washington Post (11/6, Rosenberg, 10.52M) reported "a panel of three judges appointed by Republican presidents" of the US Court of Appeals for the Fifth Circuit on Saturday "suspended the Biden administration's new vaccine requirement for private companies, delivering a major blow for one of the White House's signature attempts to increase the number of vaccinations to corral the pandemic."

The New York Times (11/6, Hirsch, Paz, 20.6M) called the order "a sign that the Biden administration may face an uphill battle in its biggest effort yet to combat the virus among the American work force." The Times says the stay "doesn't have an immediate impact" since "the first major deadline in the new rule is Dec. 5, when companies with at least 100 employees must require unvaccinated employees to wear masks indoors," and "businesses have until Jan. 4 to mandate Covid vaccinations or start weekly testing of their workers," but it "provided momentum for a wide coalition of opponents of the rule, who have argued that it is unconstitutional."

The Hill (11/6, Lonas, 5.69M) reported the ruling came "after Texas Attorney General Ken Paxton (R) filed a challenge to the mandate requiring employers with over 100

or more employees to get vaccinated or undergo frequent testing directly with the court."

Bloomberg (11/6, Brubaker Calkins, 3.57M) reported the request for the stay "came late Friday from more than two dozen private companies suing the U.S. Occupational Safety and Health Administration and Labor Department, as part of a court challenge led by Texas, Louisiana, Utah and South Carolina."

CNBC (11/6, Kimball, 7.34M) noted the judges "who issued the order – Stuart Kyle Duncan, Kurt Damian Englehardt and Edith Hollan Jones – were appointed by former Presidents Donald Trump and Ronald Reagan."

The Hill (11/7, Gangitano, Weixel, 5.69M) reports, "Administration officials insist they are on solid legal footing, but it's not stopping business groups, religious organizations and GOP state attorneys general from filing a barrage of lawsuits or Republican lawmakers from threatening legislative actions" after "the Labor Department on Thursday released the sweeping vaccine-or-test mandate, which President Biden had previously announced was coming." The Hill adds, "A coalition of 11 states led by Missouri Attorney General Eric Schmitt (R), who is running for Senate, argued the Occupational Safety and Health Administration (OSHA) doesn't have the authority to put in place such sweeping federal public health regulations and that the rule unconstitutionally infringes on states' powers." Meanwhile "a separate group led by West Virginia Attorney General Patrick Morriesy (R) filed a similar lawsuit in the 6th Circuit." On Friday, White House deputy press secretary Karine Jean-Pierre "said the administration is 'very confident' the mandate can withstand legal challenges."

Politico (11/6, Rainey, 6.73M) reported "more than two dozen states have filed multiple legal challenges in federal court against the Biden administration's vaccinate-or-test mandate for private businesses, arguing that the Occupational Safety and Health Administration doesn't have the authority to issue the requirements." Politico adds the four lawsuits "were filed by groups of 26 states in the 8th Circuit, 11th Circuit, 6th Circuit and 5th Circuit over the past few days." Politico says the lawsuits "broadly...argue that the Department of Labor lacks the authority to issue a rule and that it did not follow the proper procedure in issuing the emergency temporary standard."

The Helena (MT) Independent Record (11/5, Wilson, 97K) reported that Montana Attorney General Austin Knudsen "joined a lawsuit filed by 11 states to block the Biden administration's new rule requiring businesses with 100 or more employees to mandate COVID-19 vaccinations in the workplace or conduct weekly coronavirus testing."

Bloomberg (11/7, 3.57M) reports, "President Joe Biden's requirement for employees at companies with more than 100 workers to get Covid-19 vaccinations or regular testing is on solid legal ground, presidential adviser Cedric

Richmond said" in an appearance on CBS's "Face the Nation" on Sunday.

However, CNN (11/6, Vogue, Janfaza, 89.21M) reported that Sean Marotta, a lawyer at Hogan Lovells, "said he was surprised that the Fifth Circuit Court of Appeals acted to block the mandate on a Saturday, particularly because the mandate does not require employers or employees to act for months."

The Wall Street Journal (11/6, Kendall, Siddiqui, Subscription Publication, 8.41M) provided similar coverage as did Fox Business (11/6, 3.06M) and the San Francisco Chronicle (11/7, Said, 2.44M).

Employees Not Allowed To Self-Administer And Self-Report Tests. The Wall Street Journal (11/5, Calfas, Cutter, Subscription Publication, 8.41M) reported that under the rule, employees will be not be able to self-administer and self-report their COVID-19 tests. Instead they must be observed by the employer or another authorized person.

OSHA Seeking Comment On Whether To Extend COVID-19 Vaccine And Testing Requirement To Businesses With Fewer Than 100 Employees. Fox Business (11/5, Olson, 3.06M) reported, "The Occupational Safety and Health Administration (OSHA) says it is 'seeking comment' about whether businesses with fewer than 100 employees should be subject to the COVID-19 vaccine and testing mandate it is implementing on big businesses." The current rule "will force businesses with more than 100 employees to mandate vaccines or else their employees will need to wear masks and be tested for COVID-19 weekly."

OSHA Report Says People With Prior COVID-19 Case Less Protected Than Those With Vaccine. Fox Business (11/6, Aitken, 3.06M) reported, "The Occupational Safety and Health Administration (OSHA) has issued a report in which it explains that even if a person had COVID-19, they face significant danger from the virus." OSHA stated in the report, "A considerable number of individuals who were previously infected with SARS-CoV-2 do not appear to have acquired effective immunity to the virus."

Republicans In Congress To Use Congressional Review Act To Vote Against COVID-19 Vaccine Rule. Fox News (11/5, McFall, 23.99M) reported Rep. Fred Keller (R-PA), ranking member of the Education and Labor Subcommittee on Workforce Protections, "will take steps next week to block President Biden's 'unconstitutional' coronavirus vaccine mandate." Keller said that in cooperation with Sen. Mike Braun (R-IN), they "will attempt to use the Congressional Review Act to nullify the executive order." Braun had previously announced that he had the backing of "at least 42 other Senate Republicans in challenging the president's executive order."

Kansas Governor Opposes OSHA COVID-19 Vaccine Requirement. The AP (11/5, Field) reported that Kansas Gov. Laura Kelly (D) "grew more forceful Friday in

opposing President Joe Biden's COVID-19 vaccine mandates, saying it's 'too late' in the coronavirus pandemic to impose them after Kansas and other states tailored responses to their needs." Kelly said, "While I appreciate the intention to keep people safe, a goal I share, I don't believe this directive is the correct, or the most effective, solution for Kansas." Also on Friday, Kansas Attorney General Derek Schmidt (R), "who hopes to unseat Kelly next year, announced that Kansas is among seven states that filed a federal lawsuit with the 6th U.S. Circuit Court of Appeals in Cincinnati against a Biden mandate that applies to companies with more than 100 workers."

Fox News (11/7, Colton, 23.99M) reports that Gov. Kelly said in a statement, "States have been leading the fight against COVID-19 from the start of the pandemic. It is too late to impose a federal standard now that we have already developed systems and strategies that are tailored for our specific needs."

The Hill (11/5, Coleman, 5.69M) reported that Kelly's "disapproval of the administration's move comes as many Republican governors and attorneys general have voiced their opposition to the vaccine requirements for businesses."

Newsweek (11/5, Fung, 2.67M) reported that "Kelly questioned how effective President's Biden's vaccine mandate would be for residents of her state and said it was 'too late' for the federal government to be implementing such efforts this late into the coronavirus pandemic."

US To Lift Travel Ban On 33 Nations For People Fully Vaccinated Against COVID-19.

The CBS Weekend News (11/7, 6:04 p.m. EST, story 2, 2:10, Duncan, 9.45M) reported, "The United States reopens its borders to international visitors" on Monday, "as long as travelers are fully vaccinated" against COVID-19. It is "the first time since COVID restrictions closed crossings nearly 20 months ago." CBS (Carter Evans) reported, "US airports are facing and preparing for big crowds. [Monday] alone, United Airlines is anticipating there will be more than 30,000 inbound international travelers and it's not just by air. Our land borders are expected to be crowded as well. Excitement and relief for thousands of people on both sides of the US-Canada border. Starting Monday, after being shut down for nearly 600...days, the world's longest land border will be open to the vaccinated."

The Washington Post (11/6, Stein, 10.52M) reported, "On Monday, the United States will lift the travel ban on 33 nations – including many in Europe and several other places including China – for people who are fully vaccinated" against COVID-19 "or show a negative coronavirus test before boarding a flight." The travel ban lift "gives more families a chance to reunite and offers a boost to the world's sagging tourism industry." The United States has been criticized by

European businesses, lawmakers, and residents for months "for failing to allow vaccinated EU citizens into the country – particularly as the vaccination rates in many European countries began to eclipse rates in the United States."

The New York Times (11/7, Murphy, 20.6M) reports that under the new rules, travelers fully vaccinated against COVID-19 "will be allowed to enter the United States if they can show proof of vaccination and a negative coronavirus test taken within three calendar days of travel." Those Americans not vaccinated against COVID-19 as well as "children under the age of 18 are exempt from the requirement, but must take a coronavirus test within 24 hours of travel."

The AP (11/6, Loller) reported, "Loved ones have missed holidays, birthdays and funerals while nonessential air travel was barred from a long list of countries that includes most of Europe, Brazil and South Africa." Closed land border crossings "with Mexico and Canada have devastated the border towns where traveling back and forth, sometimes daily, is a way of life."

Biden Says US Has Secured Millions Of Doses Of Experimental COVID-19 Pill.

Reuters (11/5, Alper, Hunnicutt) reported President Biden on Friday said the US has secured millions of doses of Pfizer's investigational antiviral oral COVID-19 therapy should it receive FDA authorization.

The Hill (11/5, Samuels, 5.69M) reported Biden said: "If authorized by the FDA, we may soon have pills and may treat the virus of those who become infected. We have already secured millions of doses, and the therapy would be another tool in our toolbox to protect people from the worst outcomes of Covid."

Forbes (11/5, Kim, 10.33M) provided additional coverage.

First Lady Launches Nationwide Effort To Encourage COVID-19 Vaccine For Children 5

To 11. The Hill (11/5, Chalfant, 5.69M) reported the White House on Friday announced First Lady Dr. Jill Biden on Monday will "begin a nationwide effort by the Biden administration to encourage COVID-19 vaccinations among children ages 5 to 11 next week, after the Pfizer vaccine was approved for use in this age group." The Hill says Dr. Biden will "visit a pediatric coronavirus vaccine clinic at Franklin Sherman Elementary School. The elementary school has historical significance because it was the first to administer the polio vaccine more than half a century ago, which the White House noted." According to The Hill, the Administration "has undertaken similar campaigns to encourage vaccines among those ages 15 and up, seeking to answer questions that unsure Americans may have and to combat misinformation about the shots."

CNN (11/5, Klein, 89.21M) reported that Dr. Biden, "who is also a community college professor, has previously traveled the country to encourage vaccinations and has also visited schools, highlighting the enormous deficit Covid-19 has caused for many families and teachers and school systems. Biden's new efforts will target the nation's youngest, most newly eligible vaccine candidates."

Forbes (11/5, Bissada, 10.33M) also covered the First Lady's vaccination campaign.

Murthy On ABC's "This Week" Says Administration Will Defend COVID-19 Vaccine Requirement.

ABC's This Week (11/7, 2.23M) hosted US Surgeon General Dr. Vivek Murthy and asked him about the decision by the 5th Circuit Court of Appeals decision to issue a stay of the OSHA COVID-19 vaccine requirement. Dr. Murthy said, "The President and the Administration wouldn't have put these requirements in place if they didn't think they were appropriate and necessary. The administration is certainly prepared to defend them." Asked about the suits filed by 27 states in opposition to the rule, Murthy said, "One of the reasons why you would see thousands of companies actually moving forward with requirements even before the federal deadline is because they've realized how important and helpful it is for the health of their workers and for the health of their businesses to have everyone vaccinated."

ABC News (11/7, Demissie, 2.44M) reports that Dr. Murthy "defended the actions of the Biden administration in a Sunday interview on ABC's 'This Week' after it announced a sweeping COVID-19 vaccine mandate for companies with 100 employees or more." ABC's Martha Raddatz "pressed Murthy on the impact that this mandate could have on businesses and the economy," and Murthy responded, "I hear time and time again, from small businesses, large businesses and workers is that what's really hurting the economy is actually COVID itself."

Fox News (11/7, Aitken, 23.99M) reports that Dr. Murthy "defended the Biden administration's workplace COVID-19 vaccine mandate by insisting that it is the virus that is hurting the economy over any other factor."

Forbes (11/7, Kvetenadze, 10.33M) reports, "As the White House vowed on Sunday morning to defend its federal vaccine mandates against court challenges, Surgeon General Dr. Vivek Murthy didn't rule out the possibility of future mandates for smaller companies." On "This Week," "Murthy hinted that the administration would consider extending the decree to include those companies with fewer than 100 employees, telling" ABC's Martha Raddatz "certainly nothing is off the table."

The Hill (11/7, Mastrangelo, 5.69M) reports that "Murthy said President Biden's administration is ready to fight for the

implementation of a vaccine requirement for large businesses."

Politico (11/7, Cohen, 6.73M) reports "Murthy on Sunday defended the Biden administration's workplace rules on vaccine mandates" by "citing historical precedents dating back to George Washington during the American Revolution," and "said President Joe Biden had faith in both the legality of the mandate and the effectiveness of such requirements."

Axios (11/7, Saric, 1.26M), HuffPost (11/7, 363K), the New York Post (11/7, 7.45M), and the New York Daily News (11/7, Shahrigian, 2.51M) also provide coverage.

Schools Across US Preparing To Offer COVID-19 Vaccine To Younger Children.

The AP (11/7, Eaton-Robb) reports that following the approval of the Pfizer-BioNTech COVID-19 vaccine "for younger children, many elementary schools around the US are preparing to offer the shots, which educators see as key to keeping students learning in person and making the classroom experience closer to what it once was." However, "many school systems are choosing not to offer elementary schools as hosts for vaccination sites after some middle and high schools that offered shots received pushback."

The Washington Post (11/6, Svrluga, 10.52M) reported "the go-ahead Tuesday night from federal officials allowing the Pfizer-BioNTech shots to be given to millions of 5-to-11-year-olds had many parents in Washington dropping everything to stand in line. ... It marked a turning point for many families: a welcome sense of relief, and anticipation."

Bloomberg (11/6, Edney, 3.57M) and CBS News (11/5, Gibson, 5.39M) also covered the story.

Cities, States Incentivizing Younger Kids To Receive COVID-19 Vaccine. USA Today (11/5, Miranda, 12.7M) reported, "Children age[d] 5 to 11 are now eligible for the Pfizer-BioNTech COVID-19 vaccine and cities like New York City and Chicago are incentivizing kids to get vaccinated." Those "in New York City can either gain \$100 if they receive their first dose of the vaccine within the city or tickets to city attractions." Furthermore, "Louisiana is offering \$100 to children who get vaccinated, and in Texas, San Antonio officials announced parents can claim a \$100 gift card for H-E-B grocery stores."

Big Bird Tweeted He Got COVID-19 Vaccine.

NBC News (11/7, Kessler, 4.91M) reports, "Big Bird's seemingly innocuous – and obviously fictional – announcement Saturday that he has been vaccinated against Covid-19 caused a stir online, as Republicans like Sen. Ted Cruz of Texas accused the yellow anthropomorphic bird of tweeting 'government propaganda.'" Big Bird tweeted, "I got the COVID-19 vaccine today! My wing is feeling a little sore, but it'll give my body an extra protective boost that keeps me and others healthy." NBC also reports, "Little research has

been done about birds' getting Covid-19, but the Centers for Disease Control and Prevention says 'chickens and ducks do not seem to become infected or spread the infection based on results from studies.'"

The Hill (11/7, Schnell, 5.69M), Newsweek (11/7, Rahman, 2.67M), Fox News (11/7, Kornick, 23.99M), Forbes (11/7, Placido, 10.33M), and the New York Daily News (11/7, Feldman, 2.51M) also provide coverage

Murthy Discusses COVID-19 Vaccine With Sesame Street's Granny Bird. CNN (11/6, 384K) carried video of US Surgeon General Dr. Vivek Murthy appearing in a segment with Sesame Street's Granny Bird character to discuss the COVID-19 vaccines, their safety and efficacy. Murthy said, "this vaccine works to protect our kids against COVID. But it is also very safe. The only side effects that they're really seeing have popped up are soreness, in the arm where you get the shot, and some people have had headaches and felt a little tired afterwards."

Global COVID-19 Cases Reach 250M. Reuters (11/7, Abraham, Biswas) reports, "Global COVID-19 cases surpassed 250 million on Monday as some countries in eastern Europe experience record outbreaks, even as the Delta variant surge eases and many countries resume trade and tourism." The average of daily cases "has fallen by 36% over the past three months, according to a Reuters analysis, but the virus is still infecting 50 million people every 90 days due to the highly transmissible Delta variant."

CDC Data Show Cruises Reported Over 1,350 COVID-19 Cases Since June. The Washington Post (11/5, 10.52M) reported, "A report from the Centers for Disease Control and Prevention offers the first publicly available look at how many coronavirus cases were discovered on cruises in the United States following an industry-wide shutdown." In spite of "cruise lines' efforts to impose strict public health rules, covid-19 has still found a way on board." According to the data from the CDC, "cruise operators confirmed 1,359 cases between June 26 and Oct. 21, a roughly four-month period that represents a rebound for the industry." Many of the cases "involved breakthrough infections of people who were fully vaccinated." Furthermore, "during that time, operators reported 49 hospitalizations and 38 medical evacuations for covid-19 or covid-like illnesses. At least one person died after testing positive during a cruise."

Arizona Reports More Than 3K COVID-19 Cases For Fourth Consecutive Day. The AP (11/7) reports, "Health officials in Arizona on Sunday reported over 3,000 confirmed COVID-19 cases for the fourth consecutive day." The state's "coronavirus dashboard reported 3,231 additional cases but just one death." These newest numbers

"increased Arizona's pandemic totals to 1,189,708 cases and 21,409 known deaths."

Over 10K Patients Diagnosed With COVID-19 In US Hospital After Being Admitted For Something Else, Analysis Suggests. Kaiser Health News (11/7, Jewett) reports "more than 10,000 patients were diagnosed with" COVID-19 "in a U.S. hospital last year after they were admitted for something else, according to federal and state records analyzed exclusively for KHN." The analysis also revealed that nearly "21% of the patients who contracted covid in the hospital from April to September last year died," while "nearly 8% of other Medicare patients died in the hospital at the time."

California COVID-19 Case Rate Now Higher Than Texas, Florida. The San Jose Mercury News (11/6, Woolfolk, 432K) reported, "COVID-19 cases aren't falling in California anymore. They have climbed back up to the CDC's blood-red 'high' level of virus transmission as the highly contagious Delta variant continues to wreak havoc." The case rate in the state "is now well above Texas' and double Florida's, which along with the rest of the Gulf Coast are down to the CDC's orange 'substantial' transmission level."

Las Vegas Nursing Facility Claims It Is Not Liable In COVID-19 Death Lawsuits. The Las Vegas Review-Journal (11/7, Erickson, 372K) reports, "A Las Vegas nursing home where 30 residents died from COVID-19 is facing several negligence lawsuits, but the facility asserts it is protected from the claims because it followed federal safety directives." The facility "is arguing that a longstanding law guarantees it the same liability protections as companies like COVID-19 vaccine manufacturers, according to court documents." The home "is invoking the Public Readiness and Emergency Preparedness Act (PREP Act), a longstanding federal law that is meant to shield companies fighting public health emergencies." This January, "the U.S. Department of Health and Human Services clarified that nursing homes and other facilities are immune from liability, except for cases where virus mitigation measures were totally disregarded."

Opinion: Lower Vaccination Rates Lead To Losing Ground Against Diseases We Have Already Defeated. In an opinion piece for the Washington Post (11/7, Bhadelia, 10.52M), infectious disease physician Nahid Bhadelia writes, "As we race to build global immunity against covid-19, we're losing ground against diseases we've already defeated. There are two reasons for this: growing vaccine hesitancy as a result of the politicization of coronavirus vaccines, and the disruption of routine

childhood inoculation.” Bhadelia says, “Health officials – and the public at large – must shore up our defenses against all diseases and keep routine childhood vaccination requirements from becoming enmeshed in our growing ideological divide.” She discusses further the reasons behind growing anti-vaccination sentiment, and concludes with suggestions to combat lowering vaccination rates: “First, we must support routine childhood vaccination regardless of our political affiliations.” Additionally, “clinicians must be more vigilant about detecting vaccine-preventable diseases that might appear in our clinics with greater frequency.”

Opinion: COVID-19 Presents Opportunity For Tennessee To Expand Medicaid.

In an opinion piece for the Tennessean (11/7, 645K), Kevin Gibas, an infectious diseases physician at Vanderbilt University Medical Center, says “the COVID-19 pandemic has had a devastating impact on the health of communities across the southeastern United States, including here in Tennessee.” The impact “has been exacerbated by high numbers of residents lacking insurance in Tennessee and throughout the region.” Gibas asserts that “a critical component of any response to COVID-19 is addressing the health insurance coverage gap – a gap that is largely the result of Tennessee’s failure to expand Medicaid.” According to Gibas, “Medicaid expansion is Tennessee’s best hope of closing the state’s health care coverage gap, and COVID-19 presents a unique opportunity to act.”

Judge Orders California Officials To Provide Distance Learning For Students With Disabilities.

The Los Angeles Times (11/5, Blume, 3.37M) reported California “officials must act immediately to provide distance learning that is comparable to last year for students with disabilities and also adequate to their overall needs, a judge has ordered.” The court ruling “will provide immediate relief for 15 students...but there are broad implications for students across California.” The policies “at issue arise from Assembly Bill 130, which put in place rules meant to ensure that school districts provided and prioritized in-person instruction for all students this fall.” The alleged fallout “in this case was that families that wanted remote learning for students with disabilities...faced delays and roadblocks that kept them out of school and denied them needed services.” The judge wrote, “The Court finds that plaintiffs have demonstrated irreparable harm.”

Biden Administration’s COVID-19 Vaccine Rules Will Apply To Many Workers Who Support People With Developmental Disabilities. Disability Scoop (11/8, Diamant) reports “new Biden administration rules requiring millions of American

workers to receive COVID-19 vaccines will apply to many of those who support people with developmental disabilities.” Under a rule proposed by CMS, “workers at health care facilities that participate in the Medicare and Medicaid programs will need to be fully vaccinated by Jan. 4.” Along with “hospitals and long-term care facilities, the rule will apply to staff at intermediate care facilities for individuals with intellectual disabilities, psychiatric residential treatment facilities, home health agencies and other providers.” Meanwhile, “providers of home and community-based services could be subject to Biden’s other rule, a mandate...that workers at any business employing 100 or more people be vaccinated or be tested weekly for COVID-19.”

Lockdown In Auckland, New Zealand Likely To Be Over By Month’s End, Prime Minister Says.

The AP (11/8) reports “the lockdown of New Zealand’s largest city is likely to end later this month, with some coronavirus restrictions eased from Tuesday, Prime Minister Jacinda Ardern said.” The city of “Auckland has been in lockdown for almost three months after an outbreak of the delta variant.” The prime minister “said Monday the city’s improving vaccination rate among people age 12 and older meant it could continue cutting back on restrictions.”

Reuters (11/8, Menon) reports “a pick-up in vaccination rates and mounting pressure from critics calling for more freedoms have prompted Ardern to end the curbs.”

Columnist Calls Florida’s Surgeon General Awaiting Confirmation The “Anti-Fauci.”

New York Post (11/7, 7.45M) columnist Karol Markowicz says “America has had 20 long months with Dr. Anthony Fauci as the spokesman for the government’s COVID response, poorly explaining to us both the pandemic and the concurrent illogical restrictions.” Markowicz writes, “Perhaps it’s time for some new voices.” The media call “Joseph Ladapo, Florida’s surgeon general awaiting confirmation, a ‘firebrand’ who’s ‘controversial.’” Ladapo “opposes mask and vaccine mandates – and so is erroneously called ‘anti-vax’ by his foes.” However, Markowicz says “Ladapo came off as none of these things when I spoke to him for an exclusive interview last week.” She asserts that “in so many ways, the Nigerian-born Ladapo is the anti-Fauci.”

Residents In Major US Cities More Willing To Receive COVID-19 Vaccine Than Rest Of Country, Survey Suggests.

The New York Post (11/7, Campanile, 7.45M) reports “residents of the Big Apple metro area and other major US cities are more willing to get vaccinated against COVID-19 than the rest of the US, a new survey suggests.” The study, which was “conducted by the

CUNY School of Public Health and just published in nature.com, surveyed 6,037 Americans in mid-April with a particular focus on four large metro areas in different regions of the country: New York City, Chicago, Los Angeles and Dallas." According to the Post, "the number of people unwilling to get vaccinated in each city was: 10.1 percent in New York City; 11.2 percent in Chicago; 11.5 percent in LA and 19.7 percent in Dallas, according to the survey." However, "the resistance rate for the entire country was more than 21 percent."

Protest Against COVID-19 Safety Measures Brings Rallyists, Counter-Protestors To Boston Common.

The Hill (11/7, Beals, 5.69M) reports "a rally against COVID-19 public safety measures like mask and vaccine mandates brought dozens of protesters and even more counter-protesters to Boston Common on Sunday, where the participants breached police barricades and repeatedly clashed, according to The Boston Globe." Police officers "said two people were arrested, the Globe reported." The Globe "estimated that 'scores' of right-wing protesters turned out, while hundreds of counter-demonstrators showed up – along with hundreds of police trying to keep them apart."

Pfizer CEO Says Vaccines, Boosters Will Remain Key In Fight Against COVID-19.

CNBC (11/5, Mendez, 7.34M) reported Pfizer CEO Albert Bourla said in an interview with CNBC on Friday that while the company's investigational antiviral oral COVID-19 pill appears promising, vaccines and vaccine boosters remain key in combatting SARS-CoV-2. Bourla said, "The fact that we have a treatment is not at all a reason not to take the vaccine, in fact we should take the vaccine."

Fox Business (11/5, Kaplan, 3.06M) reported Bourla added, "People should get vaccinated and they should be able to protect themselves and their families by doing that because otherwise they can spread the disease."

UK To Begin Distributing COVID-19 Antiviral Pill Molnupiravir.

Reuters (11/7, MacLellan) reports the UK will begin rolling out Merck & Co.'s "molnupiravir COVID-19 antiviral pill through a drug trial later this month, Susan Hopkins, Chief Medical Adviser at the UK Health Security Agency said on Sunday." The UK last week "became the first country in the world to approve the potentially game-changing COVID-19 antiviral pill."

FDA Approves Two More Batches Of J&J COVID-19 Vaccine Manufactured At Emergent.

Reuters (11/5, Roy) reported the Food and Drug Administration "said on Friday it had authorized the use of two more batches of Johnson & Johnson's one-dose COVID-

19 vaccine manufactured at the problem-plagued Baltimore factory of Emergent BioSolutions Inc," bringing the total to 11 approved batches. The "authorization was based on a thorough review of facility records and quality testing by the manufacturer, and considering the current COVID-19 public health emergency, the health agency said in a statement."

Moderna Facing COVID-19 Vaccine Vial-Filling Slowdowns.

Bloomberg (11/6, Langreth, LaVito, 3.57M) reported that "more than 10 months after U.S. regulators first authorized its potent shot" against SARS-CoV-2, "Moderna is finding that inventing a Covid-19 vaccine was actually a relatively straightforward task compared with what would come next: ramping up vial-filling operations on multiple continents and distributing the vaccine to countries around the world in the middle of a devastating pandemic." Moderna on Thursday "announced it would be able to supply only 700 million to 800 million vaccine doses this year, down from its previous projection of 800 million to 1 billion doses."

In an analysis of Moderna's partnership with the National Institutes of Health, Politico (11/5, Cancryn, Oermohle, Banco, 6.73M) said the agreement the federal government "negotiated with Moderna has become a key stumbling block in the global fight against Covid-19. Armed with the generous language in its contract, the company has refused to grant access to its technology to vaccine manufacturers serving the developing world and at multiple points resisted pleas to increase aid to the neediest countries."

Researchers Explore Heart-Related Risks Tied To COVID-19 Vaccines.

The Wall Street Journal (11/7, Loftus, Subscription Publication, 8.41M) reports that as health authorities are expanding the use of COVID-19 vaccines in the US, researchers exploring heart-related risks tied to the shots, such as myocarditis or pericarditis, are following several emerging theories. The Journal says the FDA has held back on authorizing Moderna's coronavirus vaccine in adolescents while the agency investigates the risk.

Novavax To Complete Submission Of COVID-19 Vaccine Data To FDA By Year End.

On its website, CNN (11/5, Howard, 89.21M) reported Novavax CEO Stanley Erck in a Friday interview revealed the company "plans to submit complete data to the US Food and Drug Administration soon for possible emergency use authorization of its coronavirus vaccine." Erck said, "We'll have a file in the US hopefully before the end of the year." The vaccine, "called NVX-CoV2373, is made using somewhat more conventional methods than the vaccines already authorized for use in the US."

Use Of Monoclonal Antibodies In Massachusetts Has Surged In Recent Months.

The Boston Herald (11/6, Cohan, 327K) reported, "Demand for monoclonal antibodies to treat coronavirus has surged at Massachusetts hospitals in recent months as awareness about the once-little-known drug and its incredible effects is increasing, bringing patients in from a dozen different states." While the FDA authorized the therapy's use in December 2020, its use in state hospitals plateaued across the spring and summer months before increasing "dramatically" this fall.

FDA May Soon Authorize Use Of Monoclonal Antibodies As PrEP Against SARS-CoV-2.

NBC News (11/7, Ryan, 4.91M) reports, "The Food and Drug Administration could soon grant emergency authorization to monoclonal antibodies from drugmaker Regeneron for what is known as pre-exposure prophylaxis, or PrEP, against Covid-19." If authorized, the therapy could provide the "ticket back to some semblance of normalcy" for many immunocompromised patients. Still, "questions remain about how accessible such routine preventive therapy would be to any of the estimated 3 percent of Americans who have compromised immune systems."

Ocugen Submits Emergency Use Authorization To FDA For Use Of Investigational COVID-19 Vaccine BBV152 In Pediatric Patients.

The Philadelphia Business Journal (11/5, George, Subscription Publication, 875K) reported Ocugen "has submitted an emergency use authorization application to the Food and Drug Administration for use of the company's Covid-19 vaccine candidate BBV152, known as Covaxin outside the United States, in children ages 2 to 18." Ocugen is working with India-based Covaxin developer Bharat Biotech "to bring the Covid-19 vaccine to the United States under a partnership formed in February."

WPost: Manufacturers Should Focus On Sending COVID-19 Pills To Developing Nations.

A Washington Post (11/6, 10.52M) editorial was hopeful Pfizer's new COVID-19 pill could fill the gaps in protection vaccines leave such as challenges with accessibility and distribution. It could also lessen the burden on hospitals. The Post concludes that drug manufacturers should prioritize sending pills to developing nations after wealthy nations received initial shipments of vaccines.

Increased Binge-Drinking Among Some Americans Could Increase Risk From COVID-19 Breakthrough Infections, NIDA Study Finds.

In an article highlighting alcohol use trends in the US, USA

Today (11/4, Loehrke, 12.7M) reported that while "some research shows many Americans are drinking less, some are binge-drinking more," an issue that could weaken immune systems putting heavy drinkers "at risk for breakthrough infections of COVID-19." For example, one "study by the National Institute on Drug Abuse found that people who binge-drink had a higher rate of breakthrough infections of COVID-19 compared with those with no substance disorders. Binge-drinking is defined as men consuming five drinks or more and women consuming four drinks or more in a two-hour period."

Murthy Defends COVID-19 Vaccine Requirement, Vaccines For Children.

NPR (11/5, 3.69M) featured Steve Inskeep talking to US Surgeon General Vivek Murthy "about President Biden's latest vaccine rules for 100 million workers." Murthy said, "I certainly understand the frustrations people may have. But what we have to keep in mind here is that the real burden that we've been facing in workplaces has been from COVID itself. ... So if we really want to safeguard our workers, if we want to create safer workplaces, getting people vaccinated is actually the best pathway to go down." Asked, "is there a risk of another wave in the United States like they're experiencing in Europe right now?" Murthy said, "A number of times in the past 20 months, we have underestimated this virus. ... we still have 60 million-plus people who are not vaccinated. They remain vulnerable to COVID-19." Asked about COVID-19 vaccines for children, Murthy said, "No. 1 – the risk of COVID to children is not zero. It's not negligible. ... No. 2 – recognize that the vaccine is actually very effective, more than 90% effective in reducing the risk of children getting symptomatic COVID. ... And third is safety. ... There were no serious adverse effects seen."

US Secretary of Education Dr. Miguel A. Cardona, and US Surgeon General Dr. Vivek H. Murthy wrote for USA Today (11/5, 12.7M), "Between the two of us, we have kids in pre-K, elementary, middle school and high school." And "so far, we've been heartened by what we see in schools. Recent data from the Centers for Disease Control and Prevention shows that 98% of schools are fully open this fall, up from about 70% at the end of the 2020-21 school year." They added, "we're fortunate not to have to choose between reducing our kids' COVID risk and keeping them connected to their support systems in school. But we do need to support and implement the strategies backed by scientists, mental health professionals and more than eighteen months of experience."

Reviewing Applications For Exemptions To COVID-19 Vaccine Requirement Among Federal Employees May Take Months.

The

Washington Post (11/7, Rein, Duncan, Horton, 10.52M) reports, "With a Monday deadline looming, high percentages of federal workers are reporting they have been vaccinated against the coronavirus," yet, "tens of thousands of holdouts have requested exemptions on religious grounds, complicating President Biden's sweeping mandate." In addition, "A far smaller number of employees has asked for exemptions on medical grounds." The Post adds that consideration of requests for exemptions "could take months for officials designated at each agency." The Post also reports that "Jeffrey Zients, the White House's covid-19 response coordinator, said this week that religious exemptions have existed for past vaccines and pledged that federal agencies 'are following rigorous processes to evaluate religious and medical exemptions.'"

The AP (11/7, Long, Sisak) reports, "About 4 million federal workers are to be vaccinated by Nov. 22 under the president's executive order." That "deadline is the first test of Biden's push." The AP adds that "officials at Health and Human Services, U.S. Food and Drug Administration and Housing and Urban Development said they were working on getting their employees vaccinated but had no figures yet."

Defense Industry Concerned By "Ambiguities" In COVID-19 Vaccine Requirement For Federal Contractors. Roll Call (11/5, Donnelly, 130K) reported, "Defense industry executives are concerned that ambiguities in President Joe Biden's vaccine mandate for federal contractors will create financial and legal risks for some 20 percent of America's businesses." According to Roll Call, business is concerned about "how ambiguities in the order have created other forms of uncertainty for their businesses." One attorney summarized their concerns as "the loss of key employees, the lack of clarity on whether and how the contractors will be able to recover costs associated with the disruptions to their business because of the mandate, and their ability to meet delivery requirements of the customers up the federal supply chain."

Evictions Increasing, Though They Remain Below Pre-Pandemic Levels. The New York Times (11/7, Kasakove, 20.6M) reports that instead of a "sudden surge of evictions that tenants and advocates feared after the Supreme Court ruled in August that President Biden's extension of the eviction moratorium was unconstitutional," what is being seen "is a more gradual eviction crisis that is increasingly hitting communities across the country, especially those where the distribution of federal rental assistance has been slow, and where tenants have few protections." The Times adds, "the number of eviction filings remained at nearly half of prepandemic averages during the first two weeks of October," but "filings are also increasing."

COVID-19 Vaccines For Children May Be Required By Schools Soon. The Atlantic (11/5, Gutman, 3.53M) reported, that while "school mandates for new vaccines tend to lag behind CDC recommendations by about half a decade, but COVID-19 shots appear to be in the express lane." The Atlantic adds, "The kid-size COVID vaccine fights a disease that is about as dangerous as others for which schools regularly require vaccination, and, at least for now, it appears to be about as effective as these other vaccines."

Commentary: Despite Progress During Pandemic, Government Has Much Work To Do To Improve Data Handling. Ali S. Khan, dean of the College of Public Health at the University of Nebraska Medical Center, and William J. Kassler, Palantir's chief medical officer working with the U.S. government, write in an op-ed for the Los Angeles Times (11/7, 3.37M), that at the beginning of the COVID-19 pandemic, "the speed, scope and scale of the data overwhelmed the government's outdated infrastructure until it finally created new systems for functions such as disease tracking and race and ethnicity reporting." As a result, "our nation relied on academic, media and other volunteers...rather than on our nation's public health agency, the Centers for Disease Control and Prevention." Now, "the HHS Protect hub – built for the Department of Health and Human Services with technology from the company Palantir...makes available near-real-time data," but, they add, "data are still largely locked into the government's outdated legacy tech systems that can't communicate with one another." They call for more improvements in the collection and handling of data by the government.

US To Reopen To Foreign Travelers As Long As Their COVID-19 Vaccinations Are Authorized By FDA. The AP (11/7, Spike) reports, "Starting Monday, the United States plans to reopen to foreign travelers who are fully vaccinated against the coronavirus." It comes with a condition: "non-immigrant adults need to have received vaccines authorized by the Food and Drug Administration or which received an emergency use listing from the World Health Organization." The decision "leaves many hopeful travelers across the globe who [have] taken full courses of vaccines widely used in other parts of the world – Sputnik V and the China-produced CanSino jab, in particular – scrambling to get reinoculated with shots approved by US authorities."

Canada, US Opening Border, Though Canadian COVID-19 Rules Likely To Deter Many. The Washington Post (11/7, 10.52M) reports, "On Monday, for the first time in more than 19 months, fully vaccinated Canadians

will be allowed to cross the US land border for such nonessential purposes as tourism or family visits." Though "the reopening is being cheered in the tightknit communities that straddle the 5,500-mile border – and by the Canadian snowbirds who prefer to drive South to warmer climes – few are expecting an immediate flood of tourists." One reason is "because those entering Canada – including Canadians returning from even the briefest of visits on the American side – must present a negative coronavirus molecular test result within 72 hours of arrival."

Europe Again Becomes COVID-19 Epicenter.

Bloomberg (11/6, Loh, 3.57M) reported, "Europe has again become an epicenter for the coronavirus, calling into question the region's efforts to recover from the pandemic."

Russia Hits Another Single-Day High For New COVID-19 Cases.

The AP (11/6) reported, "Russia's COVID-19 cases hit another one-day record as the country struggles to contain a wave of infections and deaths that has persisted for more than a month." Russia's "national coronavirus task force on Saturday reported 41,335 new cases since the previous day, exceeding the previous daily record of 40,993 from Oct. 31." It also "said 1,188 people with COVID-19 died, just seven fewer than the daily death record reported Thursday."

Sydney To Further Ease Social Distancing COVID-19 Curbs, Officials Say.

Reuters (11/7, Kelly) reports, "Australia's largest city of Sydney will further ease social distancing curbs on Monday, a month after emerging from a coronavirus lockdown that lasted nearly 100 days, as close to 90% of people have got both doses of vaccine, officials said." Though only available for those who are fully vaccinated against COVID-19, "the relaxation in the state of New South Wales, home to Sydney, lifts limits on house guests or outdoor gatherings, among other measures." Hundreds of "people held an anti-vaccine protest on Sunday in the state's capital of Sydney, but such opposition is slight in Australia, where polls show vaccination opponents number in the single digits."

Austrian Government Bans Those Unvaccinated Against COVID-19 From Restaurants, Hotels As Coronavirus Cases Rise.

The AP (11/6) reported, "Unvaccinated people in Austria who also haven't had COVID-19 will no longer be allowed to enter restaurants, hotels and hair salons or attend public events larger than 25 people under new rules that take effect Monday, the government said." Alexander Schallenberg, Austria's chancellor, "outlined the rules Friday night after a meeting with state-level leaders to discuss the

country's response to rapidly rising coronavirus cases." Formerly, "people could enter restaurants, hotels and other areas if they were vaccinated, had recovered from the virus or could show results from a negative test."

Reuters (11/7, Revill) reports, "Austria's latest measures to tackle the spread of coronavirus will likely stay in force over Christmas and New Year, Chancellor Alexander Schallenberg was quoted as saying on Sunday, as the country posted a record number of infections." Austria "reported 9,943 new infections within a 24-hour period on Saturday, surpassing the previous worst day of Nov. 13 2020 when 9,586 cases were recorded."

Costa Rica Issues COVID-19 Vaccine Mandate For Children Aged Five And Older.

Reuters (11/6, Murillo) reported, "Costa Rican children aged five and up must get COVID-19 vaccinations, according to a new health ministry mandate, making the Central American country one of the first to adopt such a requirement for kids." The mandate "would add COVID-19 to a list of other infectious diseases in which vaccines for children have for years been required, including for polio and smallpox." According to official data, "coronavirus infection rates in Costa Rica have been trending down recently, with confirmed COVID-19 deaths totaling more than 7,000...out of a population of around 5.1 million."

People Not Vaccinated Against COVID-19 In Greece Face New Restrictions As Coronavirus Cases Soar.

Reuters (11/6, Fronista) reported, "Queues formed outside shops in Athens on Saturday on the first day of new restrictions to curb soaring coronavirus infections which require the unvaccinated to have negative COVID-19 tests." Coronavirus "infections in Greece hit a new daily high almost every day in November, prompting authorities to announce new measures on Tuesday, which also restrict access to cafes and restaurants, state services and banks to those who are either vaccinated or have a negative test." People "vaccinated against COVID-19 also have to present their vaccination certificates, triggering long lines outside shops in the capital's busiest shopping street, Ermou."

China Has Given 1.072 Billion People Complete COVID-19 Vaccine Doses, Spokesman Says.

Reuters (11/6, Liu, Stanway) reported, "China had given 1.072 billion people complete COVID-19 vaccine doses by Nov. 5, Mi Feng, spokesman at the National Health Commission, told a briefing on Saturday." Commission official Wu Liangyou said at the briefing that "a total of 37.97 million people in China had received a booster shot as of Friday." In a bulletin, the commission said "that China had administered

2.312 billion doses of COVID-19 vaccines by the end of Nov. 5, an increase of about 8.9 million from the previous day."

Bloomberg (11/6, 3.57M) reported, "China said it will continue with stringent curbs to disrupt the coronavirus's domestic transmission, suggesting the nation won't break away from its COVID-Zero strategy anytime soon."

Australia's 80-Percent Vaccination Rate Against COVID-19 A "Magnificent Milestone,"

Prime Minister Says. Reuters (11/6, Kelly) reported, "Australia reached on Saturday a full inoculation rate of 80% of those aged 16 and older, which Prime Minister Scott Morrison called a 'magnificent milestone' on the path to becoming one of the world's most vaccinated countries against COVID-19." An early advocate "of a COVID-zero strategy to manage the pandemic, the country of 25 million has moved towards living with the virus through extensive vaccinations, as the Delta variant has proven too infectious to suppress." While inoculations against COVID-19 "remain voluntary on the federal level, Australia's states and territories introduced mandatory measures for many occupations and workers."

Bloomberg (11/5, Leigh, 3.57M) reported, "Australia hit its target of having 80% of people aged 16 and older fully vaccinated against COVID-19, Prime Minister Scott Morrison said, as the country shifts from a strict containment policy toward living with the virus as endemic."

Britain's Health Ministry To Open Up Bookings For COVID-19 Booster Shots.

Reuters (11/6, Smout) reported, "Britain's health ministry on Saturday said it would open up bookings for booster shots" against COVID-19 "a month before people were eligible to receive the shots to help speed up the rollout ahead of the challenging winter months." Boris Johnson, Britain's prime minister, "is relying on booster doses as a major plank of his plan to avoid lockdown this winter through COVID-19 vaccines rather than social distancing rules or mask mandates, but has been criticized for a slow start to the program."

UK's Health Secretary Urges Eligible Residents To Get COVID-19 Booster Shots.

The New York Times (11/7, Kwai, 20.6M) reports, "Britain's health secretary is urging eligible residents to get booster shots of the coronavirus vaccine, aiming to reduce pressure on the country's health system as winter approaches." Ten million British people, most of whom are over age 50, "have received booster shots since the government began offering them, and millions more will be invited in the coming weeks to book appointments."

Politico Europe (11/7, Haeck, 15K) reports that UK Health Secretary Sajid "Javid 'strongly urged' everyone to get

a third dose of the coronavirus vaccine – saying 'immunity begins to wane after six months,' which means a booster shot is needed to keep older people and those at risk safe over the winter."

Fire In COVID-19 ICU Ward In India Kills 11.

The New York Times (11/6, Kumar, Schmall, 20.6M) reported, "Eleven people died after a fire broke out in a coronavirus intensive care unit in the western state of Maharashtra, the latest in a series of fatal disasters in COVID-19 wards in India." Shankar Misal, the fire chief in the Ahmednagar district, said "hospital staff tried to douse the fire that started Saturday morning with fire extinguishers, but the flames spread quickly in the airtight room, cutting the power out and forcing people to flee to safety." Misal said, "Most of the 11 patients who died suffocated from smoke."

The Hill (11/6, Lonas, 5.69M) reported, "Police Inspector Jyoti Karkade said 17 patients were staying in the ward at the hospital in the state of Maharashtra, with 11 of them dying, The Associated Press reported." The six survivors "are in stable condition." The cause of the fire is unknown.

Fears Grow That Diwali Gatherings In India Could Start Another COVID-19 Case Spike.

The New York Times (11/5, Yasir, 20.6M) reported, "When a devastating second wave of coronavirus infections hit India in the spring, hospitals were overwhelmed with sick patients and crematories struggled to bury the dead as the bodies piled up." India is now "celebrating one [of] its most important holidays – Diwali, the festival of lights – and many fear another wave of infections as millions gather for the celebrations." Diwali "began on Thursday, and there is no data yet on what impact the festivities might be having on the country's battle to curb the spread of the virus."

Germany Post-Merkel Will Not Reimpose Strict Pandemic Lockdowns, Coalition Partner Pledges.

Politico Europe (11/6, Bennett, 15K) reported, "Germany's expected incoming government will not reimpose strict pandemic lockdowns, a coalition partner pledged Saturday amid a record spike in COVID-19 cases." The promise "came from Christian Lindner, head of the pro-business Free Democrats (FDP), one of three parties working to form a coalition to replace outgoing Chancellor Angela Merkel's government." While the Free Democrats "is the smallest of the three parties, Lindner is seen as the front-runner to become Germany's next finance minister."

Op-Ed Criticizes Inequities In Worldwide Distribution Of COVID-19 Vaccine Doses.

Thomas J. Bollyky, director of the Global Health Program at

the Council on Foreign Relations, wrote in a Wall Street Journal (11/5, Subscription Publication, 8.41M) op-ed that more than 94% of COVID-19 vaccine doses administered worldwide to date went to just 10 nations. Canada, Japan, and the US will likely have small children vaccinated before Ethiopia and Nigeria will inoculate their healthcare workers and high-risk elderly. NIH was mentioned.

Desperate Officials In Ukraine Enact Ad Campaign To Scare People Into Receiving COVID-19 Vaccine. The Washington Post (11/5, Stern, 10.52M) reported, "Ukraine's pandemic figures are scary: skyrocketing infections, record deaths and among the lowest vaccination rates" against COVID-19 in Europe. In response, the country's "officials decided to become scary themselves with an advertising campaign aimed at young people: If you're unvaccinated, you may die." The campaign is "a noticeable escalation in the government's struggle to get the upper hand on the pandemic."

LGBTQ People More Likely To Lose Income During Pandemic, US Census Bureau Survey Suggests. Bloomberg (11/5, 3.57M) reported, "In a first-of-its-kind survey from the US Census Bureau, nearly a quarter of LGBTQ people reported losing income during the COVID-19 pandemic, a higher share than non-LGBTQ respondents." Unemployment and income statistics in the United States "don't specifically measure the LGBTQ experience." For the first time this past July, the US Census Bureau "collected information about sexual orientation and gender identity of respondents to its Household Pulse Survey."

With Bigger US Companies Relaxing COVID-19 Mask Guidelines, Employees At Other Places Wonder If They Can Do The Same. The Wall Street Journal (11/7, Smith, Subscription Publication, 8.41M) reports that watching larger American employers such as Apple, Amazon, and JPMorgan Chase & Co. eliminate mask requirements as COVID-19 vaccination rates rise and case numbers fall has workers at other companies wondering if they can do the same.

COVID-19 Vaccine Mandate Divides Small Washington State Hospital. The Washington Post (11/6, A1, Saslow, 10.52M) reported on its front page that Washington "state's vaccination deadline for health-care workers was less than a week away when Shane McGuire," the CEO of Dayton General Hospital in southeast Washington, "called his HR department for another daily update." The CEO "liked to refer to his small staff as a family, and many in fact were family, but it had been splitting in two since the beginning of the year, when exactly 50 percent of

the hospital's few hundred employees chose to be vaccinated and 50 percent refused." The prime example of the division due to the COVID-19 vaccination mandate was where McGuire's "medical director told the staff that mass vaccination was 'safe, wildly effective and absolutely necessary,'" while his director of nursing "wrote that it was 'government overreach and medical tyranny.'"

Commentary Questions If Debate Over SARS-CoV-2's Origin Is Worth Having. In a STAT (11/5, 262K) op-ed, John P. Moore, professor of microbiology and immunology at Weill Cornell Medicine in New York City, wrote, "The American public is understandably interested in how a pandemic that has killed nearly 750,000 people in this country and almost 5 million worldwide – with few signs of slowing down – emerged." Moore wrote, "But the US intelligence community has now concluded that the precise sequence of events by which SARS-CoV-2, the virus that causes COVID-19, entered the human population may never be known." Moore questioned if the hand-wringing is worth it, concluding, "Is there any point to continuing an increasingly fractious debate on the origin of COVID-19, or should we now focus on applying the lessons already learned, and think about the future?" NIAID Director Dr. Anthony Fauci and NIH were mentioned in this story.

Healthcare Professionals Urge Facebook To Disclose Its Data On COVID-19 Disinformation. USA Today (11/5, Collins, 12.7M) reported that Dr. Kavita "Patel is among the more than 500 US public health care professionals battling the ongoing spread of COVID who signed a letter demanding Facebook disclose its data regarding disinformation about the virus and the vaccine." The document "comes a week after Facebook CEO Mark Zuckerberg changed the parent company's name to Meta as a pivot toward the metaverse." The letter by the physicians, which was in the works for two months, "further gained momentum following leaked documents provided by whistleblower Frances Haugen to the Securities and Exchange Commission and Congress about the harms caused on Facebook and Instagram, and its parent company's failure to take action."

The Hill (11/5, Klar, 5.69M) reported, "Hundreds of health care professionals are urging Facebook to disclose data on the breadth of COVID-19 disinformation on the platform, piling onto the criticism the social media giant has faced in recent weeks after a whistleblower leaked internal company documents." The professionals "underscored the importance of combating false claims after the vaccine" against COVID-19 "became available to younger children." A letter written by the Doctors For America to Facebook on Thursday "was signed by more than 500 health care

professionals, including medical professors at New York University, Columbia University and Johns Hopkins Medicine."

Op-Ed: Diversion Of Resources During COVID-19 Pandemic Has Allowed Tuberculosis Death Rates To Increase For First Time In 15 Years.

In a Forbes (11/5, 10.33M) op-ed, Joshua Cohen, an independent healthcare analyst, wrote, "Last month, the World Health Organization reported that for the first time in 15 years the number of people who have died from tuberculosis has increased." Last year, worldwide, "more than 1.5 million deaths were attributed to tuberculosis; the first year-on-year increase since 2005." Cohen wrote that multiple reasons were mentioned, including "diversion of resources due to the COVID-19 pandemic." Worse, Cohen wrote, "tuberculosis is not the only disease that has been impacted, with disproportionately severe health burdens on the world's poorest populations."

Despite Signals That Things Are Improving, Americans Seem Stuck In "A Pandemic Hangover Of Pessimism."

The New York Times (11/5, A1, Healy, Burch, Mazzei, 20.6M) reported on its front page, "Despite many signals that things are improving – the stock market is hitting record highs, hiring is accelerating sharply with 531,000 jobs added in October, workers are earning more, and COVID hospitalizations and deaths are dropping from their autumn peaks – many Americans seem stuck in a pandemic hangover of pessimism." In opinion surveys, "more than 60 percent of voters...say that the country is heading in the wrong direction – a national funk that has pummeled" President Joe "Biden's approval ratings and fueled a backlash against Democrats that could cost them control of Congress in next year's midterm elections."

WPost: China's Prosecution Of Woman Who Told Truth About Wuhan And Is Now Near Death In Prison Is An "Act Of Barbarity."

The Washington Post (11/7, 10.52M) editorializes, "China committed one act of barbarity when it prosecuted the citizen journalist Zhang Zhan for her revealing look at Wuhan in the first stages of what became a global pandemic." The woman "was sentenced in December to four years in prison on the specious charge of 'picking quarrels and provoking trouble,' which China uses to suffocate free speech." The editorial board writes, "Now her health has deteriorated, and relatives say she is near death." The board concludes that "China will compound the barbarity unless it sets her free and saves her life."

Woman Who Died From Long-COVID Sparks Debate Of Whether Organs Are Safe, Healthy Enough To Be Transplanted.

The New York Times (11/7, Rabin, 20.6M) reports that a case of a 50-year-old woman ravaged by long-COVID who died "highlights an urgent debate among medical professionals about whether the organs of people who survived COVID, and even of those who died with the illness, are really safe and healthy enough to be transplanted." As a general rule, "the organs are considered safe for transplantation if the test is negative, even if the donor has recovered from COVID." There is also "no universally accepted set of recommendations regarding when organs can be safely recovered from virus-positive bodies and transplanted to patients in need." Creating more complications "is the fact that people with long COVID, whose debilitating symptoms may persist for months, mostly do not test positive for the infection."

Cruz Pushes Legislation To Block COVID-19 Vaccine Mandate For Children.

The Houston Chronicle (11/5, Wermund, 982K) reported, "US Sen. Ted Cruz is pushing legislation that would block schools from requiring students to get" vaccinated against COVID-19. Cruz "filed a bill this week that would prohibit the federal government or any entity at the federal, state or local level that receives federal funding, including school districts, from requiring COVID-19 vaccines for minors." The bill "comes as the Food and Drug Administration has authorized the emergency use of the Pfizer vaccine for children 5 through 11 years of age."

Face Masks No Longer Required In Buildings In Miami-Dade County, Mayor Says.

The Miami Herald (11/5, Cetoute, 647K) reported, "Masks will no longer be required in Miami-Dade County buildings, Miami-Dade Mayor Daniella Levine Cava said Friday." The mayor tweeted that the reversal "is due to 'enormous progress we have made against the Delta variant, and in consultation with our Chief Medical Officer.'" She "had reinstated the mask mandate in July and urged businesses to do the same due to a spike in COVID cases and deaths, which was later recognized as Florida's third wave."

Schools In Minnesota Encouraging, But Not Requiring, Children To Get Vaccinated Against COVID-19.

The Minneapolis Star Tribune (11/6, Lonetree, Klecker, 855K) reported, "Schools across Minnesota have encouraged children to get vaccinated against COVID-19 – even hosting vaccine clinics in school buildings – but so far district leaders have stopped short of requiring the immunizations." Though "all school-age children are eligible for the vaccine, state education and health officials say it's

unlikely districts will move to require students to get the shots." COVID-19 vaccine requirement decisions "are now left to individual school districts, and many school boards have already opted out of other COVID-related precautions, including requiring students to wear face masks." The FDA was mentioned in this story.

Chicago Schools Will Close Friday To Boost COVID-19 Vaccinations. The AP (11/5) reported Chicago Public Schools officials sent a letter to parents and families announcing "schools will be closed Nov. 12 for 'Vaccination Awareness Day.'" District CEO Pedro Martinez "said...Friday's canceled classes will provide 'an opportunity for parents and guardians to take their children five years of age and older to get vaccinated.'"

The Chicago Tribune (11/5, Pratt, Casanova, Yin, 2.03M) reported Chicago city employees will also "be let off work two hours early that day to get a vaccine shot or booster, an effort city officials said is aimed at increasing shots ahead of the Thanksgiving holiday."

Lawsuits Against Illinois Over Lack Of COVID-19 Vaccine Religious Exemption Accumulating.

The AP (11/7, O'Connor) reports, "Lawsuits against Illinois over required COVID-19 vaccination were accumulating and the reason was clear: the legal claims leverage of the state's Health Care Right of Conscience Act in stating religious objections to the shot and in appealing sanctions, including losing a job, that come with refusal." The Health Care Right of Conscience Act "has long been considered protection from repercussions against physicians who refuse on moral grounds to perform abortions." However, "Democrats' clarification, which has yet to be sent to [Gov.] Pritzker for his OK, would ensure that the law's prohibition on penalties for religious objections doesn't apply to COVID-19 vaccine refusal."

Los Angeles To Require Proof Of COVID-19 Vaccination To Enter Many Businesses. The AP (11/7, Weber, Dazio) reports, "Los Angeles is among a growing number of cities...requiring people show proof of [COVID-19] vaccination to enter various types of businesses and venues." However, "rules in the nation's second-most-populous city, called SafePassLA, apply to more types of businesses and other indoor locations including museums and convention centers." The order takes effect Monday, but "city officials say they won't start enforcing it until Nov. 29 to give businesses time to adjust."

The Los Angeles Times (11/7, Harris, 3.37M) reports that "if a business doesn't comply with the ordinance, the first response is a warning," and the "second violation could result

in a \$1,000 fine." Subsequent "fines can escalate up to \$5,000."

Oklahoma City School District Fires Six Teachers For Refusing To Wear Masks. The AP (11/7) reports, "The Oklahoma City school district violated state law by firing six teachers who refused to wear masks, according to Oklahoma Gov. Kevin Stitt." The governor said, "This is about a school district not following state law, this isn't a debate about masks."

Maryland Could Lose Federal Funding For Rent Because Of Its Failure To Distribute Aid.

The AP (11/6) reported, "Maryland failed to reach a federal benchmark early this fall for distributing COVID-19 relief funds it received for rent to tenants in danger of eviction, according to the US Treasury Department." The Department claims Maryland is "among the states that could lose some funding because it failed to spend 30% of the money by Sept. 30." US Sen. Chris Van Hollen (D-MD) and Sen. Ben Cardin (D-MD) wrote to "Housing & Community Development Secretary Kenneth Holt on Friday urging more disbursements." The letter indicated that "the state needs to act quickly 'to avoid having these funds revoked by the Treasury Department beginning on November 15th.'"

Kansas City Rescinds Mask Order. The AP (11/6) reported, "Business operators in Kansas City, Missouri, are hopeful that the end of the city's mask mandate will mean a return of more customers." On Thursday, city council voted "to rescind the mask order, though it kept the mandate for children and adults in school."

Florida Judge Tosses School Districts' Lawsuit Over Mask Mandate Ban. The Hill (11/5, Williams, 5.69M) reported, "A Florida judge tossed a challenge from several of the state's school districts over the Sunshine State's ban on school mask mandates." On Friday, "an administrative law judge ruled that officials from six counties failed to prove that the ban was 'an invalid exercise of delegated legislative authority.'"

Demand For COVID-19 Booster Shots Slower Than Expected In California. The Los Angeles Times (11/5, Lin, Money, Smith, 3.37M) reported, "Demand for COVID-19 booster shots in California has been slower than some health officials anticipated...sparking new concerns of at-risk populations losing immunity as the risky holiday season approaches." As of late October, health officials reported that only 20% of eligible seniors received a booster in San Francisco, and just 27% of fully vaccinated seniors in Los Angeles County have done so.

West Virginia Grandparents Who Are Primary Caregivers For Grandchildren Could Receive School Voucher If Vaccinated Against COVID-19. The AP (11/5) reported that on Friday, West Virginia Gov. Jim Justice (R) announced “grandparents who are the primary caregivers for their grandchildren age[d] 5 to 11 can receive a \$150 school voucher if they are vaccinated against COVID-19.” To receive a voucher, each “family must be enrolled in West Virginia State University’s Healthy Grandfamilies program.”

Florida Governor Bobblehead Will Help Raise Money For Fight Against COVID-19. The Orlando (FL) Sentinel (11/5, Hylton, 599K) reported that on Friday, the National Bobblehead Hall of Fame and Museum announced its newest bobblehead – Florida Gov. Ron DeSantis (R). A \$5 donation will be made to the Protect the Heroes fund for each bobblehead sold. The organization helps “raise funds for hospitals in their fight against COVID-19.”

Alabama Governor Signs Protections For Workers Unvaccinated Against COVID-19 Because Of Religious, Health Reasons. The AP (11/5, Chandler) reported that on Friday, Alabama Gov. Kay Ivey (R) “signed employment protections for workers who claim a religious or health reason for not getting vaccinated against COVID-19.” Under the new law, “state employers can’t fire workers for being unvaccinated against COVID-19 if the employee returns a new standardized state form to claim a religious, medical exemption.”

Court Ruling Accelerates End Of Palm Beach County Schools’ Mask Mandate. The Palm Beach (FL) Post (11/6, Isger, 223K) reported, “Pressed by a court ruling Friday, the mask mandate in Palm Beach County schools will fall Monday, one week earlier than an already accelerated timeline and only days after vaccines became available to the district’s youngest students.”

Nearly All Philadelphia Nursing Homes Satisfy Mandate For Staff To Be Vaccinated Against COVID-19. The Philadelphia Inquirer (11/6, Brubaker) reported, “All but one of Philadelphia’s 47 nursing homes have satisfied the city mandate to have all staff vaccinated against COVID-19 unless the workers have approved exemptions, the Philadelphia Department of Public Health said Friday.” A survey sent to these 47 nursing homes found “that 8,460 of 9,313 nursing home workers, or 90.8%, were fully vaccinated.”

Less Than Half Of San Francisco’s COVID-19 Booster Shots Administered To Seniors. The San Francisco Chronicle (11/6, Neilson, 2.44M) reported San Francisco Department of Health data show that as of November 1, “just under 23,000 of the city’s approximately 118,000 seniors eligible to receive COVID-19 booster shots – about 1 in 5 – had received them.” Additionally, the data indicate that less the half of the 52,000 booster shots administered by the city “have been given to people 65 and older.”

Mississippi Behind On Spending Federal COVID-19 Relief. The AP (11/6, Pettus) reported, “Mississippi is running behind many other states in deciding how to spend billions of federal dollars for pandemic relief.” According to Lt. Gov. Delbert Hosemann (R), it is “imperative that the money go to ‘transformational projects.’” Meanwhile, Northern District Public Service Commissioner Brandon Presley (D) “wrote in a recent newspaper column that Mississippi needs to invest pandemic relief money in projects that could boost people’s quality of life, including expansion of broadband access and improvement of water systems.”

COVID-19 Vaccine Mandates Stirring Controversy At NIH. The Wall Street Journal (11/7, Strasburg, Subscription Publication, 8.41M) reports COVID-19 vaccine mandates are causing controversy at the NIH. On December 1, the NIH’s main bioethics department will host a roundtable session over the ethics of mandates.

NIH Launches Tool Recommending When Americans Should Get Tested For COVID-19. The Daily Mail (UK) (11/5, Kekatos, 4.11M) reported, “The National Institutes of Health (NIH) has launched a new tool for Americans to use to determine whether or not they should get tested for COVID-19.” The When To Test Calculator for Individuals “uses factors such as vaccination status, protective measures taken by the user and rates of spread where a person lives to recommend testing.” The calculator “spits out results based on whether or not it determines someone is at low or high risk of contracting COVID-19 or spreading it to others.”

NIH To Study Impacts Of COVID-19 On Pregnant Women. Fox News (11/6, Musto, 23.99M) reported the NIH announced “it will study the long-term impact of COVID-19 on pregnant women.” The four-year follow-up “study will enroll some participants from an earlier study by the Maternal-Fetal Medicine Units (MFMU) Network, which was supported by NIH’s Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD).” Researchers “will also follow the offspring of those

women to see whether or not there have been long-term health effects on them.” The team “aims to understand what proportion of patients with COVID-19 in pregnancy are at risk for Long COVID, whether the severity of COVID-19 infection in pregnancy influences the likelihood of developing Long COVID and how the proportion of patients who develop Long COVID after COVID-19 in pregnancy compares to that of non-pregnant women who develop it.”

Opinion: COVID-19 Exposes How Science Vs. Faith Disclosure A False Dichotomy.

In an opinion piece for the New York Times (11/7, 20.6M), Anglican Church priest Tish Harrison Warren writes, “I have never had much interest in faith versus science debates,” but “these past two years have exposed how the science vs. faith discourse isn’t an abstract ideological debate but a false dichotomy that has disastrous real-world consequences.” Warren interviewed BioLogos President Deborah Haarsma “to better understand this cultural division.” NIH Director Francis Collins is the founder of BioLogos, “an organization that explores the relationship between faith and science.” In fact, “some questioned whether Collins’s religious faith should disqualify him from” his position at NIH. Haarsma concluded by saying “these two communities share a goal: seeking truth.”

NIAID Funding Research To Assess Antibody Response To Additional COVID-19 Vaccine Dose In People With Autoimmune Diseases.

The Triad (NC) Business Journal (11/7, Ezzone, Subscription Publication, 845K) reports the NIAID is funding a trial “which will assess the antibody response to an additional COVID-19 vaccine dose in people with an autoimmune disease who did not respond to an initial vaccine regimen.” The study “will also investigate whether pausing immunosuppressive therapy improves the antibody response to an additional COVID-19 vaccine dose.” Rho, a long-term partner of NIH, will serve “as the clinical coordinating center.”

NIH Director Says Long COVID Research Could “Improve Our Understanding Of Other Chronic Post-Viral Syndromes.”

The Washington Post (11/7, Sellers, 10.52M) reports, “The National Institutes of Health earlier this year launched a \$1.15 billion initiative to study long covid, and researchers and patients hope it will provide answers.” However, “NIH Director Francis S. Collins said the \$1.15 billion investment in research would go beyond long covid to ‘improve our understanding of other chronic post-viral syndromes and autoimmune diseases, as well as other diseases with similar symptoms.’”

FDA IN THE NEWS

New FDA Commissioner Must Restore Trust To Agency, Former FDA Officials Say.

Bloomberg Law (11/5, Castronuovo, Subscription Publication, 4K) reported “a new permanent commissioner at the” FDA “will need to bring more clarity to the government’s pandemic response and restore trust to an agency that remains without a Senate-confirmed leader nearly a year into the Biden administration, policy analysts and former FDA officials say.” President “Biden has just weeks left to name a replacement for acting Commissioner Janet Woodcock, who was ruled out for the post due to opposition on Capitol Hill.” Former FDA Deputy Chief of Staff Kalah Auchincloss said, “It’s hard to have a vision and goals that you want to achieve as an acting commissioner; you’re more just keeping the ship steered in the right direction.” Former FDA Commissioner Robert Califf “is being reported as the top contender to lead the agency once again.” Former FDA Commissioner Scott Gottlieb and Howard Sklamberg, who worked as the FDA’s deputy commissioner for global regulatory operations, were mentioned.

US House Committee’s Opioid Crisis Probe Focuses On Consulting Firm.

The New York Times (11/5, Bogdanich, Forsythe, 20.6M) reported the US House Committee on Oversight and Reform is conducting an opioid crisis-related investigation that is focused on the consulting firm McKinsey. The committee has asked the company to provide it with records that relate to this matter. On Friday, an unnamed McKinsey spokesperson said the company “will engage directly with the committee” as it handles that request. The article mentions the FDA.

Endpoints News (11/5, Brennan) reported Sen. Maggie Hassan (D-NH) “directly questioned FDA acting commissioner Janet Woodcock on Thursday about how the FDA didn’t know until recently about its conflicting work with McKinsey, which was also consulting for opioids manufacturers while working on the FDA’s track-and-trace system. But Woodcock said that she did know, and she said she didn’t think there was anything improper going on.” Woodcock said: “I was aware, at that time that McKinsey was only doing consulting work of an administrative nature for the FDA. ... Nothing to do with any product or standard or anything like that.”

Endo Pharmaceuticals Discontinues Cheaper Version Of Treatment For Central Precocious Puberty.

NPR (11/6, Lupkin, 3.69M) reported Endo Pharmaceuticals is discontinuing its medication for central precocious puberty histrelin acetate (Vantas), forcing patients

to turn to the more expensive version of the drug, Supprelin LA. The company "told NPR there was a manufacturing problem." NPR added, "It's hard to know exactly how many children will be affected by the Vantas discontinuation because data about off-label use is hard to come by." FDA spokesman Jeremy Kahn wrote in an email: "The FDA expects that manufacturers will notify the agency before a meaningful disruption in their own supply occurs."

Gottlieb Says COVID-19 Pandemic In US Could Be Over By The Time Biden's Workplace Vaccine Mandates Take Effect. CNBC (11/5, Towey, 7.34M) reported, "The COVID-19 pandemic could be over in the US by the time President Joe Biden's workplace vaccine mandates take effect in early January, Pfizer board member Dr. Scott Gottlieb told CNBC's 'Squawk Box' on Friday." OSHA's COVID-19 vaccine mandates "begin on Jan. 4 for any company with at least 100 employees."

The Hill (11/5, Sullivan, 5.69M) reported, "Former Food and Drug Administration Commissioner Scott Gottlieb said Friday that he thinks the COVID-19 pandemic in the United States could be over by January, transitioning to a lower-level 'endemic' presence." COVID-19 "is not expected to disappear entirely, but it could settle into a lower-level, constant presence that does not cause the disruption and harm of the past year and a half."

Newsweek (11/7, Zhao, 2.67M) reports Gottlieb "said Sunday that families can safely gather for the Thanksgiving holiday, with partially vaccinated children in close proximity around their vaccinated grandparents, depending on local case rates in their community."

Axios (11/7, 1.26M) also covers the story.

Gottlieb Expects "Broad Immunity" Among Younger Children As More Get Vaccinated Against COVID-19. CBS News (11/7, Hayes, 5.39M) reports, "Dr. Scott Gottlieb, the former commissioner of the Food and Drug Administration who now serves on Pfizer's board of directors, said Sunday that he expects 'broad immunity' against COVID-19 among younger children as more get vaccinated." The CDC "recommended the pediatric version of Pfizer's COVID-19 vaccine for children 5 to 11 years old" last week. Throughout the country, "vaccine clinics and pediatricians' offices...have already received shipments of the smaller vaccine and started putting shots in arms."

The Hill (11/7, Schnell, 5.69M) reports, "Gottlieb on Sunday said he is not expecting travel from outside the country to 'feed a lot of additional infections,' one day before the US is set to lift border restrictions for fully vaccinated international travelers."

MEDICAL PRODUCT SAFETY

Study Suggests MDMA May Improve Outcomes Among Individuals With Severe PTSD. TODAY (11/5, Carroll, 2.24M) reported that 67% of individuals with severe post-traumatic stress disorder (PTSD) who received MDMA "no longer qualified for a diagnosis of PTSD two months after the treatment," according to findings published in Nature Medicine. Those findings may "help get PTSD therapy with MDMA approved by the Food and Drug Administration." TODAY added, "According to the National Institute on Drug Abuse, MDMA is also in clinical trials as a possible treatment aid for anxiety in terminally ill patients and for social anxiety in autistic adults. The FDA recently classified MDMA-assisted psychotherapy for PTSD as a 'breakthrough therapy.'"

FDA Grants Breakthrough Device Designation To ReWalk For ReBoot Product. FierceBiotech (11/5, Park, 4K) reported, "ReWalk Robotics is picking up the pace on its trek down the FDA's review pathway." The FDA "has granted ReWalk's latest offering a breakthrough device designation" for Reboot, "a soft exoskeleton that's designed to be worn by patients undergoing rehabilitation for lower limb disabilities caused by stroke and other neurological injuries." This designation gives "the company access to an expedited evaluation." Additionally, "once ReWalk has submitted its regulatory application for the ReBoot system, not only will the FDA prioritize the submission, but experts from the agency will also keep in touch with ReWalk throughout the review so any issues can be addressed as quickly and efficiently as possible."

FDA Panel Rejects Nasal Spray For Treating PWS. MedPage Today (11/5, Grant, 183K) reported, "An investigational carbetocin nasal spray for treating extreme hunger and other symptoms of Prader-Willi syndrome (PWS), a rare genetic disorder, received a near-unanimous thumbs-down from FDA advisors on Thursday." For some advisors, "the decision to vote 'no' came down to how FDA framed the question at hand – in short, whether there was 'substantial evidence' of efficacy with the product." NHLBI affiliate James Troendle, PhD, "emphasized the importance of the question's wording before explaining his 'no' vote." He said, "I don't even think they even met a low bar for evidence, let alone 'substantial' evidence – it's far from that."

Novel Drug For Patients With Anemia From CKD Succeeds In Two Phase 3 Trials. FiercePharma (11/5, Liu, 12K) reported, "After the FDA's resounding rejection for AstraZeneca and FibroGen's

roxadustat, attention on novel oral anemia therapies has shifted to later players,” and “now, third-in-line GlaxoSmithKline has notched up a first-in-class success.” For “dialysis-dependent and nondialysis patients with anemia from chronic kidney disease, GSK’s daprodustat matched up to traditional erythropoiesis-stimulating agents (ESAs) in terms of heart safety, as measured by a composite marker of time to first major adverse cardiovascular event.” The data, “from two phase 3 trials, ASCEND-D and ASCEND-ND, were unveiled at American Society of Nephrology Kidney Week 2021.”

Opinion: Drug Pricing Legislation Should Preserve Savings From Generics, Biosimilars.

Association for Accessible Medicines President and CEO Dan Leonard wrote in an opinion for STAT (11/6, 262K), “Americans rightfully expect Congress to address the ever-increasing prices of brand-name prescription drugs.” However, Leonard cautioned that “the wholesale policy changes now under rushed consideration in Congress will undermine the only proven solution to this long-standing problem: competition from Food and Drug Administration-approved generic and biosimilar medicines.” He said that while “Congress is right to try to meet the expectations of a nation concerned about the high prices of brand-name drugs...policies to reform drug pricing should be focused on ensuring that Americans have access to more-affordable medicines by eliminating barriers and improving incentives for competition.”

Columnist: Science Should Not Have To Depend On 20th-Century Institutions To Guide 21st-Century Progress.

In his column for The Atlantic (11/5, 3.53M), Derek Thompson wrote, “Two stories in science are worth cheering right now: the amazing amount of knowledge humanity is gathering about COVID-19 and the quietly revolutionary ways we’re accelerating the pace of discovery.” He added that last year, George Mason University economist Tyler Cowen and Stripe CEO Patrick Collison “co-founded a new program for quickly funding scientific research on COVID-19.” Dubbed Fast Grants, they “aimed to solve the speed problem in several ways.” For example, “Its application process was designed to take half an hour, and many funding decisions were made within a few days.” Thompson wrote, “The US relies on a fleet of scientific agencies – the CDC, FDA, NIH, and NSF – that are decades old and that, in many cases, act their age.” He concluded, “We shouldn’t have to depend on 20th-century institutions to guide 21st-century progress.”

FOOD SAFETY

Tastykake Krimpets Recalled Over Possible Metal Pieces.

CBS News (11/5, 5.39M) reported, “Tastykake cake treats sold by retailers across the U.S. are being added to a recall of cupcakes in more than half a dozen states because the baked goods could contain tiny fragments of metal mesh wire, according to a notice posted by the Food and Drug Administration.” The cakes “have been added to the list of products being recalled by Thomasville, Georgia-based Flowers Foods, the maker of Tastykakes and one of the biggest U.S. producers of packaged bakery foods.”

The Miami Herald (11/7, Neal, 647K) also covers the story.

Jumbo Cooked Shrimp Recalled For Possible Listeria.

The Miami Herald (11/6, Cohen, 647K) reported, “Southeastern Grocers, parent company of Winn-Dixie, Fresco y Más and Harveys Supermarkets, has recalled its Fisherman’s Wharf brand Jumbo Cooked Shrimp that is sold frozen in 16-20 count, 16-ounce bags” for possible listeria. The issue “with these affected bags was found after routine testing detected listeria in the 16-20 count, 16-ounce bags of the jumbo cooked shrimp, according to the U.S. Food and Drug Administration’s recall alert issued on Nov. 5.”

Effects Of Poppy Seed Consumption Highlighted.

According to Inverse (11/6, Wells, 645K), “A sprinkling of poppy seeds in a muffin or over a bagel may sound utterly innocent, but the consumption of these seeds is still regularly flagged by urine tests as opium,” and “the National Institute of Drug Abuse suggests skipping a poppy seed snack altogether before a drug test, just to be safe.” Sam Houston State University assistant professor of forensic toxicology Madeleine Swortwood said, “What came to our attention a few years ago was that there were companies that were intentionally not washing the seeds, not processing the seeds, so that they could sell seeds that were essentially laced with morphine and codeine.” However, she added, “The FDA and DEA have done a really good job of shutting down some of those companies that might be selling the unwashed poppy seeds. As long as you’re buying them at grocery stores and major retailers they should be fairly safe.”

TOBACCO

Democrats’ Spending Bill Imposes Heavy Taxes On E-Cigarettes And Vapes.

Fox Business (11/4, Hasson, 3.06M) reported, “The latest version of Democrats’ Build Back Better Act is poised to cripple the multi-billion dollar American e-cigarette and vaping industry

and could drive tobacco-free nicotine pouches from the market altogether, experts warn.” The current proposal “leaves tobacco taxes untouched, while imposing heavy taxes on e-cigarettes and vapes, as well as tobacco-free nicotine pouches and lozenges.” The provision is “intended to help fund Democrats’ massive proposed social spending package by trying to bring taxes on tobacco-free nicotine products in line with the tax rates on tobacco products.” However, critics find the method “ill-conceived in its attempt to treat tobacco products and tobacco-free alternatives the same,” and say “many tobacco-free products will be at a severe disadvantage to similar tobacco products.”

Reason (11/5, 350K) reported, “The proposed nicotine tax would raise about \$10 billion at most” and possibly “much less given the Food and Drug Administration’s current crackdown on which vaping products are allowed in the market.”

FDA Rescinds MDO For Humble Juice E-Liquids.

The Tobacco Reporter (11/5) reported, “The U.S. Food and Drug Administration has rescinded the marketing denial order (MDO) issued Sept. 15, 2021, for Humble Juice Co.’s flavored e-liquid products, the company announced on Nov. 5.” In October, Humble filed a petition “challenging the FDA’s decision and seeking to have the MDO vacated.” The FDA stated “that upon further review it identified information contained in Humble’s PMTA that requires additional evaluation such as ‘randomized controlled trials comparing tobacco-flavored ENDS to flavored ENDS as well as several cross-sectional surveys evaluating intentions to use or likelihood of use in current smokers, current ENDS users, former tobacco users, and never users.’”

Vuse Closing Market-Share Gap With Juul. The Winston-Salem (NC) Journal (11/5, Craver, 226K) reported, “The market-share gap tightened slightly again between top-selling electronic cigarette Juul and No. 2 Vuse of R.J. Reynolds Vapor Co. in the latest Nielsen analysis of convenience store data.” In its report covering “the four-week period ending Oct. 23,” Nielsen found “Vuse had a 34.3% market share, up from 33.3% in the previous report.” Juul was “at 40.6%, down from 41% in the previous report.” Sales of e-cigarette products “overall have slumped since February 2020, when the Food and Drug Administration implemented its latest round of heightened regulations on the products.”

Baby Monkey Forced To Smoke Cigarette For Public Health Campaign, Sparking Backlash.

The Daily Mail (UK) (11/5, Davies, 4.11M) reported a Chinese zoo “has sparked outrage after forcing a baby monkey to smoke a cigarette on camera as part of a supposed public health campaign.” In a promotional video, a monkey is seen smoking and “a woman off-screen removes the cigarette and

tells off the animal, saying smoking is harmful.” The video sparked backlash with many saying “the video was cruel.” The FDA has “said animals exposed to cigarette smoke have been found to be at higher risk of illnesses such as pneumonia, eye problems, heart disease, and cancer.”

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Biogen Declines To Say If It Seeks IG Advice On Aduhelm Giveaway

By [Gabrielle Wanneh](#) / September 9, 2021 at 10:42 AM

Biogen is giving free Aduhelm to patients at Florida-based First Choice Neurology, and that arrangement is at risk of being considered a kickback, a former HHS Inspector General official said. However, the company said its program is common practice and declined to say whether it seeks an advisory opinion from the IG that would help shield it from enforcement action.

"Biogen is committed to ensuring that appropriate patients have access to any Biogen medicines prescribed by their physicians," Biogen said. "It is common for pharmaceutical companies, including Biogen, to offer patient assistance programs designed to help remove non-clinical barriers to access to care, and Biogen designs and administers its access programs to comply with applicable laws and regulations."

Reuters reported that Biogen is providing its Alzheimer's drug for free while Medicare determines whether, and under what circumstances, to cover Aduhelm. FDA granted Aduhelm an accelerated approval in June against the advice of independent agency advisers. It's the first new

Alzheimer's treatment in nearly two decades, but many insurers and doctors don't believe there is sufficient evidence that the drug works to outweigh the risk of serious side effects.

It's common for companies to offer free medications, and the legality of patient assistance programs depends on their details. Companies may seek advice from the IG on their programs, but they aren't required to get advisory opinions, and advisory opinions are not legally binding, according to Jennifer Michael, former chief of the Industry Guidance Branch at the IG.

Based on the description of Biogen's program in the Reuters article, Michael says there's a chance the IG would consider the arrangement to be a 'seeding' program. Seeding refers to the practice of giving free drugs to a subset of eligible patients to induce those patients to use drugs that federal health programs will eventually pay for.

"The government might be concerned that those types of seeding programs could result in the potential for overutilization or inappropriate utilization," Michael told *Inside Health Policy*. -- Gabrielle Wanneh (gwanneh@iwpnews.com)

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Premier Acquires Stake In Specialty Drug Maker To Ensure Supply Of At-Risk Drugs

By [Gabrielle Wanneh](#) / September 9, 2021 at 2:39 PM

The group purchasing organization Premier and 11 leading health systems [have invested a small stake in specialty drug maker Exela Pharma Sciences](#) to ensure the supply of essential drugs that face possible shortages due to sourcing problems spurred by the COVID-19 pandemic.

The participating health systems are expected to secure an uninterrupted supply of 19 pharmaceutical products, including generic injectables that often appear on the FDA drug shortage list and 503B compounded pharmaceutical products provided by Exela, which primarily sources active ingredients domestically and from Europe, through its manufacturing facility in North Carolina.

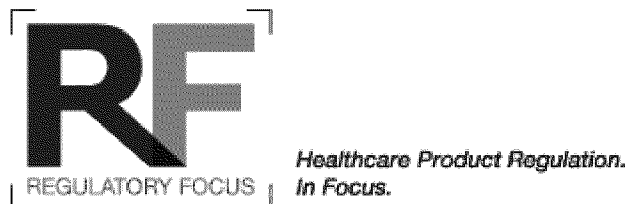
The move is a part of [a longtime initiative of Premier's ProvideGx and is similar to actions by other hospital groups](#) like CivicaRx that aim to avert drug shortages and prevent price gouging that often follows a drug scarcity. For more than a decade, several drug shortages have contributed to \$230 million in additional drug costs as well as poorer health outcomes for patients, according to a 2018 study conducted by researchers at the University of Pittsburgh.

"Leveraging Exela's significant production capacity, this collaboration is expected to give Premier's participating members uninterrupted access to high-quality, U.S.-made pharmaceutical products and safety stock," Premier President and CEO Michael Alkire said in a statement Wednesday (Sept. 8). "With this investment, we are committing to support the domestic production of vital medicines for our members and the market and to bring much-needed solutions to help eliminate drug shortages."

The participating health systems have signed a multi-year commitment to buy a portion of their drugs from Exela through Premier's GPO. They include Baystate Health of Springfield, MA; Genesis Health System of Davenport, IA; Henry Ford Health System of Detroit, MI; McLaren Health Care of Grand Blanc, MI; Memorial Healthcare System of Hollywood, FL; OSF HealthCare of Peoria, IL; Riverside Health System of Newport News, VA; Saint Francis Health System of Tulsa, OK; Texas Health Resources of Arlington, TX; and Universal Health Services, Inc. Of King of Prussia, PA. -

- Gabrielle Wanneh (gwanneh@iwpnews.com)

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FDA updates guidance on generic drug development during COVID

Posted 09 September 2021 | By [Michael Mezher](#)

The US Food and Drug Administration (FDA) on Wednesday revised its questions and answers guidance on generic drug development and abbreviated new drug application (ANDA) submissions during the COVID-19 public health emergency.

The revised guidance updates the agency's previous guidance on generic drug development during the COVID-19 pandemic that was released in April 2021. (RELATED: [FDA offers guidance on generic drug development during COVID](#), *Regulatory Focus* 5 April 2021)

The revisions include a clarification about when FDA will accept requests to receive an ANDA with "less than the full complement of recommended stability data," and information on how to handle expired batches of test product in interrupted bioequivalence studies. FDA also says it has made some minor updates throughout the frequently asked questions section.

"FDA recognizes that the COVID-19 public health emergency may impact the development of generic drug products, interrupt or delay ANDA applicants' bioequivalence studies, and impact the submission of ANDAs to FDA for assessment. The ongoing development of generic drugs submitted as ANDAs to the FDA for assessment helps Americans continue to have access to safe, effective generic drugs," FDA says.

In the revised guidance, FDA now explains that it will "generally accept requests to receive an ANDA with less than the full complement of recommended stability data if the drug product is on FDA's Drug Shortage List or FDA confirms that the drug product is vulnerable to shortage." The agency also says it will also generally do so for products that meet its criteria for priority review to help address the COVID-19 pandemic.

FDA is also granting generic drugmakers additional flexibility when it comes to resuming bioequivalence studies that were interrupted due to the COVID-19 public health emergency. The revised guidance now provides recommendations allowing prospective applicants to potentially use a test product batch that is beyond the established expiration dating period to resupply bioequivalence studies, "as long as sufficient evidence can be provided to demonstrate the acceptability of the test product batch."

FDA further explains that applicants who wish to follow this approach "should submit data to ensure the test batch samples stored at the long-term stability conditions beyond the current expiration date are in compliance with the finished drug product shelf-life specifications at the time of conducting the [bioequivalence] BE study."

[FDA](#)

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Kaleido warned; FDA not persuaded by 'medical food' claim

The US Food and Drug Administration (FDA) recently issued a [warning letter](#) to Kaleido Biosciences for administering an investigational new COVID-19 treatment to patients without obtaining an investigational new drug (IND) application.

The company argued that the product under investigation was a food and not a drug, and therefore not subject to IND filing requirements. Yet the agency disagreed, saying that the endpoints used in the clinical trials went beyond 'dietary management' of COVID-19 and were more aligned with drug trials in assessing effectiveness of the treatment.

The 26 August warning letter was sent over "objectionable conditions" observed following a mid-February to early March inspection of Kaleido's Lexington, MA facility, conducted under the auspices of FDA's bioresearch monitoring program. At issue was the firm's clinical investigation of a molecule called KB109 for treating COVID-19. The treatment is a microbiome metabolic therapy, a synthetic glycan that is orally administered for treating COVID-19.

The warning letter focused on two protocols, Protocol K031-120, and Protocol K032-120. The first trial was a randomized, open-label prospective parallel group study to assess the natural history of COVID-19 and compare the effect of the treatment in patients undergoing supportive self-care (SSC). The other trial was an exploratory open-label clinical study to evaluate how the treatment affected the gut microbiota of adult patients with mild to moderate COVID-19.

Between the two multi-site trials, 399 patients were randomized.

Kaleido argued it was not obligated to submit an IND before initiating these clinical investigations because KB109 is a food rather than a drug. Specifically, Kaleido argued that the investigations were conducted "to evaluate the effect of KB109 on the microbiome" and to "determine the safety and tolerability of KB109 in the disease population," according to FDA's characterization of the firm's position.

These arguments failed to persuade FDA, which concluded that the "evidence collected during the inspection showed that KB109 was intended for use as a drug and not as a food."

FDA maintained that "even if there were distinctive nutritional requirements established for COVID-19, Protocols K031-120 and K032-120 went beyond 'dietary management' of COVID-19 to evaluate KB109's ability to treat COVID-19 by measuring the investigational product's effects on fever, oxygen saturation, viral load, and other biomarkers of COVID-19 infection, among other things."

Kaleido's argument that KB109 is a medical food fails on other counts as well, wrote FDA. Since no particular nutritional requirements that might help manage COVID-19 have yet been discovered, KB109 cannot be used in the dietary management of the disease, as would be required for a product classified as a medical food.

Also, the fact that an article has been established to be generally recognized as safe (GRAS) does not preclude its use as a drug. FDA clarified that "an article may be a drug as used in a clinical trial, even if it is considered a food as used in other contexts."

The company has 15 days to file a response.

Daniel Menichella, Kaleido's chief executive officer, told *Focus* in an email that "we are reviewing the letter currently

and look forward to providing a response within the given timeframe and continuing our discussions with the FDA.”

[Kaleido warning letter](#)

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FDANEWS

Advocacy Group Files Lawsuit Seeking FDA Action on Propecia

September 10, 2021

A new lawsuit filed against the FDA this week contends that the male pattern baldness drug Propecia (1 mg) should either be taken off the market or its prescribing information should include warnings about the risk of long-term erectile dysfunction, depression and suicide.

The consumer watchdog group Public Citizen filed the suit Sept. 8 in the U.S. District Court for the District of Columbia on behalf of the Post-Finasteride Syndrome Foundation (PFSF). The complaint asks the FDA to act on a petition the foundation filed in 2017, which has gone unanswered.

The case has more merit than it did when originally filed, Philip Roberts, PFSF’s patient manager, told *FDAnews*. “Much more scientific information has come to light since then,” he said, including two studies by Harvard investigators confirming the risk of long-term erectile dysfunction, depression, suicidal ideation and suicide; and epidemiologic data showing that the drug alters the expression of more than 3,700 genes in men who take the drug. The drug mimics a male hermaphroditism gene; people who have this are genetic males who sometimes look female and who never go bald.

The 2017 petition asked FDA to either withdraw 1 mg oral Propecia from the market or include a Black Box warning and send out a “Dear Healthcare Provider” letter, alerting clinicians to the risks associated with the medication. The petition included information on about a dozen men whose suicides were considered related to Propecia use and contraindications for patients with a history of sexual dysfunction and depression.

Since then, PFSF has amassed more data, including 17,953 adverse event reports filed in the International Drug Monitoring database. These include reports of erectile and sexual dysfunction (5,234) and 4,313 reports of psychiatric disorders, including anxiety (1,756) and depression (1,386). The database includes 88 cases of completed suicides, 39 attempts and 384 cases of suicidal ideation.

In light of Propecia’s enormous global market (almost 9 million users in 2018, according to the website ClinCalc.com), the numbers are very small. Nevertheless, they represent an unnecessary tragedy that needs to be addressed, said Michael Kilpatrick, the Public Citizen attorney who filed the suit.

“It’s a small percentage, but the number of prescriptions is so high that it’s still a large number of people,” he told *FDAnews*. “We think patients should know this. It’s a drug for a cosmetic issue. If people knew there was even a slight change you could develop a significant, life-altering syndrome and even think about suicide, they might not want to take it.”

The FDA has been sitting on the matter far too long, he said.

“A four-year wait is unreasonable. Our goal is to compel FDA to issue a decision on the original petition. We think it’s a strong one and that the decision should be for granting a black box warning or even withdrawing the drug. This delay is in violation of the Administrative Procedure Act and given the seriousness of the issue and the quality of the petition, it should be resolved.”

Merck didn’t reply to a request for comment. The company has moved all of its Propecia patient and prescribing data to a new company spinoff, Organon, launched on June 3.

Read the PFSF complaint here: www.fdanews.com/09-09-21-Complaint.pdf. — Michele G. Sullivan

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Humanigen’s Lenzilumab for COVID-19 Draws FDA Complete Response Letter

September 10, 2021

The FDA has declined to grant Humanigen an emergency use authorization (EUA) for its antibody lenzilumab for treatment of hospitalized COVID-19 patients.

The agency instead sent the Burlingame, Calif.-based company a Complete Response Letter (CRL), in which it said it was “unable to conclude that the known and potential benefits of lenzilumab outweigh the known and potential risks of its use as a treatment for COVID-19,” Humanigen said.

But the agency also said in the letter that it is committed to working with Humanigen to develop lenzilumab and has invited the company to submit additional data as it becomes available, the company said.

Humanigen CEO Cameron Durrant said the National Institutes of Health’s ACTIV-5/BET-B study, which is enrolling up to 500 patients, is expected to provide further data that may support a new EUA request. The company said it is also committed to completing the regulatory processes it has under way to seek a marketing authorization for lenzilumab to treat hospitalized COVID-19 patients in the UK and other territories.

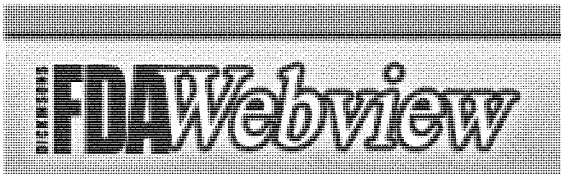
Humanigen is focused on preventing and treating an immune hyper-response called ‘cytokine storm,’ which has been a major problem for critically ill COVID-19 patients. Lenzilumab neutralizes granulocyte-macrophage colony-stimulating factor (GM-CSF) that plays a role in inflammation that can lead cytokine storm.

The company said its phase 3 LIVE-AIR study “suggests early intervention with lenzilumab may prevent consequences of a full-blown cytokine storm in hospitalized patients with COVID-19.”

Humanigen is also studying the antibody for other inflammatory conditions such as acute graft versus host disease in patients undergoing allogeneic hematopoietic stem cell transplantation, eosinophilic asthma, and rheumatoid arthritis.

— Martin Berman-Gorvine

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Apellis Pharma Plan NDAs for Geographic Atrophy

09/09/2021

Apellis Pharmaceuticals says it plans to file an NDA in the first half of 2022 after reporting top-line results from the Phase 3 DERBY and OAKS studies evaluating intravitreal pegcetacoplan, an investigational targeted C3 therapy, in 1,258 adults with geographic atrophy secondary to age-related macular degeneration. Geographic atrophy is a leading cause of blindness that affects about one million people in the U.S.

In the studies, pegcetacoplan demonstrated a clinically meaningful slowing of disease progression with an even stronger effect in geographic atrophy patients with extrafoveal lesions, the company says. "Monthly and every-other-month treatment with pegcetacoplan met the primary endpoint in OAKS, significantly reducing [geographic atrophy] lesion growth by 22% ($p=0.0003$) and 16% ($p=0.0052$), respectively, compared to pooled sham at 12 months," it says. "DERBY did not meet the primary endpoint, showing a reduction in GA lesion growth of 12% ($p=0.0528$) and 11% ($p=0.0750$) with monthly and every-other-month treatment, respectively, compared to pooled sham at 12 months."

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Myfembree sNDA for Endometriosis Pain

09/09/2021

FDA has accepted for review a Myovant Sciences and Pfizer supplemental NDA for Myfembree (relugolix 40 mg, estradiol 1 mg, and norethindrone acetate 0.5 mg) for managing moderate to severe pain associated with endometriosis. The agency set a user fee review action target date of 5/6/2022.

The submission is based on data from the Phase 3 SPIRIT program, which included two multinational, replicate pivotal clinical studies (SPIRIT 1 and SPIRIT 2) in over 1,200 women with pain associated with endometriosis for 24 weeks, and an open-label extension study for eligible women through one year.

Endometriosis is an estrogen-dependent, inflammatory disease in which tissue similar to the uterine lining is found outside the uterine cavity, which results in chronic inflammation and can cause scarring and adhesions.

Myfembree is currently approved for managing heavy menstrual bleeding associated with uterine fibroids in premenopausal women, with a treatment duration of up to 24 months.

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New FDA Jardiance Breakthrough Therapy Designation

09/09/2021

FDA has granted breakthrough therapy designation to Boehringer Ingelheim and Lilly's Jardiance (empagliflozin) for treating heart failure with preserved ejection fraction. An *American Journal of Managed Care* online [post](#) says Jardiance is the first drug in its class to gain the designation. The post says results from the EMPEROR Preserved trial showed that Jardiance, a sodium glucose co-transporter 2 inhibitor reduced cardiovascular death and hospitalization for heart failure by 21%.

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FDA Rejects Lenzilumab EUA for Hospitalized Covid Patients

09/09/2021

FDA has rejected a Humanigen emergency use authorization (EUA) request for the use of its lenzilumab in newly-hospitalized Covid-19 patients. FDA reportedly told the company it was unable to conclude that the known and potential benefits of lenzilumab outweigh the known and potential risks of its use as a treatment for Covid-19.

The company [says](#) the agency committed to working with it in the development of lenzilumab and invited Humanigen to submit additional data as they become available. Humanigen says the National Institutes of Health ACTIV-5/BET-B study is expected to provide further data that may support a new EUA request.

Humanigen describes lenzilumab as a first-in-class antibody that binds to and neutralizes granulocyte-macrophage colony-stimulating factor.

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Congress Must Regulate CBD Since FDA Won't: Steve Mister

09/09/2021

Responding to an FDA objection to a new dietary ingredient notification submitted by Charlotte's Web to demonstrate that its full-spectrum hemp extract is reasonably expected to be safe, Council for Responsible Nutrition CEO **Steve Mister** says Congress must step in and regulate the CBD (cannabidiol) marketplace. Mister appeared with Charlotte's Web CEO **Deanie Elsner** in a "Short Legal Briefs" [video](#) posted by *Natural Products Insider*.

In the video, Elsner said she was disappointed in the way the agency made its decision to object to the notification for two reasons: that CBD products are excluded from the definition of a dietary supplement and that there were concerns about the sufficiency of the safety evidence.

She indicated that her company met multiple times with FDA officials before the notification was submitted to share data. The agency "ignored big parts of our application," she said.

Mister said the FDA denial letter showed that the agency is not going to solve the problem with CBD and thus Congress must act. He said the industry should turn its attention to persuading Congress to intervene. Regulation is needed, he said, because reputable companies that are concerned about the quality of their products are stuck in a "quagmire" with other companies that are not as concerned about quality, and consumers are being hurt.

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FiercePharma

No dice: Court tells AbbVie it can't force Takeda to supply a drug that's in shortage worldwide

by [Eric Sagonowsky](#) | Sep 9, 2021 9:28am

Amid the global shortage of prostate cancer drug Lupron, AbbVie took its partner Takeda to court over alleged failures to live up to supply agreements. Now, a court has denied the company's plea to force Takeda to fill orders.

In an [opinion](#) (PDF) this week, Delaware Chancery Court Vice Chancellor Sam Glasscock III denied AbbVie's request to compel Takeda to fill supply orders from its plant in Hikari, Japan.

Under a deal between the companies, AbbVie sells the drug in the U.S. and Canada. Because Takeda is the sole supplier of the drug worldwide, AbbVie relies on the Japanese drugmaker for its product.

Problem is, the med has been in short supply worldwide after the FDA and Takeda in 2019 found "protocol violations" at the plant, Glasscock wrote in this week's opinion. Thanks to "remediation efforts" including a weeks-long production hold global supply hiccups and shortages have persisted to this day, Glasscock wrote.

AbbVie had asked the court to find that Takeda breached its contract plus issue damages and "injunctive relief," or an order forcing Takeda to supply the drug. In his new opinion, Glasscock said an injunction would be "unworkable" because it'd force the court to oversee Takeda's operations and would "inevitably lead to contempt hearings" over Takeda's ability to fulfill orders.

"In other words, even if I find that Takeda has breached its contract with AbbVie and that, as a result, AbbVie faces irreparable harm, the injunctive relief sought is unavailable," Glasscock wrote.

The Lupron saga started in November 2019, when FDA inspectors traveled to Japan to review production processes at the Hikari site. In a June 2020 warning letter, the FDA slapped Takeda for poor equipment maintenance, faulty documentation and quality shortfalls. The company has been working to resolve those concerns, but the Lupron problem has dragged on throughout this year.

Right now, the FDA [lists](#) most Lupron Depot dose forms as "available" in the U.S., but there's one dose that's not expected to become available until next January.

While Glasscock decided against an injunction forcing Takeda to fill orders, he hasn't made a ruling on whether the company breached its contract. He plans to issue a decision on that issue in "due course," he said, and another trial phase on damages could follow.

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WCLC: Jazz charts new path for small cell lung cancer drug Zepzelca after late-stage flop

by [Angus Liu](#) | Sep 9, 2021 11:00am

Jazz Pharmaceuticals wasn't able to ride a breakthrough FDA nod to a pivotal trial win for conditionally approved small cell lung cancer (SCLC) drug Zepzelca. But after talking with the FDA, the company and partner PharmaMar see a new path forward.

In previously treated SCLC, the combination of Zepzelca and doxorubicin failed to extend patients' lives over physician's choice of doxorubicin-based chemo or Hycamtin, according to data presented at the 2021 World Conference on Lung Cancer virtual meeting.

Jazz and PharmaMar first unveiled the phase 3 ATLANTIS failure in December. Now we know patients who mostly got the Zepzelca regimen as their second-line treatment lived a median 8.6 months, versus 7.6 months in the control arm.

If successful, the trial could have served as the confirmatory trial to convert Zepzelca's accelerated approval earned last June into a full one. In a previous single-arm study, Zepzelca monotherapy shrunk tumors in 35% of relapsed SCLC patients as measured by investigator assessment. The response lasted a median 5.3 months.

Pascal Besman, chief operating officer at PharmaMar, noted that the ATLANTIS study used a 2-mg/m² dose of Zepzelca, which is lower than the FDA-approved dose of 3.2-mg/m². Although the Zepzelca-doxorubicin combo didn't improve survival, "the overall results support the activity and tolerability of Zepzelca monotherapy in second-line therapy," Besman said in an email interview.

As ATLANTIS investigator Luis Paz-Ares, M.D., Ph.D., noted in a prepared presentation, the research team ran a so-called exposure-response analysis to gauge Zepzelca's contribution on patient survival. The model predicted that single-agent Zepzelca, at the 3.2-mg/m² dose, would have had a statistically significant advantage over the control arm at prolonging the lives of patients in the ATLANTIS study.

Jazz stands "firmly behind the favorable benefit/risk profile of monotherapy Zepzelca" in second-line SCLC as shown in its phase 2 trial, the company's chief medical officer, Rob Iannone, M.D., said in a separate email interview.

For now, Zepzelca will remain on the market. Jazz and PharmaMar have agreed "in principle" with the FDA to run another phase 3 as the confirmatory trial, Besman said.

The three-arm trial will test Zepzelca monotherapy at 3.2 mg/m², and a second experimental arm will combine the drug at 2 mg/m² with Pfizer's Camptosar (irinotecan).

Second-line patients in the control arm will get physician's choice or either Camptosar or Hycamtin. The trial, expected to launch this year, will also support a regulatory filing to the European Medicines Agency, Besman added.

In addition, Jazz is collecting data from an observational phase 4 trial to understand Zepzelca's real-world profile in second-line SCLC, Iannone noted.

Meanwhile, Jazz is also looking to move Zepzelca earlier in the treatment line. Through a partnership with Roche, a phase 3 trial dubbed ImForte will test Zepzelca with immunotherapy as a first-line maintenance therapy for patients

with extensive-stage SCLC after induction chemotherapy. If successful, the new regimen could pressure AstraZeneca and its Imfinzi, which is currently dominating the front-line SCLC space.

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WCLC: AstraZeneca's detailed Imfinzi, tremelimumab data in metastatic NSCLC are far from a clear-cut win

by [Angus Liu](#) | Sep 9, 2021 11:00am

After two high-profile late-stage trial flops, AstraZeneca has finally scored a victory for Imfinzi and ill-fated tremelimumab in newly diagnosed metastatic non-small cell lung cancer (NSCLC). But in a lucrative field where immuno-oncology rivals Merck and Bristol Myers Squibb have established a solid presence, a detailed look at the data reveals a win that's perhaps too little, too late.

A cocktail of Imfinzi, investigational CTLA-4 inhibitor tremelimumab and chemotherapy pared down the risk of death by 23% over chemo alone in previously untreated patients, AZ announced Thursday at the 2021 World Conference on Lung Cancer virtual meeting.

The phase 3 POSEIDON trial came after two prior phase 3 flops for Imfinzi in metastatic NSCLC and marked the first positive pivotal readout for treme after many more failures in multiple indications. But the data don't look favorable compared to Merck's Keytruda or Bristol Myers' Opdivo, and efficacy in some patient subgroups showed imbalances that could limit an eventual FDA approval if AZ gets one.

First off, the combination of Imfinzi and chemo failed to significantly outdo chemo alone at extending patients' lives. That pairing only reduced the risk of death by 14% over chemo alone. That already makes Imfinzi look inferior to current standard-of-care Keytruda.

Keytruda's combo with chemo is well known for its impressive 51% death risk reduction over chemo alone in front-line non-squamous NSCLC. Those were the results at an interim analysis in the phase 3 Keynote-189 trial, while the final analysis at 31 months of follow-up showed a 44% risk decrease. As for front-line squamous cell NSCLC, Keytruda and chemo reduced death risk by 36% at interim and 29% at final analysis in the KEYNOTE-407 trial.

Luckily for AZ, treme finally pulled through to help Imfinzi. The CTLA-4 agent has a bad track record with Imfinzi and has been marred with failures in NSCLC as well as small cell lung, head and neck and bladder cancers. In front-line NSCLC alone, the dual immunotherapy combo of Imfinzi and treme flopped the phase 3 MYSTIC trial in a broad patient group and the NEPTUNE trial in patients with high tumor mutation burden.

Now that the Imfinzi-treme-chemo doesn't appear likely to shake up Keytruda's dominance, what about less competitive Opdivo? Data don't look very promising for AZ.

For one thing, while the chemo-free regimen of Imfinzi and treme failed, Opdivo's own CTLA-4 combo with Yervoy is approved for front-line NSCLC patients whose tumors express PD-L1.

For a better apple-to-apple comparison, the Opdivo-Yervoy-chemo combo won an FDA nod last year for front-line NSCLC patients regardless of PD-L1 status. In the CheckMate 9LA trial, Opdivo, Yervoy and chemo were able to show a survival benefit early, having cut patients' risk of death by 31% over chemo after a minimum follow-up of 8.1 months. After a longer follow-up of at least 24.4 months, the rate stood at 28%, as patients on the Opdivo-Yervoy-chemo regimen lived a median 15.8 months versus 11 months for the solo chemo group.

Imfinzi-treme-chemo's 23% death reduction looks slightly weaker than Opdivo-Yervoy-chemo's 28%, but cross-trial comparisons have their problems. In POSEIDON, patients on the AZ therapy lived a median 14 months, versus 11.7 months for those who took chemo alone.

To further complicate the front-line NSCLC landscape, Roche's PD-L1 inhibitor Tecentriq is allowed as a monotherapy for PD-L1-high patients thanks to data from the IMpower110 trial. And its combo with Avastin and chemo has an approval for non-squamous disease after showing it reduced the risk of death by 22% over Avastin and chemo.

Sanofi and Regeneron's Libtayo also bears a monotherapy nod in front-line PD-L1-high NSCLC. The companies recently found that adding Libtayo on top of chemo could further prolong the lives of first-line patients in the phase 3 EMPOWER-lung 3 trial.

Eli Lilly and Innovent Biologics have a front-line non-squamous NSCLC filing for their China-approved PD-1 inhibitor Tyvyt under review at the FDA. The application includes data from a China trial that showed a combination of Tyvyt, Lilly's Alimta and platinum chemo could stave off tumor progression or death over chemotherapy alone.

For AZ, non-squamous NSCLC might be just what it could eventually reach for with the Imfinzi-treme-chemo pairing.

As POSEIDON lead investigator Melissa Johnson, M.D., noted during a prepared presentation Thursday, among patients with the squamous histology, progression-free survival (PFS) "and overall survival were poor across all treatment arms, with little separation of the curves for PFS or overall survival."

In the squamous subgroup, Imfinzi, trema and chemo only managed to reduce the risk of death by 12% over chemo.

What's more, the AZ therapy also showed some worrisome signs in other patient subgroups. Notably, women and Asian patients didn't seem to experience any additional survival benefits from Imfinzi and trema.

The one upside? The efficacy of Imfinzi, trema and chemo didn't seem to be bound by a patient's PD-L1 expression level.

Still, with the mixed bag of data and a poor track record, AZ could face a limited FDA label and a hard time winning over market share in front-line NSCLC. Imfinzi is currently approved to treat stage 3 NSCLC and extensive-stage small cell lung cancer. In the first half of 2022, AZ plans to read out data from PEARL, an IMpower110 counterpart trial that evaluates Imfinzi monotherapy against chemo as front-line treatment for PD-L1-high patients.

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Biogen CEO blames Aduhelm's rocky launch on 'too much confusion, misinformation and controversy'

by [Eric Sagonowsky](#) | Sep 9, 2021 1:44pm

Before Biogen scored an FDA approval for Alzheimer's drug Aduhelm, the company probably hoped its launch would go much smoother than the last three months have been. It turns out Biogen's CEO Michel Vounatsos is willing to say so publicly.

Speaking at a Morgan Stanley healthcare event Thursday, Vounatsos said there's "clearly too much confusion, misinformation and controversy surrounding our data and the approval process." The launch has been "even more gradual than we had expected," he added.

The company is facing "near-term challenges," Vounatsos told the virtual audience, noting that "everybody can see that." Now, months after the drug's approval, he said it's "imperative" that "society moves its attention [from] the clinical data to the patients in need."

After Aduhelm's accelerated approval in June, a firestorm quickly engulfed the new Alzheimer's drug. Lawmakers and medical experts have questioned the data Biogen used to support an approval plus the FDA's procedures that led to the accelerated nod.

The criticism grew so intense that the FDA and Biogen, in an unusual move, walked back Aduhelm's broad label just weeks into the launch. The FDA originally endorsed the drug for all Alzheimer's patients but later restricted the label to those with mild cognitive impairment or mild dementia.

In another big setback for the rollout, the Department of Veteran's Affairs last month decided against covering the drug, citing a "risk of significant adverse drug events" and a "lack of evidence of a positive impact on cognition."

Looking forward, one key question mark for the rollout remains in the Centers for Medicare & Medicaid Services' coverage decision on the drug. That's expected in January and will determine whether federal insurance programs get behind the treatment.

Meanwhile, the company is working to improve the public's understanding of the clinical data and on generating additional data, Vounatsos said. It's also aiming to bring more treatment centers online and negotiate reimbursement deals to "maximize access," he added.

Biogen's drug launched with big sales expectations, but its initial stumbles have dampened some of the enthusiasm on Wall Street. In a note about Vounatsos' Thursday presentation, Jefferies analysts said the Street is "very negative on the early launch." Analysts still expect the drug to bring in multiple billions of dollars per year once it gets rolling.

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Despite mixed phase 3 showing, Apellis hopes to push eye disorder drug past FDA's finish line

by [Noah Higgins-Dunn](#) | Sep 9, 2021 4:05pm

Apellis Pharmaceuticals' pegcetacoplan posted mixed results in two late-stage trials studying the C3 inhibitor in patients living with geographic atrophy (GA). Despite the mixed showing, CEO Cedric Francois thinks the med's performance was strong enough to warrant an FDA nod.

Apellis studied pegcetacoplan, the active ingredient in rare disease drug Empaveli, in two phase 3 trials, known as Oaks and Derby, as a monthly and every-other-month treatment in 1,258 patients with geographic atrophy (GA) secondary to age-related macular degeneration (AMD). For the studies, investigators weighed efficacy by measuring change in the total area of GA lesions from baseline.

In Oaks, pegcetacoplan met its primary endpoint in both dosing groups, significantly reducing GA lesion growth by 22% in the monthly arm and 16% in the every-other-month group compared with sham injections after 12 months, Apellis said.

The drug failed to do the same in Derby, reducing lesions by just 12% and 11%, respectively. The drug didn't produce any significant safety concerns in both trials, although its use was associated with slightly higher rates of exudation, or emitted fluid.

Despite the conflicting readouts, Apellis is "unbelievably happy" with the results given the totality of pegcetacoplan clinical trial performance, CEO Cedric Francois said in an interview. The company plans to file the med with the FDA in the first half of 2022.

There are no FDA-approved treatments for the condition, an advanced form of AMD that eventually leads to vision loss and blindness in older people.

"This is a drug that, in our opinion, has absolutely no doubt around its efficacy and comes out with a safety profile that I think exceeds our own and other people's expectations," Francois said.

Meanwhile, the drug posted another late-stage upside, Francois argued. In a prespecified analysis of the combined trials, pegcetacoplan was more effective in patients with extrafoveal lesions at the start of the trials, decreasing GA lesion growth in that group by as much as 26% in the monthly arm and 23% in the every-other-month group.

GA patients typically present first with extrafoveal lesions, which then progress toward the fovea where central vision is impacted, Apellis says. That's important because the disease is like a forest fire in the retina, continuously destroying photoreceptor cells until someone's blind with no way to turn it around, Francois said.

There are roughly 1 million Americans living with the condition and 5 million people globally. If Apellis is able to do what others have failed, it could be looking at \$3 billion in peak sales, Jefferies analysts recently predicted.

Pharma has tried and failed to push GA treatments over the FDA's finish line in recent years, including an effort by Roche on its complement-inhibiting drug lampalizumab. That drug, however, crashed out of two phase 3 trials in 2017.

This has already been a busy year for Apellis, which scored its first-ever FDA nod in May for pegcetacoplan in patients with the rare blood disorder paroxysmal nocturnal hemoglobinuria (PNH). That has teed-up a showdown between Apellis' C3 inhibitor and Alexion's heavyweight C5 inhibitors Soliris and its follow-up Ultomiris, which target a different enzyme.

"If this drug gets approved, it will be the first time that complements control would get approved in a large speciality, rather than in a rare disease like PNH," Francois said. "I think it opens up a new era for what a complement can do in many of these conditions across multiple therapeutic areas."

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Room Available In US Pharma Industry For OTC Switch Leader: Has Perrigo Made A Reservation?

09 Sep 2021

ANALYSIS

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Executive Summary

Acquiring HRA Pharma, Perrigo could take OTC switch lead, likely starting with HRA's daily oral contraceptive before moving to numerous other potential candidates. Pharmas previously busy in switch have other priorities for their consumer health businesses.

The US pharma industry has a vacancy for an OTC switch leader as longtime sponsors of applications to make additional ingredients available nonprescription prioritize reorganizing their consumer health businesses over expanding their product portfolios.

By acquiring HRA Pharma, Perrigo Company PLC could fill the space in the pharma industry. Its switch proposals likely will start with HRA's daily oral contraceptive before moving to numerous other potential candidates.

"There aren't a lot of switch powerhouses out there. I think there's an opportunity for a new player to emerge as a switch powerhouse, given the changing market dynamics," says pharma industry consultant Susan Levy .

"I think this alignment, HRA with Perrigo's scale, is just what the industry needs," said switch consultant Susan Lavine Coleman, president of NCI Consulting in Moorestown, NJ.

Levy and Coleman spoke with HBW Insight following Perrigo's 8 September announcement of an agreement to acquire Paris-based HRA, listed in France as Héra SAS, for €1.8bn (\$2.1bn) in a cash deal.

Perrigo's predominant revenue stream has been private label/store brand OTC drugs, although it has added brands with its 2014 move into Europe's consumer health market and by acquiring the Prevacid 24 HR (lansoprazole/15 mg) proton pump inhibitor in 2019 and licensing the Burt's Bees brand in May to make and market a line of organic baby formulas and supplements for babies and children.

"I think there's an opportunity for a new player to emerge as a switch powerhouse, given the changing market dynamics." – Susan Levy

But while it consistently is first to launch generic equivalents for brand switches, its name hasn't been linked to submitting proposals for making additional drug ingredients available OTC.

Bringing HRA, including management, under its roof changes the switch outlook for Perrigo.

"These are pros. They know what they're doing," Perrigo president and CEO Murray Kessler said about HRA's management during a briefing about the deal. "They've all agreed to stay on with Perrigo."

Slow Time For Switch NDAs

The HRA acquisition isn't the only reason Perrigo could become a top OTC switch sponsor in the US. Other pharmas are leaving the top spot open.

Looking over the current lineup of major OTC marketers in the US, Levy, founder and principal of Susan B. Levy Consulting LLC in Cranford, NJ, doesn't see any interested in sponsoring new drug applications for OTC switches.

"If you look at who has historically been a driver for OTC switches, you've got GSK, Novartis and Pfizer, but they're all one [consumer health] company now, and it's being spun out. It's kind of unclear to what extent they are positioned right now to invest in expensive growth programs," she said.

GlaxoSmithKline plc is the majority owner of a joint venture with Pfizer Inc. marketing the two firms' consumer health product lines. GSK, with products including brands it acquired after previously operating a consumer health JV with Novartis AG, plans to spin out its current consumer health business. (Also see "McNamara To Lead GSK's Standalone Consumer Healthcare Business" - HBW Insight, 23 Jul, 2021.)

"Then you've got Sanofi," Levy added.

The French firm is known to be considering proposing switches for flu treatment Tamiflu (oseltamivir) and erectile dysfunction drug Cialis (tadalafil), but it too looks to separate its consumer health business. (Also see "Sanofi To Divest 60% Of Brands In Consumer Healthcare Overhaul" - HBW Insight, 10 Feb, 2021.)

"It's unclear where they want to put their bets right now as a result of being spun out independently," she said.

Bayer AG earlier in 2021 received Food and Drug Administration approval for OTC sales of Astepro Allergy (azelastine hydrochloride nasal spray, 205.5 mcg per spray). Bayer plans to launch the first-in-class switch, the first ingredient available OTC that is a first-generation H1 (histamine receptor inhibitor) antihistamine, in 2022. (Also see "Market Exclusivity Decision Pending For Bayer's First-In-Class OTC Switch Astepro Allergy" - HBW Insight, 24 Jun, 2021.)

Levy pointed out the previous OTC switch for a product the German firm markets in the US was Oxytrol for Women overactive bladder treatment, a first-in-class switch when it was approved in 2013 when US

firm Merck & Co., Inc. owned the brand. After acquiring Merck & Co.'s consumer business in 2014, Bayer relinquished OTC rights to the 3.9mg oxybutynin transdermal patch to Allergan PLC less than a year later.

J&J Large In Consumer, Not In Switch

Johnson & Johnson may be synonymous with OTC drugs due to its market-leading brands, but like Perrigo it hasn't been a switch driver.

"Who could be the powerhouses? ... J&J has not historically been a switch player. They don't have an experienced team of switch professionals," Levy said.

"It speaks to the fact that there is a gap in the marketplace because these switch programs are very expensive for an OTC company. They are somewhat risky, and they're much longer-term than typical OTC innovation. ... If you're heading up a consumer health care company and thinking about where you're going to put your R&D bets, much more incentives are there to do some of the shorter-term, easier innovation."

The FDA is encouraging firms to study making available nonprescription drug ingredients indicated for chronic conditions; in 2012 it opened a docket for recommendations on changing its OTC switch NDA rules to allow for "novel switches" targeting chronic conditions, indications more complicated than most currently available OTC drugs have.

The agency launched the initiative expecting labeling for novel switches to be subject to the same limits as other OTCs, with all necessary information available on a product's Drug Facts label. However, it has since indicated in a 2018 guidance that switch sponsors could tap into digital media and other extra-label tools to help demonstrate that consumers can assess whether an OTC drug is appropriate for them and they can use the product safely. (Also see "Before Asking FDA For Novel OTC Switch, Be Sure DFL Alone 'Can't Get There'" - HBW Insight, 23 May, 2019.)

In a recent FDA workshop conducted online, industry experts and other stakeholders said some problems with OTC DFLs are worse than others and some likely can be corrected with minor changes to the format, but other changes, notably for supporting OTC labeling for drugs that currently are available Rx-only and are indicated for chronic conditions aren't minor and won't happen within the limits of one-dimensional text media printed on nonprescription drug containers, packages and inserts. (Also see "Progress On Adding OTC Drugs To US Market? Room To Move But Label Format Limits Options" - HBW Insight, 18 Jun, 2021.)

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Sept. 9 Quick Takes: Obsidian readies for IPO with \$115M crossover round

PLUS: VENTURE ROUNDS FOR LEXEO, EVOMMUNE, ALAMAR, AND SETBACKS FOR HUMANIGEN, SANOFI AND MORE

BY BIOCENTURY STAFF

SEP 9, 2021 | 9:44 PM EDT

Cell and gene therapy play Obsidian Therapeutics Inc. looks set to seek a public listing after raising \$115 million in a series B round led by The Column Group Crossover Fund. Other new investors included RA Capital, Surveyor Capital, Cowen Healthcare Investments, Deep Track Capital, Logos Capital, Pivotal BioVenture Partners, Samsara BioCapital and Soleus Capital. Existing investors Atlas Venture, Vertex Ventures HC, Amgen Ventures, Bristol Myers Squibb Co. (NYSE:BMJ) and Vertex

Pharmaceuticals Inc. (NASDAQ:VRTX) also participated. Obsidian's lead tumor infiltrating lymphocyte (TIL) program, cytoTIL15, is expected to enter the clinic next year.

\$100M SERIES B TO SUPPORT LEXEO'S GENE THERAPIES

Adding to an \$85 million January series A, Lexeo Therapeutics raised \$100 million in a series B led by D1 Capital Partners and Eventide Asset Management. Also backing the round were CAM Capital, Verition Fund Management, Laurion Capital Management, Gray's Creek Capital Partners, and existing investors Longitude Capital, Omega Funds, Lundbeckfonden Ventures, PBM Capital, Janus Henderson Investors, Woodline Partners LP, Invus Capital and Alexandria Venture Investments. The company has three lead AAV-mediated gene therapy programs: LX2006, which is in preclinical testing for cardiomyopathy associated with Friedreich's ataxia, and LX1004 for CLN2 Batten disease and LX1001 for APOE4-associated Alzheimer's disease, both of which are in Phase I/II testing. The company is also developing recently acquired cardiac gene therapies from Stelios Therapeutics Inc. and additional preclinical programs targeting Alzheimer's disease.

EVOMMUNE RAISES \$83M IN SERIES A

Evommune Inc., which was founded in 2020 by Dermira Inc. alums, raised \$83 million in series A round with participation from Andera Partners, LSP, global syndicate of investors and Pivotal BioVenture Partners, the seed investor, to enable development of therapies to treat inflammatory diseases through industry and academic collaborations. Evommune has exclusive rights to three of the four candidates in its pipeline, from Dermira, now a Eli Lilly and Co. (NYSE:LLY) company.

SHERPA LEADS ALAMAR'S \$80M SERIES B

An \$80 million series B round led by Sherpa Healthcare Partners will enable Bay Area-based proteomics company Alamar Biosciences Inc. to accelerate the development of its NULISA and Attobody technology platforms to deliver single-digit attomolar detection sensitivity. NULISA (NUcleic acid-Linked Immuno-Sandwich Assay) seeks to provide "ultra-high sensitivity and massively parallel scale" to proteomic analysis, while Attobody, the company says, yields highly specific picomolar affinity antibodies. Other firms participating in the round included new investors Morningside Ventures and Samsara Biocapital and existing investors Qiming Venture Partners, which lead the series A, and Illumina Ventures. The company has now raised \$110 million.

LILLY TAKES EQUITY STAKE IN LYCIA AS PART OF \$70M SERIES B

New Lycia Therapeutics, Inc. partner Eli Lilly and Co. (NYSE:LLY) has invested in the South San Francisco company's \$70 million series B, which Redmile Group led. Founding investor Versant Ventures and new investors Cowen Healthcare Investments, Invus, RTW Investments and Alexandria Venture Investments also participated. The financing comes two weeks after Lycia picked Lilly as its collaborator to develop LYTACs against targets implicated in the pharma's areas of focus.

FIRST APPROVAL FOR PFIZER'S ORAL ATOPIC DERMATITIS THERAPY COMES IN U.K.

The U.K.'s MHRA approved Cibinqo abrocitinib from Pfizer Inc. (NYSE:PFE) to treat atopic dermatitis, marking the first global approval for the JAK1 inhibitor. FDA pushed back the therapy's PDUFA date due to a class-wide safety review. Last month, the oral therapy demonstrated superiority to leading atopic dermatitis therapy Dupixent dupilumab in the Phase III JADE DARE trial.

CENTESSA REPORTS FIRST POSITIVE DATA READOUT SINCE FORMATION

Asset-centric play Centessa Pharmaceuticals plc (NASDAQ:CNTA) had its first positive data readout since the company was formed this year by Medicxi. Centessa subsidiary ApcinteX Ltd. said that in the Phase IIa portion of a Phase I/IIa trial, the high dose of SerpinPC over six months led to an 88% reduction in annualized bleeding rate compared with a pre-exposure observation period in patients with either hemophilia A or hemophilia B. The PROC inhibitor was well tolerated with no cases of sustained elevation of D-dimer, a marker of excess thrombin generation. Data from an open-label extension of the study are expected next year. Centessa rose \$1.21 to \$22.26

HUMANIGEN SINKS AFTER FDA DECLINES TO ISSUE EUA

Shares of Humanigen Inc. (NASDAQ:HGEN) shed nearly half their value Thursday after the biotech said FDA declined to grant an emergency use authorization for lenzilumab, its mAb targeting GM-CSF, to treat hospitalized patients with COVID-19. Humanigen said FDA could not yet conclude that lenzilumab's benefits outweigh its risks, but the biotech believes the NIH-funded ACTIV-5/BET-B study may generate data sufficient to support the mAb's emergency authorization. Humanigen lost \$7.14 (47%) to \$7.97 on Thursday.

ACQUIRED IN PRINCIPIA DEAL, SANOFI'S BTK MISSES IN PEMPHIGUS

Sanofi (Euronext:SAN; NASDAQ:SNY) said Btk inhibitor rilzabrutinib failed to significantly improve remission rates in the Phase III PEGASUS trial to treat pemphigus, missing the study's primary endpoint. The pharma gained the molecule via its \$3.4 billion buyout of Principia Biopharma Inc. (NASDAQ:PRNB) last year. Rilzabrutinib is also in a Phase III study to treat chronic immune thrombocytopenia.

AZ-AVILLION COMBO MEETS MARK IN ASTHMA READOUTS

AstraZeneca plc (LSE:AZN; NYSE:AZN) and Avillion LLP said PT027, the partners' fixed-dose combination of albuterol and budesonide, met the primary endpoints in the Phase III DENALI and MANDALA trials to treat asthma. The treatment significantly improved lung function compared with each of its components in DENALI, and significantly reduced exacerbations compared with albuterol alone as a rescue medicine in MANDALA. Blackstone Life Sciences, Abingworth and Royalty Pharma plc (NASDAQ:RPRX) are backing the PT027 development program in a risk-sharing arrangement.

POSITIVE READOUT FOR A SUBCUTANEOUS GENMAB BISPECIFIC

Epcoritamab, a subcutaneously administered CD3 x CD20 bispecific antibody from Genmab A/S (CSE:GMAB; NASDAQ:GMAB), led to an 88% ORR in relapsed or refractory diffuse B cell lymphoma patients, providing support that the formulation may compete with more burdensome IV infusions on efficacy. Among eight patients who received the recommended Phase II dose of 48 mg, 38% achieved a complete response. ORR was 90% and CR was 50% in the 10 follicular lymphoma patients treated across dose ranges in the dose-escalation study. There was no Grade 3 or higher cytokine release syndrome.

Odronextamab (REGN1979), a CD3 x CD20 bispecific from Regeneron Pharmaceuticals Inc. (NASDAQ:REGN), led to an ORR of 92.9% in follicular lymphoma patients in a Phase I readout last year, though a partial clinical hold was placed on the program in December due to incidence of Grade 3 and higher CRS during step-up dosing.

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Cancer leads way to routine use of accelerated approval: Data Byte

AN ANALYSIS FROM BIOCENTURY'S 2021 BACK TO SCHOOL PACKAGE

BY SELINA KOCH, EXECUTIVE EDITOR

SEP 9, 2021 | 7:08 PM EDT

Accelerated approval is becoming the *de facto* route for approvals of new molecular entities in cancer.

In three of the past five years, the number of new cancer drugs granted accelerated approval by FDA has outnumbered those given full approval. Halfway through this year, the count stood at eight accelerated to four standard approvals.

BioCentury's annual Back to School project explored cancer as a case study of the promise and problems of accelerated approval, and argued for changes that could usher in routine use of the pathway for all serious diseases with unmet needs.

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COVID-19

Biden Calls On Governors To Mandate COVID-19 Vaccinations For School Workers.

The [[HYPERLINK](#)

"<https://www.nytimes.com/2021/09/09/us/politics/biden-schools-vaccines-testing-covid.html>"] (9/9, Green, 20.6M) reports "President Biden on Thursday amplified calls for states to take more aggressive measures to keep children in school amid surging coronavirus cases, calling on governors to require vaccinations for all school employees and for districts to implement more regular testing." In the most recent "iteration of the White House's plan to combat the coronavirus pandemic, the administration will also require teachers and other employees of schools run by federal agencies to be vaccinated as part of his broad push to get the federal work force protected." That rule "would apply to those who teach in Head Start programs, Department of Defense Schools, and schools operated by the Bureau of Indian Education."

[[HYPERLINK](#) "[https://www.usnews.com/news/education-news/articles/2021-09-09/biden-to-raise-pressure-on-schools-](https://www.usnews.com/news/education-news/articles/2021-09-09/biden-to-raise-pressure-on-schools-to-require-vaccinations)

[to-require-vaccinations](#)"] (9/9, Camera, 1.91M) says "the pressure from Biden...signals a new strategy for the administration, which has been trying to push states and school districts to adopt on their own the suite of safety measures recommended by the" CDC.

Biden Says He Will Take On GOP Governors Attempting To Stop COVID-19 Measures In Schools. [[HYPERLINK](#)

"<https://thehill.com/homenews/administration/571595-biden-vows-to-take-on-gop-governors-over-schools>"] (9/9, Gangitano, 5.69M) reports "President Biden said Thursday he will take on Republican governors who are trying to prevent COVID-19 protocols in schools, vowing to 'get them out of the way.'" Biden said, "Right now, local school officials are trying to keep children safe in a pandemic while their governor picks a fight with them and even threatens their salaries or their jobs. Talk about bullying in schools." He added, "If they'll not help, if these governors won't help us beat the pandemic, I'll use my power as president to get them out of the way."

Los Angeles School District Makes Student COVID-19 Vaccinations Mandatory.

The [[HYPERLINK](#) "<https://www.latimes.com/california/story/2021-09-09/los-angeles-school-officials-approve-vaccine-mandate>"] (9/9, Gomez, 3.37M) reports that all children 12 and older in

Los Angeles public schools "must be fully vaccinated against COVID-19 by January to enter campus." LAUSD interim Superintendent Megan K. Reilly "said the student mandate was the next logical step to keep children, staff and community members safer from a COVID-19 pandemic that still poses significant risks."

The [HYPERLINK "https://www.nytimes.com/2021/09/09/us/la-vaccine-mandate-students-schools.html"] (9/9, Goldstein, 20.6M) reports the district's Board of Education "voted, 6-0, to pass the measure on Thursday afternoon," which "would eventually apply to more than 460,000 students, including some enrolled at independent charter schools located in district buildings."

The [HYPERLINK "https://www.washingtonpost.com/education/2021/09/09/los-angeles-school-district-student-vaccine-mandate/"] (9/9, Meckler, Knowles, 10.52M) reports the new regulations require "students 12 and older who participate in in-person extracurricular programs to receive their first shot of the Pfizer-BioNTech vaccine by Oct. 3 and their second by the end of that month." All other eligible "students must get their first dose by Nov. 21 and their second by mid-December."

The [HYPERLINK "https://www.wsj.com/articles/los-angeles-school-district-requires-students-to-get-covid-19-vaccine-11631230734"] (9/9, Koh, Subscription Publication, 8.41M) reports students who are not vaccinated by January 10 will not be allowed on campus and must enroll in virtual learning.

Among other outlets covering the story are the [HYPERLINK "https://apnews.com/article/health-education-los-angeles-coronavirus-pandemic-school-boards-3e6d32b5545965d20128953478ee144d"] (9/9, Taxin, Antczak), the [HYPERLINK "https://playvideo.bulletinintelligence.com/7c4067035bc34e0aa530049580736604?pubid=fda"] (9/9, 6:35 p.m. EST, story 2, 2:30, O'Donnell, 3.48M), [HYPERLINK "https://playvideo.bulletinintelligence.com/23a98a412dac426ea54a62f6956dd88c?pubid=fda"] (9/9, 6:34 p.m. EST, story 2, 1:50, Holt, 4.78M), [HYPERLINK "https://r.bulletinintelligence.com/7ed9ea90906e47d39ef8421d0c252c5c"] (9/9, Allen, O'Brien), [HYPERLINK "https://www.cnn.com/2021/09/09/us/los-angeles-schools-vaccine-mandate-students/index.html"] (9/9, Andone, Chan, Mossburg, 89.21M), [HYPERLINK "https://thehill.com/homenews/state-watch/571557-los-angeles-school-board-to-vote-on-vaccine-mandate-for-students"] (9/9, Choi, 5.69M), [HYPERLINK "https://www.foxnews.com/politics/los-angeles-public-school-board-votes-to-implement-student-vaccine-mandate"] (9/9, 23.99M), [HYPERLINK "https://www.forbes.com/sites/kimberleespeakman/2021/09/09/la-school-district-will-require-eligible-students-get-

vaccinated-against-covid-19/"] (9/9, Speakman, 10.33M), and [HYPERLINK "https://thehill.com/policy/healthcare/571614-los-angeles-school-board-mandates-covid-vaccines-for-all-eligible-students"] (9/9, Vakil, 5.69M).

FDA Rejects Humanigen's Monoclonal Antibody As COVID-19 Treatment. [HYPERLINK

"https://www.reuters.com/business/healthcare-pharmaceuticals/us-fda-declines-emergency-use-approval-humanigens-covid-19-drug-2021-09-09/"] (9/9, Bhalla) reports the FDA "declined Humanigen Inc's request for emergency use authorization (EUA) of its lenzilumab drug to treat newly hospitalized COVID-19 patients, the company said on Thursday." In a statement, Humanigen said, "In its letter, FDA stated that it was unable to conclude that the known and potential benefits of lenzilumab outweigh the known and potential risks of its use as a treatment for COVID-19." The company "expects an ongoing study to provide additional safety and efficacy data to support its new EUA request."

Among other sources covering the story are [HYPERLINK "https://thehill.com/policy/healthcare/571546-emergency-use-authorization-for-humanigen-covid-19-drug-declined"] (9/9, Schnell, 5.69M), [HYPERLINK "https://www.fiercebiotech.com/biotech/fda-slams-door-humanigen-s-face-rejects-emergency-use-green-light-wants-more-data"] (9/9, Adams, 4K), [HYPERLINK "https://www.statnews.com/2021/09/09/fdas-rejection-of-humanigen-drug-highlights-problems-in-developing-covid-19-treatments/"] (9/9, Herper, Subscription Publication, 262K), and [HYPERLINK "https://endpts.com/humanigen-crashes-as-fda-shoots-down-its-covid-19-drug-eua/"] (9/9, Brennan).

US COVID-19 Cases More Than 10 Times Higher Than What Is Needed To End Pandemic, Fauci Says. The [HYPERLINK

"https://www.nytimes.com/2021/09/09/health/fauci-covid-cases.html"] (9/9, Anthes, Slotnik, 20.6M) reports, "Coronavirus infections are more than ten times higher than they need to be in order to end the pandemic, Dr. Anthony S. Fauci, the nation's top infectious disease doctor, told the political news site Axios." He said, "In a country of our size, you can't be hanging around and having 100,000 infections a day. ... That's not even modestly good control." Fauci added, "You've got to get well below 10,000 before you start feeling comfortable."

[HYPERLINK "https://thehill.com/policy/healthcare/public-global-health/571468-fauci-covid-19-infections-10-times-higher-than-needed"] (9/9, Choi, 5.69M) and [HYPERLINK "https://www.axios.com/fauci-no-control-covid-a2572210-

dbdb-48b0-aa2e-92e8e9fcd69a.html?stream=top"] (9/9, Drage O'Reilly, 1.26M) also cover the story.

TSA Doubles Fines For People Who Refuse To Wear Masks On Public Transit.

The [HYPERLINK "https://apnews.com/article/health-coronavirus-pandemic-transportation-6c88f60f19e6bce843c6332456584488"] (9/9) reports the Transportation Security Administration "is doubling the fine for people who break the rule requiring masks on planes, trains and other forms of public transit to slow the spread of COVID-19, with President Joe Biden warning Thursday that violators should 'be prepared to pay.'"

The [HYPERLINK "https://www.washingtonpost.com/transportation/2021/09/09/tsa-fines-wear-mask-airports/"] (9/9, Aratani, 10.52M) says, "Beginning Friday, the fine for refusing to wear a mask will increase to a range of \$500 to \$1,000 for first offenders," and "penalties for a second offense will range from \$1,000 to \$3,000." Meanwhile, the Department of Homeland Security extended the mask mandate until January 18.

Among other outlets covering the story are [HYPERLINK

"https://www.bloomberg.com/news/articles/2021-09-09/maskless-airline-passengers-face-doubled-fines-under-biden-plan?srd=premium"] (9/9, Laing, 3.57M), [HYPERLINK "https://www.reuters.com/world/us/us-doubling-fines-travelers-not-wearing-masks-2021-09-09/"] (9/9, Shepardson), [HYPERLINK "https://www.cnn.com/2021/09/09/politics/biden-mask-flight-attendants-respect/index.html"] (9/9, Wallace, Sullivan, 89.21M), and [HYPERLINK "https://www.forbes.com/sites/joewalsh/2021/09/09/tsa-doubles-mask-wearing-fines-as-biden-administration-ramps-up-covid-fight/"] (9/9, 10.33M).

Government Counts Of COVID-19 Deaths In Nursing Homes Likely Missed More Than 16,000 Fatalities, Study Indicates.

[HYPERLINK "https://www.usatoday.com/story/news/investigations/2021/09/09/study-16-k-coronavirus-nursing-home-deaths-missing-official-count/5780082001/"] (9/9, Stein, 12.7M) reports, "Government counts of the devastation from coronavirus among the most vulnerable elderly likely missed more than 16,000 COVID-19 deaths in U.S. nursing homes during the early months of the pandemic, an academic study" has found. Additionally, researchers "estimated that 68,000 additional coronavirus cases – representing nearly 12% of last year's total nursing home cases – were omitted before a federal reporting requirement took effect in late May 2020."

[HYPERLINK "https://thehill.com/policy/healthcare/571538-research-federal-tally-missing-16k-nursing-home-covid-deaths"] (9/9,

Weixel, 5.69M) reports the Trump Administration "first imposed a requirement for nursing homes to report weekly cases and deaths on May 24, 2020, through the Centers for Disease Control and Prevention's National Healthcare Safety Network (NHSN) system" but "nursing homes were not required to report retroactive data, and it's not known how many facilities chose to do so."

[HYPERLINK "https://www.forbes.com/sites/jemimamcevoy/2021/09/09/thousands-more-nursing-home-residents-died-from-covid-than-acknowledged-by-government-study-says/"] (9/9, McEvoy, 10.33M) also reports on the story.

WHO Warns COVID-19 Vaccine Supply In Africa Will Drop By 25% Partly Due To Booster Shots For Developed Countries.

[HYPERLINK "https://www.axios.com/who-africa-vaccine-supply-0b835175-4efc-4072-ba04-a6bedbff745d.html"] (9/9, Knutson, 1.26M) reports, "The World Health Organization warned on Thursday that Africa will receive 25% fewer doses of the coronavirus vaccine than expected by the end of the year, in part because of the approval of booster shots in developed countries." The WHO's regional director for Africa, Matshidiso Moeti, "criticized developed countries for wasting vaccine doses and specifically referenced the more than 15 million doses that have been discarded in the U.S. since March." According to data from the Africa CDC, "just over 3% of the continent's population has been fully vaccinated against the virus."

The [HYPERLINK "https://apnews.com/article/africa-health-coronavirus-pandemic-united-nations-kenya-94980903d9d697a74d91183e8a4ced7b"] (9/9, Anna) reports, "The Africa CDC says 145 million vaccine doses have been procured across the continent of 1.3 billion people, and 111 million of them, or 77%, have been administered."

[HYPERLINK "https://www.forbes.com/sites/tommybeer/2021/09/09/despite-desperate-need-africa-will-receive-25-fewer-covid-vaccine-doses-than-anticipated-who-says/"] (9/9, Beer, 10.33M) reports, "Responding to Wednesday's announcement that the United Nations-backed COVAX program has again been forced to reduce its delivery projections, Matshidiso Moeti, the WHO's Africa Director, said the goal of vaccinating 10% of people in Africa by the end of September is no longer a realistic target."

UK's MHRA Approves COVID-19 Boosters, But Government Will Make Final Decision On Implementing Program.

[HYPERLINK "https://www.reuters.com/world/uk/uk-medicine-regulator-says-pfizer-astrazeneca-covid-19-shots-may-be-used-2021-09-09/"] (9/9, Smout) reports Britain's MHRA "on Thursday gave the go-ahead for Pfizer and AstraZeneca's COVID-19

vaccine to be used as booster shots." While "the final decision on whether there should be booster shots is for the government," the agency's "decision clears one hurdle along that path." Meanwhile, the UK's Joint Committee for Vaccination and Immunisation (JCVI) is also "discussing whether booster shots for the elderly and vulnerable are needed, with planning underway for a booster programme that could begin this month."

Among other sources covering the story are [HYPERLINK

"https://www.bloomberg.com/news/articles/2021-09-09/eu-regulator-set-to-decide-on-next-generation-covid-shots"] (9/9, Ring, 3.57M), [HYPERLINK "https://thehill.com/policy/healthcare/571566-uk-medical-regular-approves-coronavirus-booster-shots"] (9/9, Lonas, 5.69M), the [HYPERLINK "https://www.dailymail.co.uk/news/article-9973503/Britains-watchdog-approves-AstraZeneca-Pfizers-jabs-used-doses.html?ito=1490"] (9/9, Craig, 4.11M), [HYPERLINK "https://www.theguardian.com/world/2021/sep/09/uk-medicines-regulator-approves-covid-vaccines-for-use-as-booster-shots"] (9/9, Walker, Davis, 5.53M), and [HYPERLINK "https://www.bbc.co.uk/news/health-58499863"] (9/9, 876K).

Microsoft Employees' Return To Offices In US Delayed Indefinitely As COVID-19 Cases Surge.

The [HYPERLINK "https://apnews.com/article/technology-business-health-public-health-coronavirus-pandemic-6d93405e5f9fb9ac7c45acc023cab0e2"] (9/9) reports, "Microsoft told employees Thursday that it has indefinitely delayed their return to U.S. offices until it's safer to do so." The software maker "had already postponed its planned return to the workplace from September to no earlier than Oct. 4, but now says the re-opening won't be next month."

[HYPERLINK "https://www.bloomberg.com/news/articles/2021-09-09/linkedin-lets-you-search-for-remote-jobs-with-latest-update?srnd=premium"] (9/9, Bass, 3.57M) reports, "'Given the uncertainty of Covid-19, we've decided against attempting to forecast a new date for a full reopening of our U.S. work sites in favor of opening U.S. work sites as soon as we're able to do so safely based on public health guidance,' the Redmond, Washington-based software maker said in a blog post Thursday. 'From there, we'll communicate a 30-day transition period that provides time for employees to prepare.'"

Among other news outlets covering the story are the [HYPERLINK "https://www.wsj.com/articles/microsoft-delays-full-headquarters-reopening-to-undetermined-future-date-11631199600"] (9/9, Tilley, Subscription Publication, 8.41M), the [HYPERLINK

"https://www.nydailynews.com/news/national/ny-microsoft-coronavirus-delta-variant-reopening-20210910-vwoelh5ku5ccfozw27hocvxs4-story.html"] (9/10, Sapienza, 2.51M) and the [HYPERLINK "https://www.ft.com/content/945ab1d1-0c4e-439c-9e1b-c517d64cb01b?FTCamp=engage/CAP/webapp/Channel_Bulletin/B2B"] (9/9, Wells, Subscription Publication, 1.48M).

US Could Easily Reach Some Level Of Normalcy If All Residents Did Their Part, Expert Says.

[HYPERLINK "https://us.cnn.com/2021/09/09/health/us-coronavirus-thursday/index.html"] (9/9, Caldwell, 89.21M) reports COVID-19 "hospitalizations for those 17 and under peaked in late August at a rate approaching twice that of this year's early winter surge, according to CDC data." The rise "in pediatric cases is alarming some school officials, although one health expert said Wednesday that the best way to turn the corner was to keep taking the pandemic seriously." Dimitri Christakis, Director of the Center for Child Health, Behavior and Development at Seattle Children's Hospital, said, "People in this country have no appreciation of how fortunate we are and how easily we could return to some level of normalcy if everybody would just do their part, and I think it is unfortunately going to take sticks because we're all out of carrots." To end the pandemic, "preemptive vaccination remains the key, US Surgeon General Dr. Vivek Murthy told CNN's Jim Acosta."

Analysis: Pandemic Aid Provides Framework For Ending Hunger In The US.

In an approximately 3,300-word article, [HYPERLINK "https://www.politico.com/news/2021/09/09/covid-end-hunger-america-509226"] (9/9, Bottemiller Evich, 6.73M) writes that "the pandemic triggered a country-wide policy experiment aimed at keeping families fed and financially afloat. There have been big increases in food stamps and unemployment benefits. Three rounds of stimulus checks. Universal free meals at schools and new grocery benefits for kids who are learning virtually, or out of school during the summer. Hundreds of millions of food boxes flooded into churches and other nonprofits." Politico continues by saying the "latest tranche of aid may carry the biggest bang yet: six monthly child tax credit payments that will be dispersed through the end of the year. The first two rounds of payments that went out in July and August fueled a dramatic reduction in the rate of American households with kids who report sometimes or often not having enough to eat in the past week, according to the Census Bureau."

Scientific Guidance On Protecting Babies From Coronavirus Variants Highlighted.

[HYPERLINK

"<https://www.npr.org/sections/health-shots/2021/09/09/1035274872/babies-covid-delta-variant-need-to-know>"] (9/9, Simmons-Duffin, 3.69M) reports, "Babies can't get vaccinated against COVID-19 yet – and the youngest age included in current vaccine clinical studies is six-months-old." NPR highlights the latest scientific guidance "on how to protect a new baby from all variants of the coronavirus." The NIH is mentioned.

Analysis: Vaccine Mandates Are "American Tradition," But So Is Public Anger Over Them.

In an analysis, the [[HYPERLINK "https://www.nytimes.com/2021/09/09/us/politics/vaccine-mandates-history.html"](https://www.nytimes.com/2021/09/09/us/politics/vaccine-mandates-history.html)] (9/9, Astor, 20.6M) says that from 1898 to 1903, as smallpox "and death reigned around them, some Americans declared that they would never get vaccinated and raged at government efforts to compel them." If that "sounds familiar, well, there is nothing new under the sun: not years that feel like centuries, not the wailing and gnashing of teeth over masks, and not vaccine mandates either." As COVID-19 spreads, "the same pattern is emerging." According to the Times, vaccine mandates "are a time-honored American tradition," but "so is public fury over them."

NIH Director Discusses Biden Administration's COVID-19 Vaccination Effort, Disease's Impact On Children During Interview.

During an interview on [[HYPERLINK "https://playvideo.bulletinintelligence.com/23a9de7e9bc34a7291a363c309dfe18d?pubid=fda"](https://playvideo.bulletinintelligence.com/23a9de7e9bc34a7291a363c309dfe18d?pubid=fda)] (9/9, 251K), NIH Director Francis Collins discussed the Biden Administration's COVID-19 vaccination effort and how the disease is impacting children. Collins said, "If you're unvaccinated and you are therefore much more likely to get infected with Delta and be asymptomatic quite likely for a couple of days, you are spreading that around to others around you, including kids under 12 who can't be vaccinated. So it's not just about you." He added, "If we really want to get through this, we've got to figure out how those 80 million people who still haven't rolled up their sleeves can see this as an opportunity and a responsibility, not just about them." Collins also stated that "if you want kids to be safe right now, the best thing to do is surround them with other vaccinated people."

Fauci Discusses Biden's Six-Point Plan To Combat COVID-19 During Interviews.

In an interview on [[HYPERLINK "https://playvideo.bulletinintelligence.com/01c51095a85a40c490e1b9c95f7ec0ce?pubid=fda"](https://playvideo.bulletinintelligence.com/01c51095a85a40c490e1b9c95f7ec0ce?pubid=fda)] (9/9, 840K), NIAID Director Anthony Fauci discussed President Biden's new six-point program to combat COVID-19. Fauci said, "The data are

overwhelming to show why it is so important if we want to get this outbreak under control in this country to get vaccinated. And the President is understandably frustrated, and that's the reason why he came out with his six-point plan." During the interview, Fauci was asked, "The President outlined an aggressive new testing plan, but just yesterday your colleague Dr. Francis Collins, Director of the NIH, said there's still a problem distributing tests. How can that be?" He responded, "No, what the President was saying is that he's using the Defense Production Act to get literally millions and millions of tests, many of them free for individuals. So the distribution will come."

[[HYPERLINK "https://www.pbs.org/newshour/show/dr-fauci-on-vaccine-mandates-reopening-schools-booster-shots"](https://www.pbs.org/newshour/show/dr-fauci-on-vaccine-mandates-reopening-schools-booster-shots)] (9/9, 792K) reports on a different interview in which Fauci stated, "We have tried everything we can...to get people vaccinated. We have the solution within our grasp, within our power. We just need to implement it. And that is what the president has said tonight in his speech, that we are going to implement that. And if that means more mandates, so be it." He added that he "would respectfully object to that particular appreciation or interpretation of this as being too late. It isn't. It's something that is important. And I'm very glad that the president did it when he did it."

Biden Promises To Fill Gaps In School Funding That Result From Districts Opposing States To Bolster COVID-19 Measures.

[[HYPERLINK "https://www.npr.org/sections/back-to-school-live-updates/2021/09/09/1035600582/biden-dangles-new-federal-funds-for-schools-that-defy-mask-mandate-bans"](https://www.npr.org/sections/back-to-school-live-updates/2021/09/09/1035600582/biden-dangles-new-federal-funds-for-schools-that-defy-mask-mandate-bans)] (9/9, Kamenetz, 3.69M) reports President Biden "announced new steps to encourage K-12 schools to mandate masks for all, require vaccines for employees and step up testing for COVID-19." Some school "districts are opposing their states to strengthen COVID-19 safety measures." The President "promises to fill in any gaps in funding that result." According to the White House, "Local school districts will be able to apply to the Department of Education in the coming weeks to restore funding withheld by state leaders...when a school district implemented strategies to help prevent the spread of COVID-19 in schools." NPR adds that "teachers and staff directly employed or funded by the federal government – including those at... Head Start and Early Head Start preschool programs for children in poverty – are now subject to a vaccine requirement."

Biden Rails Against "Pandemic Politics."

[[HYPERLINK "https://playvideo.bulletinintelligence.com/0b0f8097cc14429e8e0c469bf69db284?pubid=fda"](https://playvideo.bulletinintelligence.com/0b0f8097cc14429e8e0c469bf69db284?pubid=fda)] (9/9, 6:38 p.m. EST, story 2, 1:10, Muir, 6.32M) reported President Biden "railed against

what he called pandemic politics – the fights against masks and vaccinations.” Biden took “aim at local officials that he said are actively working to undermine the fight against COVID by opposing masks and vaccination mandates.” Biden stated, “I’ll get them out of the way.”

Biden Urges Large Entertainment Venues To Require COVID-19 Vaccination As Condition Of Entry. [[HYPERLINK](#)

"<https://www.usatoday.com/story/entertainment/2021/09/09/pr-ident-biden-urges-movies-concerts-require-covid-vaccinations-testing/8266041002/>"] (9/9, Della Cava, Ruggieri, 12.7M) reports President “Biden called upon the nation’s entertainment-industry executives Thursday to do their part to help beat back the raging COVID-19 pandemic by adopting admission requirements that could cut into their bottom line and spark partisan pushback.” Biden said during a speech, “To those of you running large entertainment venues, from sports arenas to concert venues to movie theaters, please require folks to get vaccinated or show a negative test as a condition of entry.” However, “it remains to be seen if Biden’s words will turn into action at those venues that have stood their ground in declining to require audiences to be vaccinated, with most simply suggesting that masks be worn during the show.”

Democratic Representative Proposes Proof Of COVID-19 Vaccination Or Negative Test Mandate For Airplane, Train Passengers. The [[HYPERLINK](#)

"https://www.washingtonpost.com/local/beyer-vaccines-travel/2021/09/09/540faedc-118f-11ec-bc8a-8d9a5b534194_story.html"] (9/9, Flynn, Aratani, 10.52M) reports that on Thursday, Rep. Don Beyer (D-VA) introduced legislation “to require all domestic travelers to show proof of vaccination or a negative coronavirus test at airports or for Amtrak trips, seeking to revive largely stagnant discussions among federal officials about more restrictive pandemic travel requirements.”

[[HYPERLINK](#) "<https://thehill.com/homenews/house/571570-house-democrat-proposes-vaccine-or-testing-mandate-for-trains-and-planes>"] (9/9, Castronuovo, 5.69M) reports the legislation, named the Safe Travel Act, also applies to “all employees, contractors and subcontractors of Amtrak and commercial airlines.”

Department Of Education To Launch Program To Help Schools Penalized For COVID-19 Mitigation Policies. [[HYPERLINK](#)

"<https://www.bloomberg.com/news/articles/2021-09-09/biden-administration-moves-to-protect-schools-in-mask-fight>"] (9/9, Querolo, Moran, 3.57M) reports, “The U.S. Department of

Education will launch a program aimed at protecting school districts that have been penalized by their state over Covid-19 mitigation policies like mask mandates.” It “will allow local school districts to apply for grants to restore funding withheld as punishment for implementing strategies to prevent the spread of the coronavirus that are consistent with guidance from the Centers for Disease Control and Prevention.”

Opinion: Democrats Need To Focus On GOP’s “Extremist And Destructive” Behavior During COVID-19 Surge. In an opinion piece for the [[HYPERLINK](#)

"<https://www.washingtonpost.com/opinions/2021/09/09/dccc-memo-republicans-exploit-covid/>"]’s (9/9, 10.52M) The Plum Line, Greg Sargent writes, “Judging by the GOP’s continuing slide into extremist and destructive behavior in the face of a surging covid-19, electing more Republicans to positions of responsibility right now would likely mean more economic malaise, sickness, misery and death.” He adds, “A confluence of new factors is making it obvious that Democrats need to take on this argument much more forcefully, not just for the good of the party, but for the good of the country.” Sargent concludes, “Democrats should engage this forcefully...because the majorities who favor sensible collective action on behalf of public health need to hear officials giving voice to their aspirations for recovery.”

Psaki Defends Fauci, Denies Allegations He Lied About Wuhan Research Support. [[HYPERLINK](#)

"<https://www.forbes.com/sites/jemimamcevoy/2021/09/09/psa-ki-dismisses-gop-accusation-fauci-lied-to-congress-about-coronavirus-research-in-wuhan/>"] (9/9, McEvoy, 10.33M) reports that on Thursday, White House Press Secretary Jen Psaki defended NIAID Director Dr. Anthony Fauci against new “attacks inflamed by a report published” Monday by The Intercept, which reveals “two previously unpublished grant proposals from the U.S.-based health organization EcoHealth Alliance that were funded by the NIAID to conduct bat coronavirus research at the Wuhan Institute of Virology in China.” Critics now “accuse Fauci of lying when he previously told Congress the NIAID had not funded ‘gain-of-function’ research at the Chinese lab, referencing a controversial method that involves researchers modifying a virus to predict its evolutionary trajectory.”

[[HYPERLINK](#) "<https://www.foxnews.com/media/psaki-defends-fauci-allegations-lied-to-congress-doocy>"] (9/9, Flood, 23.99M) correspondent Peter Doocy questioned “Psaki about the report during Thursday’s press briefing.” She responded, “NIH has never approved any research that would make a coronavirus more dangerous to humans, a

reminder that there are previous and different coronaviruses than the existing one we're battling."

Rep. Mike Gallagher Calls On Fauci To Resign.

[[HYPERLINK "https://www.foxnews.com/media/rep-mike-gallagher-on-why-dr-anthony-fauci-must-resign"](https://www.foxnews.com/media/rep-mike-gallagher-on-why-dr-anthony-fauci-must-resign)] (9/9, Nelson, 23.99M) reports that during "America's Newsroom" on Thursday, Rep. Mike Gallagher (R-WI) "called on Dr. Anthony Fauci to resign after newly released documents showing the nation's top infectious disease expert lied about the U.S. support for gain-of-function research."

Major News Outlets Not Reporting On Recent Allegations Made About Fauci.

[[HYPERLINK "https://www.foxnews.com/media/cnn-msnbc-avoid-the-intercept-anthony-fauci-wuhan-lab-funding"](https://www.foxnews.com/media/cnn-msnbc-avoid-the-intercept-anthony-fauci-wuhan-lab-funding)] (9/9, Wulfsohn, 23.99M) reports recent "bombshell revelations" about NIAID Director Anthony Fauci "have received zero coverage on CNN, MSNBC, ABC, CBS and NBC throughout Tuesday and Wednesday, according to Grabien transcripts." In addition, "The New York Times, The Washington Post, USA Today, and The Associated Press also appeared to skip the major development."

CDC Changes Definitions Of "Vaccine" And "Vaccination."

[[HYPERLINK "https://www.mcclatchydc.com/news/coronavirus/article254111268.html"](https://www.mcclatchydc.com/news/coronavirus/article254111268.html)] (9/9, Camero, 29K) reports, "Social media is calling bluff on the Centers for Disease Control and Prevention for modifying its definition of the words 'vaccine' and 'vaccination' on its website." Vaccination was defined as "the act of introducing a vaccine into the body to produce immunity to a specific disease," but the CDC changed the word "immunity" to "protection." Originally, a vaccine was "a product that stimulates a person's immune system to produce immunity to a specific disease," but it is now "a preparation that is used to stimulate the body's immune response against diseases." People are claiming the alterations are the "CDC's attempt to hide the fact COVID-19 vaccines are not 100% effective at preventing coronavirus infection," but "a CDC spokesperson told McClatchy News the 'slight changes in wording over time...haven't impacted the overall definition.'"

Some Democrats Believe COVID-19 Could Be Winning Issue In Upcoming Elections.

[[HYPERLINK "https://www.politico.com/news/2021/09/09/biden-presidency-bottlenecked-covid-pandemic-510798"](https://www.politico.com/news/2021/09/09/biden-presidency-bottlenecked-covid-pandemic-510798)] (9/9, Korecki, Siders, 6.73M) reports, "The resurgence of Covid-19 is rapidly dominating Joe Biden's administration, putting into question the future of his broader legislative agenda and increasingly steering Democrats into treacherous political territory" as midterm elections approach. However, "Democrats in some

state races still believe Covid could prove a winning issue, painting their Republican challengers as dangerous because of their opposition to vaccinations and mask mandates – which polling shows a majority of Americans favor."

Biden's Coronavirus Response Coordinator Discusses President's New COVID-19 Plan.

[[HYPERLINK "https://www.npr.org/2021/09/09/1035726474/white-house-coronavirus-response-coordinator-on-bidens-new-covid-plan"](https://www.npr.org/2021/09/09/1035726474/white-house-coronavirus-response-coordinator-on-bidens-new-covid-plan)] (9/9, Chang, Handel, Yu, 3.69M) reports hosts Ailsa Chang and Scott Detrow spoke with White House Coronavirus Response Coordinator Jeff Zients on Thursday after President Biden "announced a variety of new rules, including several mandating vaccinations in many workplaces." Zients walked them "through the administration's strategy."

Fauci Says Government May Need To Eventually Put Pressure On People To Receive COVID-19 Vaccine.

[[HYPERLINK "https://playvideo.bulletinintelligence.com/5018268b72ce4c0ea32bd3839866f1ee?pubid=fda"](https://playvideo.bulletinintelligence.com/5018268b72ce4c0ea32bd3839866f1ee?pubid=fda)] (9/9, 6:36 p.m. EST, story 3, 2:15, Holt, 4.78M) interviewed NIAID Director Dr. Anthony Fauci about President Biden's "sweeping new [COVID-19] requirements." He hopes people will start getting vaccinated against COVID-19 willingly, but said "there will have to be things that will essentially have to put pressure" on those who refuse. He mentioned the inability to work at certain places or attend certain schools without vaccination as a way to motivate people.

Surgeon General, Education Secretary Discuss COVID-19's Effects On Children.

[[HYPERLINK "https://www.foxnews.com/media/surgeon-general-vaccine-hesitancy-fertility-natural-immunity"](https://www.foxnews.com/media/surgeon-general-vaccine-hesitancy-fertility-natural-immunity)] (9/9, Halon, 23.99M) reports that on Thursday, US Surgeon General Vivek Murthy and Education Secretary Miguel Cardona joined "Special Report" to discuss "some of the looming questions surrounding the [COVID-19] vaccine and President Biden's updated COVID-19 guidance." They addressed the effects COVID-19 has on children, natural immunity, and the science behind masks, among other topics.

CNN's Jake Tapper Says Biden Is Not Addressing COVID-19 Misinformation Problem.

[[HYPERLINK "https://www.foxnews.com/media/cnn-jake-tapper-biden-covid-misinformation"](https://www.foxnews.com/media/cnn-jake-tapper-biden-covid-misinformation)] (9/9, Wulfsohn, 23.99M) reports, "CNN anchor Jake Tapper expressed his frustration with President Biden following a White House address laying out his administration's six-pronged plan to tackle the coronavirus pandemic." He "seemed to suggest that the actions that the Biden administration is taking don't solve the

real 'problem.'" Tapper said, "One of the biggest problems that the United States is having right now getting past this pandemic is that tens of millions of Americans are being lied to, are being misinformed, are being told a whole bunch of things that are contrary to what the medical and scientific establishment say is true in terms of what's the best way to combat this virus."

CDC Predicts COVID-19 Deaths Likely To Increase Over Next Four Weeks.

[[HYPERLINK "https://www.newsweek.com/covid-deaths-potentially-increase-710k-early-october-cdc-1627607"](https://www.newsweek.com/covid-deaths-potentially-increase-710k-early-october-cdc-1627607)] (9/9, Khaled, 2.67M) reports, "The Centers for Disease Control and Prevention (CDC) on Wednesday said that COVID-19 deaths in the U.S. are likely to increase over the next four weeks to reach between 683,000 and 710,000 by October 2." The agency "also said new daily COVID-19 hospitalizations are expected to remain stable or have an uncertain trend over the upcoming four weeks with 6,400 to 19,500 newly confirmed hospitalizations likely to be reported on October 4."

North Carolina Passes 15K COVID-19 Deaths.

The [[HYPERLINK "https://www.newsobserver.com/news/coronavirus/article254101628.html"](https://www.newsobserver.com/news/coronavirus/article254101628.html)] (9/9, Sessoms, 396K) reports, "North Carolina added 110 deaths due to COVID-19 to the state's tally on Thursday, bringing the total over the pandemic to 15,004."

Louisiana COVID-19 Cases "Exorbitantly High," State Health Officer Says.

The [[HYPERLINK "https://www.theadvocate.com/baton_rouge/news/article_59ae2036-11cf-11ec-b0b0-ef46588dff60.html"](https://www.theadvocate.com/baton_rouge/news/article_59ae2036-11cf-11ec-b0b0-ef46588dff60.html)] (9/9, Sentell, 255K) reports, "While COVID-19 cases in Louisiana remain 'exorbitantly high,' the state appears to have peaked in its fourth surge of the deadly disease, state health officer Dr. Joseph Kanter said Thursday." He added, "The challenge here is given the disruption that [Hurricane Ida] presented us there is no guarantee we continue on the downward trajectory."

Washington State Requires Masks At Large Outdoor Events.

The [[HYPERLINK "https://apnews.com/article/health-coronavirus-pandemic-washington-jay-inslee-f6672a71400bbdfabacd2c5e8a5fb9d8"](https://apnews.com/article/health-coronavirus-pandemic-washington-jay-inslee-f6672a71400bbdfabacd2c5e8a5fb9d8)] (9/9, Corte) reports that on Thursday, Washington Gov. Jay Inslee (D) announced "that starting next week, the state's indoor mask mandate will be expanded to include outdoor events with 500 or more attendees, regardless of vaccination status."

Maryland School System Tightens COVID-19 Vaccination Requirements For Employees.

[[HYPERLINK "https://www.washingtonpost.com/local/education/montgomery-county-schools-staff-covid-vaccine-mandate/2021/09/09/97f5cf8c-11c1-11ec-882f-2dd15a067dc4_story.html"](https://www.washingtonpost.com/local/education/montgomery-county-schools-staff-covid-vaccine-mandate/2021/09/09/97f5cf8c-11c1-11ec-882f-2dd15a067dc4_story.html)] (9/9, St. George, 10.52M) reports, "Maryland's largest school system tightened coronavirus vaccine requirements for its 24,000 employees, deciding on Thursday to fully mandate the shots rather than allow the option of weekly testing." Employees must receive the first dose of the vaccine by the end of September and the second dose by October 29.

Iowa Judge Considers Whether To Stop School Mask Mandate Ban.

The [[HYPERLINK "https://apnews.com/article/council-bluffs-health-education-coronavirus-pandemic-kim-reynolds-bd31ad3ccb545f7fe47b04990e0f75e6"](https://apnews.com/article/council-bluffs-health-education-coronavirus-pandemic-kim-reynolds-bd31ad3ccb545f7fe47b04990e0f75e6)] (9/9, Pitt) reports that on Thursday, a lawyer representing a mother and her sons argued "Iowa Republican legislators and the governor prohibited mask mandates in schools for purely political reasons to appease complaining constituents." The hearing focused on whether the judge "should issue a temporary injunction to prevent the state from enforcing the law while the case proceeds." Frances Parr, the mother, "wants a court to order the state to issue a universal mask mandate for all students and school personnel until a voluntary plan can be implemented that separates mask-wearing students and staff from those who refuse."

West Virginia Democratic Senators Oppose Special Session On COVID-19 Regulations.

The [[HYPERLINK "https://apnews.com/article/health-coronavirus-pandemic-wv-state-wire-stephen-baldwin-4d5c53381137cb5d84ca82f3f7ad5865"](https://apnews.com/article/health-coronavirus-pandemic-wv-state-wire-stephen-baldwin-4d5c53381137cb5d84ca82f3f7ad5865)] (9/9) reports that on Thursday, West Virginia Democratic Senate leaders announced "they're against a special session to consider bills that would bar schools and businesses from requiring COVID-19 vaccinations or other measures."

New Mexico's \$100 COVID-19 Vaccine Incentive's Deadline Is Friday.

The [[HYPERLINK "https://apnews.com/article/health-new-mexico-coronavirus-pandemic-coronavirus-vaccine-351b9437271921bb7b30de2d9e3642e9"](https://apnews.com/article/health-new-mexico-coronavirus-pandemic-coronavirus-vaccine-351b9437271921bb7b30de2d9e3642e9)] (9/9) reports, "The New Mexico Department of Health says people who got a COVID-19 vaccine shot between Aug. 2-31 must sign up by Friday if they want to claim the \$100 incentive being offered to entice people to get inoculated."

Democratic Lawmakers Question Amazon's Algorithms Promoting COVID-19 Misinformation.

[[HYPERLINK](#)]

"<https://www.npr.org/2021/09/09/1035559330/democrats-slam-amazon-for-promoting-false-covid-cures-and-anti-vaccine-claims>"] (9/9, Bond, 3.69M) reports, "Amazon is under pressure from Democrats in Congress over how its algorithms promote hoax COVID-19 cures, including the livestock dewormer ivermectin, as well as anti-vaccination claims and other medical misinformation." This week, Rep. Adam Schiff (D-CA) and Sen. Elizabeth Warren (D-MA) sent letters "to CEO Andy Jassy pressing for information on Amazon's misinformation policies and what the company is doing to stop its systems from recommending books and other products linked to falsehoods about the pandemic and vaccines." Amazon spokesperson Tina Pelkey said, "We are constantly evaluating the books we list to ensure they comply with our content guidelines, and as an additional service to customers, at the top of relevant search results pages we link to the CDC advice on COVID and protection measures."

Mississippi Records 72 Stillbirths In Pregnant Women Unvaccinated Against COVID-19. The [[HYPERLINK](#)

"<https://www.washingtonpost.com/health/2021/09/09/pregnant-t-covid-mississippi/>"] (9/9, Shammas, 10.52M) reports, "Mississippi has recorded 72 fetal deaths in unvaccinated pregnant women infected with the coronavirus, state health officials announced Wednesday, sounding the alarm on the virus's danger in pregnancy." Mississippi State Health Officer Thomas Dobbs and other state health leaders are urging pregnant women to receive the vaccine. CDC Director Rochelle Walensky said, "The vaccines are safe and effective, and it has never been more urgent to increase vaccinations as we face the highly transmissible Delta variant and see severe outcomes from covid-19 among unvaccinated pregnant people."

[[HYPERLINK](#) "https://www.forbes.com/sites/carlieporterfield/2021/09/09/a-real-tragedy-mississippi-stillbirths-double-during-coronavirus-pandemic/"] (9/9, Porterfield, 10.33M) also covers the story.

CNN Crew Tells Story Of Spending Time In Rural Missouri County With 27% COVID-19 Vaccination Rate. [[HYPERLINK](#)

"<https://www.cnn.com/2021/09/09/us/ozarks-missouri-covid-vaccine/index.html>"] (9/9, Reeve, Guff, Brunswick, 89.21M) chronicles the story of a CNN crew that spent five days in Carter County, Missouri. The county has around 6,000 people and a COVID-19 vaccination rate of 27%. Brandon Helvey said, "Everybody's scared. Everybody's coming down with it. And it's almost like a plague." Yet neither he, nor the majority of residents, will get vaccinated because things are still "unproven." The crew spoke with other locals to

understand why they refuse to get vaccinated when the virus is prevalent in the community.

GOP Senator Recommends Sanctions, Immigration Control Over COVID-19 Spread. [[HYPERLINK](#)

"<https://www.newsweek.com/gop-senator-wants-ban-potentially-pandemic-pathogen-research-avoid-another-covid-1627673>"] (9/9, Ellington, 2.67M) reports that on Thursday, Senator Roger Marshall (R-KS) "released an eight-point strategy regarding the COVID-19 pandemic" and "recommended sanctions and immigration control due to 'substantial evidence that COVID-19 was spreading throughout China in September or October of 2019.'"

Columnist: Masks Are More About Politics Than Pandemic. In his column for the [[HYPERLINK](#)

"<https://nypost.com/2021/09/09/masks-are-here-to-stay-as-long-as-libs-need-to-shame-their-political-enemies/>"] (9/9, 7.45M), Glenn Reynolds writes, "Masks, as Twitter wags have noted, are by now the equivalent of MAGA hats for Blue America." He adds, "We're so politically tribalized that even our response to the pandemic says more about politics than about anything else. That's especially true when it comes to masking." Reynolds concludes, "A more mature society, with a more responsible media and more sensible politicians, would address matters more rationally and less tribally. We do not live in such a society."

Former HHS Emergency Preparedness Chiefs Suggest Integrating Public Health System Into Medical Care Delivery. Behind a paywall, [[HYPERLINK](#)

"<https://news.bloomberglaw.com/pharma-and-life-sciences/medical-care-public-health-need-to-be-welded-for-next-crisis>"] (9/9, Baumann, Subscription Publication, 4K) reports, "The U.S. must better integrate the public health system into the delivery of medical care as the nation looks to overhaul its response to future outbreaks in light of the Covid-19 pandemic, former HHS emergency preparedness chiefs said."

Researchers At Harvard, BU Receive NIH Award To Study Links Between COVID-19 Vaccines, Changes In Menstruation. The [[HYPERLINK](#)

"<https://r.bulletinintelligence.com/ae0332430d7242968dfdc53c7459b02d>"] (9/9, Finucane, 1.04M) reports Harvard Medical School and the Boston University School of Public Health are among "five institutions...awarded a total of \$1.67 million by the National Institutes of Health to look into...anecdotal reports from women earlier this year that after they were vaccinated [against COVID-19], they saw changes, including earlier, heavier, and more painful periods." NIH Institute of Child

Health and Human Development Director Diana Bianchi said, "Our goal is to provide menstruating people with information, mainly as to what to expect, because I think that was the biggest issue: Nobody expected it to affect the menstrual system because the information wasn't being collected in the early vaccine studies." The National Institute of Child Health and Human Development and the NIH's Office of Research on Women's Health are funding the research.

Pandemic Causing Disability Service Providers In Many States To Close, Survey Finds. [HYPERLINK

"<https://www.disabilityscoop.com/2021/09/10/disability-service-providers-shuttering-in-many-states/29480/>"] (9/10, Diament) reports, "Half of states are reporting that Medicaid home and community-based services providers have closed since the start of the pandemic, with many seeing multiple types of services shut their doors." A survey, conducted through mid-July, of Medicaid home and community-based services programs in 41 states found "that the pandemic exacerbated existing workforce shortages at group homes and for services provided in individuals' homes, but there were also impacts on providers from people with disabilities and their families declining to allow workers into their homes due to coronavirus concerns."

Maryland Permits Seniors In Some Group Homes To Receive COVID-19 Booster Shot. [HYPERLINK

"<https://www.disabilityscoop.com/2021/09/10/covid-19-vaccine-boosters-okd-some-group-home-residents/29479/>"] (9/10, Wood) reports, "Maryland is moving forward with allowing COVID-19 vaccine booster shots for older adults and others living in nursing homes and other group settings, even though the federal government has not yet issued formal rules for when most Americans should get them." Gov. Larry Hogan (R) announced everyone aged 65 or older living "in nursing homes, assisted living centers, residential treatment centers and group homes for people with disabilities" is automatically eligible for the booster shot.

US Officials Reauthorize Use Of Lilly's COVID-19 Antibody Combination Therapy. [HYPERLINK

"<https://endpts.com/as-delta-takes-control-us-resumes-use-of-lillys-covid-19-mab-combo-nationwide/>"] (9/9, Brennan) reports in continuing coverage that US officials have reauthorized Lilly's COVID-19 antibody combination therapy bamlanivimab and etesevimab nationwide. According to the article, "Based on the most currently available data, bamlanivimab and etesevimab are now authorized in all U.S. states, territories, and jurisdictions," the FDA said yesterday,

noting in a fact sheet that the combo retained activity against the Delta variant."

EMA Approves German Sites In Boost To Pfizer COVID-19 Vaccine Supply. [HYPERLINK

"<https://www.reuters.com/world/europe/eu-boosts-pfizer-vaccine-supply-with-german-site-approvals-2021-09-09/>"] (9/9, Aripaka) reports that the European Medicines Agency "on Thursday approved an increase in manufacturing capacity for the COVID-19 vaccine from Pfizer/BioNTech, which would help produce up to 50 million additional doses this year." Both of the sites approved by the EMA are located in Germany.

Sinovac To Trial COVID-19 Vaccine In South African Children Aged 6 Months To 17 Years As Part Of Global Study. [HYPERLINK

"<https://www.reuters.com/world/africa/sinovac-trial-covid-19-vaccine-south-african-children-global-study-2021-09-09/>"] (9/9, Winning) reports, "China's Sinovac Biotech...will trial its COVID-19 vaccine in children and adolescents" aged 6 months to 17 years "in South Africa as part of a global Phase III study, Sinovac and local partner Numolux Group said on Thursday."

AstraZeneca Amends COVID-19 Vaccine License Deal With Chinese Biotech. [HYPERLINK

"<https://endpts.com/astrazeneca-amends-license-deal-with-chinese-biotech-chinese-protein-based-vaccine-gets-manufacturing-ok/>"] (9/9, Sullivan) reports, "AstraZeneca has amended its license agreement with" Chinese biotech Biokangtai to commercialize its AAV Covid-19 vaccine "in mainland China, Pakistan and Indonesia, Biokangtai announced Wednesday." According to the article, "Biokangtai will market, manufacture and distribute the drug to ease the shortened supply and make the vaccine more accessible."

Physician's Group Suing Over FDA's Revoked EUA For Hydroxychloroquine Loses Appeal. [HYPERLINK

"<https://news.bloomberglaw.com/us-law-week/doctors-group-suing-over-hydroxychloroquine-access-loses-appeal>"] (9/9, Barker, Subscription Publication, 4K) reports the US Court of Appeals for the Sixth Circuit has ruled that the Association of American Physicians & Surgeons cannot sue over the scope of the FDA's "now-revoked emergency use authorization for hydroxychloroquine." According to Bloomberg Law, "The association's claims that members couldn't prescribe, and that patients were unable to obtain, the drug for use as a prophylaxis or as an initial treatment after a Covid-19 diagnosis don't plausibly plead any injury to its members, the court said."

EMA Flags AstraZeneca COVID-19 Vaccine For Rare Guillain-Barre Syndrome. [[HYPERLINK](#)

"<https://www.fiercepharma.com/pharma/astrazeneca-s-covid-19-shot-joins-list-vaccines-flagged-for-rare-guillain-barre-syndrome>"] (9/9, Dunleavy, 12K) reports that the European Medicines Agency has flagged the AstraZeneca COVID-19 vaccine for the rare nerve disorder Guillain-Barre syndrome (GBS). The EMA "will now list GBS as a possible and 'very rare' side effect of Vaxzevria, which is administered as a two-dose regimen." According to FiercePharma, "Of the 592 million doses of the vaccine administered worldwide through the end of July, 833 cases of the nerve-damaging condition were reported."

CMS Expands COVID-19 Vaccine Mandate To All Facilities Participating In Medicare And Medicaid. [[HYPERLINK](#)

"<https://www.fiercehealthcare.com/hospitals/cms-announces-bid-to-release-emergency-rule-for-covid-19-vaccine-mandate-for-hospital>"] (9/9, King, 150K) reports, "The Centers for Medicare & Medicaid Services announced Thursday its intention to release an interim final rule in October to require hospitals, dialysis facilities, ambulatory surgical centers and other facilities to vaccinate their staff" against COVID-19 "as a condition for participating in Medicare and Medicaid." The announcement comes as "more than 100 hospitals and health systems have already implemented vaccination mandates for staff" but "lower vaccination rates in several other facilities prompted the agency to expand the mandate."

Southeastern Missouri Now "Epicenter" Of State's COVID-19 Outbreak. The [[HYPERLINK](#)

"<https://apnews.com/article/health-coronavirus-pandemic-st-louis-missouri-sikeston-751d639ad894fb105703358e939e50aa>"] (9/9) reports, "The epicenter of Missouri's COVID-19 outbreak has shifted to the state's southeast, and no place is getting hit harder than Sikeston." State tracking showed "eight of the nine hardest-hit counties over the past week are in the southeastern corner of the state."

Researchers Examine The First COVID-19 Deaths In The US. The [[HYPERLINK](#)

"<https://www.nytimes.com/2021/09/09/health/first-covid-deaths.html>"] (9/9, Mueller, 20.6M) reports that last year, the CDC kicked off a "quiet, yearlong campaign at the agency to check and recheck the country's first suspected Covid-related deaths in the uncertain days of early 2020." Research shows "at least four possible Covid-19 deaths from January 2020 have survived" the vetting and they "have begun drawing more attention as scientists and intelligence officials try to unravel how the pandemic began."

Younger Californians Who Are Unvaccinated Are Increasingly Getting Hospitalized With COVID-19. The [[HYPERLINK](#)

"<https://www.latimes.com/california/story/2021-09-09/younger-unvaccinated-californians-increasingly-hospitalized-with-covid-19>"] (9/9, Lin, Money, 3.37M) reports that in California, "younger people are being vaccinated" against COVID-19 at lower rates and the "age gap is causing growing concern among healthcare professionals and bringing new calls to get younger people vaccinated." These "unvaccinated people who are younger and otherwise healthier" are "filling hospital beds." While some "demographic groups that were hit hard earlier in the pandemic" like Native Americans are "now reporting higher vaccination rates," younger people are still proving more hesitant.

Kentucky's COVID-19 Surge Is Pushing Hospitals To Their Limits. [[HYPERLINK](#)

"<https://www.cnn.com/2021/09/09/us/kentucky-hospital-overwhelmed-covid/index.html>"] (9/9, Vera, Marquez, Kapp, 89.21M) reports that because of vaccine hesitancy, "Covid-19 has run rampant in Kentucky, where nearly all of the state's 120 counties are in the 'red' Covid-19 zone and hospitals" are "on the brink of being overwhelmed, according to Gov. Andy Beshear." State lawmakers voted to extend Kentucky's state of emergency until January due to the surge. On Thursday, the governor "said 60 of the state's 96 hospitals are reporting staff shortages, and there are only 90 available adult ICU beds statewide, the fewest amount available throughout the pandemic." The surge "illustrates that big, urban hospitals aren't the only ones being bombarded by patients."

The [[HYPERLINK "https://apnews.com/article/health-coronavirus-pandemic-kentucky-8ac1d921eb0aaace148b1ce05e92288b"](https://apnews.com/article/health-coronavirus-pandemic-kentucky-8ac1d921eb0aaace148b1ce05e92288b)] (9/9, Blackburn) reports the governor "added the limited number of ICU beds also will put those in non-virus related emergencies in danger of not receiving care, such as people in bad car accidents."

North Carolina Health Officials Say Middle And High School Sports Are Leading To COVID-19 Clusters. [[HYPERLINK](#)

"<https://www.cnn.com/2021/09/09/us/north-carolina-covid-school-sports/index.html>"] (9/9, Simon, 89.21M) reports, "Forty-five percent of all Covid-19 clusters in North Carolina's middle and high schools since July have been among sports teams, according to the North Carolina Department of Health and Human Services (NCDHHS)." The department "said the numbers underscored an urgent need for vaccination among school children as soon as possible." The announcement comes as the "Centers for Disease Control and Prevention and the American Academy of Pediatrics (AAP) have advised

that sports can play a role in the spread of Covid-19 among students, especially those who are unvaccinated."

Idaho Hospitals May Be Forced To Ration Healthcare As Students Return To School.

The [[HYPERLINK "https://apnews.com/article/coeur-dalene-ap-top-news-education-public-health-coronavirus-pandemic-0fa72d0302063e07ed45159a3aeffea3"](https://apnews.com/article/coeur-dalene-ap-top-news-education-public-health-coronavirus-pandemic-0fa72d0302063e07ed45159a3aeffea3)] (9/9, Boone) reports, "When Idaho public health officials announced this week that northern hospitals were so crowded with coronavirus patients that they would be allowed to ration health care, roughly 11,000 kids" were preparing for their first day of school. Vaccination rates in Idaho "remain among the lowest in the U.S., and coronavirus cases have grown by 44% in the last two weeks as the highly contagious delta variant burns through the population." State officials expressed concern that there will not be enough beds for the growing surge.

San Francisco Schools "Defy National Trends," See No COVID-19 Outbreaks.

The [[HYPERLINK "https://www.sfchronicle.com/sf/article/New-S-F-data-shows-children-haven-t-been-hard-16446656.php"](https://www.sfchronicle.com/sf/article/New-S-F-data-shows-children-haven-t-been-hard-16446656.php)] (9/9, Tucker, 2.44M) reports, "No COVID-19 outbreaks have occurred in San Francisco schools since they reopened to in-person learning in mid-August and case rates have remained steady among young children in recent months, even as the highly contagious delta variant has spread, according to data released Thursday by the Department of Public Health." The numbers "defy national trends that have shown large rises in cases and hospitalizations among school-aged children overall during the delta surge."

Colorado Sees Rise In Children's COVID-19 Cases Since Beginning Of School Year.

The [[HYPERLINK "https://www.denverpost.com/2021/09/09/colorado-children-covid-infections/"](https://www.denverpost.com/2021/09/09/colorado-children-covid-infections/)] (9/9, Seaman, 660K) reports, "COVID-19 cases in Colorado's school-aged children more than doubled in the past three weeks as a new school year began, with the increase most 'notable among children ages 6 to 11 – a group that is not yet eligible for a COVID-19 vaccine.'" Even though "most children don't experience severe COVID cases, there is a concern more infections will mean more hospitalizations at a time when hospitals are already 'under significant strain' with an uptick in other respiratory viruses that don't normally circulate this time of year."

Pediatric Hospitalizations Increase Across US, Overwhelm Children's Hospitals.

The [[HYPERLINK "https://www.nytimes.com/interactive/2021/09/09/us/covid-children-cases-icu.html"](https://www.nytimes.com/interactive/2021/09/09/us/covid-children-cases-icu.html)] (9/9, Leatherby, Walker, 20.6M)

reports, "Just as millions of families around the United States navigate sending their children back to school at an uncertain moment in the pandemic, the number of children admitted to the hospital with Covid-19 has risen to the highest levels reported to date." An increase in infections has driven pediatric hospitalizations "across the country, overwhelming children's hospitals and intensive care units in states like Louisiana and Texas." This strain on resources "has prompted doctors and hospital executives to plead with adults to get vaccinated and return to mask wearing and social distancing to protect children, especially those under 12, who cannot yet be vaccinated."

Rep. Schiff Calls On Facebook, Amazon To Provide Details About Efforts To Curb COVID-19 Vaccine Misinformation.

[[HYPERLINK "https://www.reuters.com/business/healthcare-pharmaceuticals/schiff-presses-facebook-amazon-efforts-curb-covid-19-vaccine-misinformation-2021-09-09/"](https://www.reuters.com/business/healthcare-pharmaceuticals/schiff-presses-facebook-amazon-efforts-curb-covid-19-vaccine-misinformation-2021-09-09/)] (9/9, Shepardson, Heavey) reports, "U.S. Democratic Representative Adam Schiff called on Facebook Inc and Amazon.com Inc on Thursday to provide a more thorough explanation of their efforts to mitigate the spread of COVID-19 vaccine misinformation." Some "other companies, including Twitter Inc and Alphabet Inc's Google and YouTube, have also faced criticism for allowing false information on COVID-19, including vaccines, to proliferate."

Battle Over COVID-19 Policies Brewing At UT Austin.

The [[HYPERLINK "https://www.wsj.com/articles/covid-19-policies-ignite-battle-at-ut-austin-11631179800"](https://www.wsj.com/articles/covid-19-policies-ignite-battle-at-ut-austin-11631179800)] (9/9, Belkin, Subscription Publication, 8.41M) reports that because of prohibition by Republican state policy makers, the University of Texas at Austin does not require masking, surveillance testing, or vaccines. Professors and graduate students are unhappy about the situation. Hundreds of them signed a petition demanding that the school impose tougher COVID-19 protection measures.

Qantas To Require International Passengers Be Vaccinated Against COVID-19.

The [[HYPERLINK "https://www.nytimes.com/2021/09/09/travel/qantas-vaccine-mandate-international.html"](https://www.nytimes.com/2021/09/09/travel/qantas-vaccine-mandate-international.html)] (9/9, Murphy, 20.6M) reports, "Qantas, Australia's largest airline, will require that all passengers on international flights are vaccinated against the coronavirus when it restarts worldwide operations in December, its chief executive said Wednesday, making it one of the first airlines in the world to require proof of vaccination for everyone on board." The airline, "which is headquartered in Sydney, suspended international operations during the

pandemic – but did resume flights to New Zealand in April this year before suspending them again on July 31.” Qantas “plans to restart flights abroad in December.”

“Remarkable” Ability Of Coronavirus Delta Variant To Replicate Tossing New “Twists” Into The Pandemic. [[HYPERLINK](https://www.statnews.com/2021/09/09/covid19-delta-variant-transmission/)

“https://www.statnews.com/2021/09/09/covid19-delta-variant-transmission/”] (9/9, Joseph, 262K) reports, “One of the key reasons the Delta variant has ignited new surges of COVID-19 infections across the United States is its remarkable ability to make copies of itself.” That remarkable ability “has helped make Delta far more transmissible than any other iteration of the coronavirus seen thus far.” The ability to replicate “could also be at the heart of the other twists Delta has thrown into the pandemic, including the increase in breakthrough infections with the variant and why it potentially causes severe COVID-19 more often.”

“Religious” Exemptions Add Potential Legal Obstacles To Looming COVID-19 Vaccine Mandates. [[HYPERLINK](https://khn.org/news/article/religious-exemptions-add-legal-thorns-to-looming-vaccine-mandates/)

“https://khn.org/news/article/religious-exemptions-add-legal-thorns-to-looming-vaccine-mandates/”] (9/9, Kreidler) reports, “With workplace vaccine mandates in the offing, opponents are turning to a tried-and-true recourse for avoiding a COVID-19 shot: the claim that vaccination interferes with religious beliefs.” Legal experts say if “a person claims their privately held religious beliefs forbid vaccination, that defense is unlikely to hold up in court if challenged.” But the EEOC “grants broad leeway to what constitutes a sincerely held religious belief,” meaning “some experts predict most employers and administrators won’t want to challenge such objections from their employees.”

Analysis: Average COVID-19 Hospitalization Costs Medicare 150 Times More Than It Does To Vaccinate One Individual. [[HYPERLINK](https://www.cnn.com/2021/09/09/health/covid-19-hospitalization-cost-vaccination/index.html)

“https://www.cnn.com/2021/09/09/health/covid-19-hospitalization-cost-vaccination/index.html”] (9/9, McPhillips, Sealy, Andone, 89.21M) reports, “A Medicare beneficiary hospitalized with Covid-19 costs the health insurance program about 150 times more than it does to fully vaccinate that individual, a CNN analysis shows.” The “vaccines are provided at no direct cost to residents of the United States, but Medicare reimburses health care providers up to \$150 to fully vaccinate beneficiaries with both doses of the two-dose regimen vaccines by Pfizer/BioNtech and Moderna, per billing documents published by the US Centers for Medicare and Medicaid Services. That’s \$40 for each dose administered and \$35 to administer the shots at the individual’s home or group living setting.” On the other hand, “the average cost to

hospitalize a Medicare beneficiary with COVID-19 is \$21,752 over an average stay of 9.2 days, according to research published in August in the Annals of Internal Medicine.”

Trump Urges Americans To Consider Getting COVID-19 Vaccines. [[HYPERLINK](https://www.foxnews.com/media/trump-gutfeld-fabulous-ratings-biden-afghan-crisis)

“https://www.foxnews.com/media/trump-gutfeld-fabulous-ratings-biden-afghan-crisis”] (9/9, Creitz, 23.99M) reports during an interview with Fox News host Greg Gutfeld, former President Trump “urged Americans to consider getting vaccinated, after Gutfeld told him his wife remains hesitant as many others in the country do.” Trump said, “I got vaccinated and I feel very good, and I think we saved millions and millions of people worldwide [with the vaccine development]...comparing the COVID-19 death toll to the 1918 Spanish Flu pandemic, which he said killed 100 million people.”

More Patients With COVID-19 Report Suffering From Long-Term Symptoms, Study Indicates. [[HYPERLINK](https://thehill.com/policy/healthcare/571477-cdc-study-confirmed-covid-19-patients-reported-long-term-symptoms-15-times)

“https://thehill.com/policy/healthcare/571477-cdc-study-confirmed-covid-19-patients-reported-long-term-symptoms-15-times”] (9/9, Coleman, 5.69M) says a CDC study indicates “people who tested positive for COVID-19 reported having long-term symptoms 1.5 times more frequently than those who tested negative.” Almost “66 percent of people who tested positive for the virus reported at least one of their COVID-19-related symptoms lasted longer than four weeks, compared to 42.9 percent of those who always tested negative,” said a survey conducted by Porter Novelli Public Services from April 9-23.

Job Postings With COVID-19 Vaccination Requirements Doubled In August, Indeed Says.

[[HYPERLINK](https://thehill.com/policy/finance/571510-job-postings-with-covid-19-vaccine-requirements-doubled-in-august) “https://thehill.com/policy/finance/571510-job-postings-with-covid-19-vaccine-requirements-doubled-in-august”] (9/9, Lane, 5.69M) reports, “Job postings that require applicants to be vaccinated against COVID-19 skyrocketed in August amid a sharp increase in coronavirus cases, according to data released Thursday by hiring website Indeed.” In the week “ending Aug. 30, the share of job postings on Indeed that required COVID-19 vaccinations rose 119 percent from the same period in July. Job postings that mentioned a general vaccination requirement, but not COVID-19 specifically, rose by 242 percent over the same period.”

[[HYPERLINK](https://www.usnews.com/news/national-news/articles/2021-09-09/study-job-postings-requiring-vaccinations-doubled-in-august) “https://www.usnews.com/news/national-news/articles/2021-09-09/study-job-postings-requiring-vaccinations-doubled-in-august”] (9/9, 1.91M) says, “According to Indeed, a small batch of job postings recommend vaccinations but stop short of requiring them, while other postings ask potential employees to verify their

vaccination status by uploading their vaccination card, and others have offered vaccine bonuses to new hires."

Workers Slow To Return To In-Person Jobs, Bureau of Labor Statistics Data Show.

The [[HYPERLINK "https://www.pewtrusts.org/en/research-and-analysis/blogs/stateline/2021/09/09/in-person-workers-are-slow-to-return-to-jobs-data-shows"](https://www.pewtrusts.org/en/research-and-analysis/blogs/stateline/2021/09/09/in-person-workers-are-slow-to-return-to-jobs-data-shows)] (9/9, 2K) reports, "Hospitality workers and others who can't telework are key to job recovery," however, "despite a sudden spike in demand for their services in a reopened economy, many are reluctant to return to the workplace." Jobs are "down about 9% in Connecticut and Vermont from pre-pandemic levels as of July, and down significantly in Hawaii (8.1%), Illinois (6.9%), Maryland (6.7%), New Jersey (6.1%) and Rhode Island (6.1%), according to a Stateline analysis of Bureau of Labor Statistics data." In The US, "hospitality job growth halted in August and is still down 1.7 million jobs from before the pandemic, according to a federal jobs report released Sept. 3."

New Mexico Health Officials Investigating Possible Fatal Poisoning From Ivermectin Used To Treat COVID-19.

The [[HYPERLINK "https://www.lcsun-news.com/story/news/local/new-mexico/2021/09/08/new-mexico-investigating-suspected-fatal-poisoning-ivermectin-covid-19/5777497001/"](https://www.lcsun-news.com/story/news/local/new-mexico/2021/09/08/new-mexico-investigating-suspected-fatal-poisoning-ivermectin-covid-19/5777497001/)] (9/8, D'Amassa, 85K) reports, "New Mexico's acting Health Secretary came down hard Wednesday on unfounded claims that the drug Ivermectin is a suitable treatment for COVID-19 disease." State Human Services Secretary Dr. David Scrase, "temporarily heading the state health department as well, said" during a weekly update on COVID-19 cases and response that "clinicians were investigating what was likely the state's first fatal case of an individual dosing themselves with the drug, while a suspected second case was in critical condition." Scrase "stressed that it might take weeks to confirm that ivermectin was the cause of the individual's death, but his expectation was that it would be confirmed, while another severely ill patient was suspected of taking the drug."

Pair Of Penn Scientists Wins \$3M Breakthrough Prize For RNA Research That Led To Creation Of COVID-19 Vaccines.

The [[HYPERLINK "https://www.inquirer.com/health/coronavirus/kariko-weissman-breakthrough-prize-covid19-vaccine-biontech-20210909.html"](https://www.inquirer.com/health/coronavirus/kariko-weissman-breakthrough-prize-covid19-vaccine-biontech-20210909.html)] (9/9, Avril) reports, "Sixteen years after their research at the University of Pennsylvania paved the way for billions to be vaccinated against COVID-19, two scientists have been honored with a \$3 million Breakthrough Prize."

The prize "for Katalin Karikó and Drew Weissman, one of five such honors announced Thursday for achievements in science and math, recognizes their success in modifying the genetic molecule called messenger RNA (mRNA) so it can instruct human cells to make customized proteins."

[[HYPERLINK "https://www.forbes.com/sites/alexknapp/2021/09/09/2022-breakthrough-prizes-announced-mrna-vaccine-pioneers-awarded-3-million/"](https://www.forbes.com/sites/alexknapp/2021/09/09/2022-breakthrough-prizes-announced-mrna-vaccine-pioneers-awarded-3-million/)] (9/9, Knapp, 10.33M) reports "Weissman isn't resting on his accomplishments." The "team is working to understand how the coronavirus that causes Covid-19 is changing so that pharmaceutical companies are better able to vaccinate against future variants of the disease," and "is also focused on developing a vaccine that could help prevent future coronavirus pandemics."

Latinos Have Higher COVID-19 Vaccination Rates In Major Metropolitan Areas.

[[HYPERLINK "https://www.axios.com/latino-vaccination-rates-white-new-york-city-029a11be-532b-4e92-b0c5-c089eefad9b3.html"](https://www.axios.com/latino-vaccination-rates-white-new-york-city-029a11be-532b-4e92-b0c5-c089eefad9b3.html)] (9/9, Gonzalez, 1.26M) reports, "The number of Latinos getting vaccinated against the coronavirus in some of the most populated cities in the U.S. is slowly increasing." In New York City, "Latinos have surpassed whites in the percentage of fully vaccinated adults." In Houston, "the Houston Health Department has administered approximately 32% of its vaccines to Latinos and 28% to whites, as of Wednesday."

Review Gives Prisons In Arizona An F For COVID-19 Pandemic Response.

The [[HYPERLINK "https://www.azcentral.com/story/news/local/arizona-health/2021/09/09/study-arizona-prisons-get-f-grade-for-covid-19-response/5776046001/"](https://www.azcentral.com/story/news/local/arizona-health/2021/09/09/study-arizona-prisons-get-f-grade-for-covid-19-response/5776046001/)] (9/9, Jenkins, 1.05M) reports, "Arizona gets an F for its response to the COVID-19 pandemic in state prisons." At least, "according to a recent nationwide review conducted by the Prison Policy Initiative, a nonpartisan advocacy group." Wanda Bertram, a Prison Policy Initiative spokesperson, "says Arizona was one of 13 states that did not adopt any policies aimed at reducing prison populations, such as accelerating release dates for those near the end of their sentence or expanding compassionate and medical releases."

Parents Of Children Who Need Home Care Have Tough Time Finding Nurses Vaccinated Against COVID-19.

[[HYPERLINK "https://www.bloomberg.com/news/articles/2021-09-09/for-the-sickest-kids-a-vaccinated-home-nurse-is-hard-to-find?srnd=premium"](https://www.bloomberg.com/news/articles/2021-09-09/for-the-sickest-kids-a-vaccinated-home-nurse-is-hard-to-find?srnd=premium)] (9/9, Edney, 3.57M) reports, "A shortage of vaccinated home-care nurses has some parents of children with conditions like cerebral palsy or severe epilepsy giving up sleep and their own mental health to

ensure their kids keep breathing at night." An industry group "estimates a vaccination rate as low as 40% among the industry's 2 million nurses, who work in a broad variety of specialties." A small number "of states require in-home nurses to be vaccinated against COVID-19, and the Biden administration hasn't created a federal mandate."

As COVID-19 Surges, NFL Opens Regular Season To Full Stadiums.

The [[HYPERLINK "https://apnews.com/article/sports-nhl-mlb-nba-nfl-ac987b4210af44ab7cd14f81f378fafb"](https://apnews.com/article/sports-nhl-mlb-nba-nfl-ac987b4210af44ab7cd14f81f378fafb)] (9/9, Maaddi) reports, "When Tom Brady runs out from the tunnel, screaming 'Let's Go!' and leading the defending Super Bowl champion Buccaneers onto the field Thursday night against the Dallas Cowboys, a full stadium will greet players for the first time since COVID-19 upended the world and changed the way sports were viewed." Fans are returning "as COVID-19 surges, with about 150,000 new cases daily." The coronavirus "delta variant is filling hospitals, children are getting sick, and some schools are abruptly switching back to remote learning because of outbreaks."

Commentary Criticizes News Outlets For Running Phony Story About Ivermectin Overdoses In Oklahoma.

Erik Wemple writes in the [[HYPERLINK "https://www.washingtonpost.com/opinions/2021/09/09/bogus-oklahoma-ivermectin-story-was-just-too-good-check/"](https://www.washingtonpost.com/opinions/2021/09/09/bogus-oklahoma-ivermectin-story-was-just-too-good-check/)] (9/9, 10.52M) that "a number of prominent news outlets couldn't resist a report last week from Oklahoma City-based TV station KFOR with the headline: 'Patients overdosing on ivermectin backing up rural Oklahoma hospitals, ambulances.'" Media outlets such as The Hill, the Guardian, Rolling Stone, DailyMail.com, the New York Daily News and MSNBC's Rachel Maddow "bit hard." But "after the story went viral, two hospitals in eastern Oklahoma – Northeastern Health System-Sequoyah and Integris Grove Hospital – issued statements denying that they had been overrun by ivermectin users." Wemple says while Fox News "dominates the playing field of false, racist and damaging news coverage in the United States," there "should be no minimizing the two-step horror of what...Maddow-Daily News-Rolling Stone and others did in this case."

Connecticut Officials Express Concern About Inconsistent Mask Wearing After Latest COVID-19 Infections.

The [[HYPERLINK "https://apnews.com/article/health-coronavirus-pandemic-connecticut-ned-lamont-hartford-75c8c8195f1dec213d2753747e2096d4"](https://apnews.com/article/health-coronavirus-pandemic-connecticut-ned-lamont-hartford-75c8c8195f1dec213d2753747e2096d4)] (9/9) reports, "Connecticut officials expressed concerns Thursday about inconsistent mask wearing and people shunning coronavirus

vaccines after 37 people became infected in outbreaks at a summer camp, group home and a party." Acting Health Commissioner Deidre Gifford and Gov. Ned Lamont "said most of the people who became infected were not vaccinated and some of them spread the virus to relatives who did not go to those locations." Each of the outbreaks occurred in August.

Hospital Leaders In Michigan Renew Plea For People To Get Vaccinated Amid Staff Shortages, COVID-19 Deaths.

The [[HYPERLINK "https://apnews.com/article/business-health-pandemics-coronavirus-pandemic-michigan-a44e7d8a733a5ce0b5e3054d83d9d8a9"](https://apnews.com/article/business-health-pandemics-coronavirus-pandemic-michigan-a44e7d8a733a5ce0b5e3054d83d9d8a9)] (9/9, Eggert) reports, "Michigan health and business officials on Thursday renewed their plea for people to get vaccinated against COVID-19, citing hospital workforce shortages, unnecessary deaths and concerns that end-of-summer travel and the return to school could fuel a surge in cases." Some "59% of Michigan residents ages 12 and older have been fully vaccinated and 65% have gotten at least one dose." These figures "trail behind the national rates of 62% and 73%," respectively.

The [[HYPERLINK "https://www.freep.com/story/news/health/2021/09/09/michigan-hospitals-staffing-shortage-workers-covid-19-patients/5783044001/"](https://www.freep.com/story/news/health/2021/09/09/michigan-hospitals-staffing-shortage-workers-covid-19-patients/5783044001/)] (9/9, 2.16M) reports, "Michigan's hospitals face a critical staffing shortage that has neared a crisis point as they also struggle to manage a growing number of patients with COVID-19, the CEO of the state's hospital association said Thursday."

Some Students In Pennsylvania Using Loophole To Avoid Masking.

The [[HYPERLINK "https://apnews.com/article/health-education-pennsylvania-coronavirus-pandemic-school-boards-64a8099221c5971194d49e0f4d94054a"](https://apnews.com/article/health-education-pennsylvania-coronavirus-pandemic-school-boards-64a8099221c5971194d49e0f4d94054a)] (9/9, Rubinkam, Scoloro) reports, "An apparent loophole in Pennsylvania's mask mandate for schools is making it easier for some students to go to class without having to cover their faces, even as state education regulators sought to make an example of one openly defiant school board." The order by the state health secretary "requiring masks to be worn inside K-12 school and child care facilities, which took effect Tuesday, includes an exemption for students who claim it would cause or worsen a medical condition."

Iowa To Use Federal COVID-19 Relief Funds For \$3.7M Ad Campaign To Promote The State.

The [[HYPERLINK "https://apnews.com/article/business-health-media-coronavirus-pandemic-iowa-ad448e4e40480af335301b79bc1e23ad"](https://apnews.com/article/business-health-media-coronavirus-pandemic-iowa-ad448e4e40480af335301b79bc1e23ad)] (9/9) reports, "Iowa will use federal coronavirus relief funds to pay for a \$3.7

million national ad campaign that promotes the state as a destination for visitors and workers, an agency spokeswoman said Thursday." The advertisement "is part of a larger 'This Is Iowa' promotional campaign and will air on cable television, online on social media sites and through streaming services, said Staci Hupp Ballard, spokeswoman for Iowa Economic Development Authority." Ballard said "funding for the campaign will come from federal American Rescue Plan funding."

Kentucky Legislators Move Closer To Scrapping Statewide Mask Mandate In Public Schools.

The [[HYPERLINK "https://apnews.com/article/health-education-coronavirus-pandemic-kentucky-bills-4a1cbda3c8f67b884758e4d739f55b13"](https://apnews.com/article/health-education-coronavirus-pandemic-kentucky-bills-4a1cbda3c8f67b884758e4d739f55b13)] (9/9, Schreiner) reports GOP "lawmakers in Kentucky moved closer Thursday to scrapping a statewide mask mandate in public schools, advancing a measure to shift masking decisions to local school boards as a surging COVID-19 outbreak threatens to overwhelm hospitals."

Editorial Criticizes Florida Governor's Comments About Vaccines.

The [[HYPERLINK "https://www.orlandosentinel.com/opinion/editorials/os-op-ron-desantis-vaccines-science-personal-choice-20210909-cfinaqxavhirkwsbavji6vx3i-story.html"](https://www.orlandosentinel.com/opinion/editorials/os-op-ron-desantis-vaccines-science-personal-choice-20210909-cfinaqxavhirkwsbavji6vx3i-story.html)] (9/9, 599K) editorializes, "For a guy who claims to follow the science, Gov. Ron DeSantis has a shaky grasp of how vaccines work. Consider his recent boneheaded statement about vaccines at a news conference last week: 'It's about your health and whether you want that protection or not. It really doesn't impact me or anyone else.'" The paper adds that NIAID Director Dr. Anthony Fauci, "a real scientist who[m] DeSantis mocks on campaign merchandise, had this to say in response: 'Yeah, that's not true at all.'"

Republicans On Michigan House Oversight Committee Say State Unemployment Agency Failed Residents Amid Pandemic.

The [[HYPERLINK "https://apnews.com/article/business-health-coronavirus-pandemic-michigan-covid-19-pandemic-a9db2d4a690eae4fb514f9763a32a4cb"](https://apnews.com/article/business-health-coronavirus-pandemic-michigan-covid-19-pandemic-a9db2d4a690eae4fb514f9763a32a4cb)] (9/9, Nichols) reports, "Republicans in the Michigan House Oversight committee criticized the state's Unemployment Insurance Agency acting director on Thursday, asking her to address how the agency has failed residents during the COVID-19 pandemic." Rep. Steve Johnson, the committee chair, "citing what he's heard from his constituents, went back and forth with Estlund Olson throughout the meeting to ask her to address what he calls 'failures' by the agency." The reported failures "include slow payment of benefits and the nearly

700,000 residents who received notifications that they may have to repay their Pandemic Unemployment Assistance from the past 18 months."

Japan Extends COVID-19 State Of Emergency Until End Of September.

The [[HYPERLINK "https://apnews.com/article/business-health-japan-tokyo-yoshihide-suga-650bf457d155dd600ff5b498555a905"](https://apnews.com/article/business-health-japan-tokyo-yoshihide-suga-650bf457d155dd600ff5b498555a905)] (9/9, Yamaguchi) reports, "Japanese Prime Minister Yoshihide Suga on Thursday announced an extension of a coronavirus state of emergency in Tokyo and 18 other areas until the end of September, saying healthcare systems remain under severe strain, and that the continuing challenges of fighting the virus had led to his decision not to seek another term." Originally scheduled to end Sunday, "the state of emergency...was issued first in Okinawa in May and gradually expanded and extended as the country prepared to host the Olympics."

[[HYPERLINK "https://thehill.com/policy/international/asia-pacific/571496-japanese-covid-19-emergency-extended"](https://thehill.com/policy/international/asia-pacific/571496-japanese-covid-19-emergency-extended)] (9/9, Castronuovo, 5.69M) reports the "pace of vaccinations in Japan has ramped up in recent months, with about 56 percent of the country's total population fully vaccinated."

Italian Anti-Terrorism Police Raid Homes Of People Advocating Violence Over Government COVID-19 Vaccine Requirements.

The [[HYPERLINK "https://apnews.com/article/europe-health-police-violence-coronavirus-pandemic-d44faa4b47d337b52211e25cff8ee63c"](https://apnews.com/article/europe-health-police-violence-coronavirus-pandemic-d44faa4b47d337b52211e25cff8ee63c)] (9/9, D'Emilio) reports, "Italian anti-terrorism police on Thursday raided the homes of eight people who allegedly advocated violence, particularly against journalists, in upcoming protests in Rome and elsewhere against the government's COVID-19 vaccine requirements, officials said." These people "had formed a group called 'the warriors' on the Telegram messaging app, police said," adding that "they are under investigation but have not been arrested or formally charged."

COVID-19 Incidence Drops Below "High Risk" Level In Spain, Data Show.

[[HYPERLINK "https://www.reuters.com/world/europe/spains-covid-19-incidence-drops-below-high-risk-level-2021-09-09/"](https://www.reuters.com/world/europe/spains-covid-19-incidence-drops-below-high-risk-level-2021-09-09/)] (9/9) reports, "Spain's coronavirus incidence slipped below 150 cases per 100,000 people – a threshold the Health Ministry considers a 'high risk' of contagion – for the first time in more than two months on Thursday." Health ministry data show that "the indicator, measured over the past 14 days, fell to around 140 cases from 150 the previous day, a dramatic change from a record 900 cases per 100,000 people at the end of January." ICU occupancy rates "also slipped to 13.7%

compared to 14.36% reported on Wednesday and 21.98% just a month ago."

Experts Suggest "Bolder" COVID-19 Strategy As Singapore Halts Reopening. [[HYPERLINK](https://www.reuters.com/world/asia-pacific/singapore-pauses-reopening-experts-suggest-bolder-covid-19-strategy-2021-09-09/)

"<https://www.reuters.com/world/asia-pacific/singapore-pauses-reopening-experts-suggest-bolder-covid-19-strategy-2021-09-09/>"] (9/9, Aravindan) reports, "Singapore is ramping up COVID-19 testing and has warned it would not rule out reimposing tighter curbs as infections rise – moves some experts see as too cautious for the vaccine frontrunner that is preparing to live with the virus as an endemic disease." Upset "by the fast pace of transmission, the government has said it would hold off on reopening measures and urged people to reduce social activities, sparking a debate among experts on if Singapore could 'be bolder' given it has one of the highest vaccination rates in the world."

Italy Adds More Job Groups For Which COVID-19 Vaccination Will Be Required. [[HYPERLINK](https://www.reuters.com/world/europe/italy-adds-more-job-groups-requirement-covid-vaccination-2021-09-09/)

"<https://www.reuters.com/world/europe/italy-adds-more-job-groups-requirement-covid-vaccination-2021-09-09/>"] (9/9) reports, "The Italian government ruled on Thursday that catering and cleaning staff in schools and nursing homes can only work if they have proof of COVID-19 immunity, extending mandatory vaccination and the use of the so-called 'Green Pass' document." The document "was already required for teachers in Italy, while mandatory vaccination for health workers was introduced in March." The Italian "government said on Thursday that under the new rules people working in schools in any capacity must have the health document, and that all nursing home staff will have to be vaccinated."

COVID-19 Variants In Africa Could Worsen Pandemic, Scientists Warn. [[HYPERLINK](https://www.bloomberg.com/news/articles/2021-09-09/africa-variants-may-derail-global-covid-19-fight-scientists-say?srnd=premium)

"<https://www.bloomberg.com/news/articles/2021-09-09/africa-variants-may-derail-global-covid-19-fight-scientists-say?srnd=premium>"] (9/9, Sguazzin, 3.57M) reports, "The proliferation of COVID-19 variants in Africa, partly attributed to the low rates of vaccination on the continent, could lead to vaccine-evading mutations that complicate attempts to end the pandemic, a group of 112 African and 25 international organizations said." Published in the journal Science on Thursday was "a study of genomes from 33 African nations and two 'overseas territories'" that "tracks the evolution of the pandemic across the continent and the emergence of a number of so-called Variants of Concern and Variants of Interest."

Analysis Examines Why COVAX Has Fallen Behind In Its Efforts To Distribute COVID-19 Vaccines To Poorer Countries. [[HYPERLINK](https://time.com/6096172/covax-vaccines-what-went-wrong/)

"<https://time.com/6096172/covax-vaccines-what-went-wrong/>"] (9/9, Ducharme, 18.1M) reports, "In March 2020, as the COVID-19 virus continued to spread across the planet, Dr. Richard Hatchett wrote a white paper laying the groundwork for a solution: a global initiative to support the development of COVID-19 vaccines through advance agreements with numerous pharmaceutical companies, and to distribute shots to countries around the world, whether or not those nations could pay for them." But Hatchett's concept had flaws. "The very issues that Hatchett feared – vaccine nationalism, self-interest, unequal access to limited supply – soon scarred COVAX's idealistic facade." Earlier this week, COVAX announced it would miss its COVID-19 vaccine goal for this year by about 500 million doses.

Australia's Victoria State Records Highest Single-Day Rise In COVID-19 Cases As Regions Exit Lockdown. [[HYPERLINK](https://www.reuters.com/world/asia-pacific/covid-19-cases-rise-australias-victoria-regions-exit-lockdown-2021-09-10/)

"<https://www.reuters.com/world/asia-pacific/covid-19-cases-rise-australias-victoria-regions-exit-lockdown-2021-09-10/>"] (9/10, Jose) says, "Australia's Victoria state reported its biggest single-day rise in COVID-19 cases this year, the majority in Melbourne, as most other regions in the state exited lockdown on Friday." Plans were also announced "to bring Melbourne and Sydney out of extended lockdowns in coming weeks, despite infections continuing to rise in both of the country's two biggest cities." The strategy shift "of living with, rather than suppressing, the virus after hitting national vaccine coverage of 70-80% is part of a four-stage national reopening plan unveiled by the federal government in July."

Attorney Demands Kansas School District Change Its COVID-19 Policies. The [[HYPERLINK](https://apnews.com/article/health-lawsuits-coronavirus-pandemic-kansas-city-kansas-a9a036244eed425919dc9e475399ede8)

"<https://apnews.com/article/health-lawsuits-coronavirus-pandemic-kansas-city-kansas-a9a036244eed425919dc9e475399ede8>"] (9/9) reports that a lawyer "who has filed lawsuits challenging mask mandates in two Kansas counties is demanding that a Kansas City-area school district revise its COVID-19 policies, arguing that they violate state and federal laws." Attorney Ryan Kriegshauser "said continuing to enforce the policies would be 'evidence of willful illegal conduct' against students."

Florida Governor's Lawyers File Emergency Appeal On School Mask Mandates. [[HYPERLINK](https://www.cnn.com/2021/09/09/us/florida-desantis-appeal-schools-mask-mandate/index.html)

"<https://www.cnn.com/2021/09/09/us/florida-desantis-appeal-schools-mask-mandate/index.html>"] (9/9, Simon, 89.21M) reports that lawyers for Gov. Ron DeSantis (R-FL) "have filed an emergency appeal with the First District Court of Appeals in Florida in hopes of halting school mask mandates while the case goes through the court system." DeSantis' lawyers "argue they 'have a high likelihood of success on appeal."

Therefore, the trial court abused its discretion in vacating the automatic stay, and this Court should reinstate the stay pending review,' according to a court document."

Also reporting is [[HYPERLINK "https://www.usatoday.com/story/news/health/2021/09/09/florida-quickly-files-emergency-motion-mask-battle-appeal-leon-county-desantis-lawsuit/8262972002/"](https://www.usatoday.com/story/news/health/2021/09/09/florida-quickly-files-emergency-motion-mask-battle-appeal-leon-county-desantis-lawsuit/8262972002/)] (9/9, Saunders).

California Lawmakers Fail To Introduce Measures To Mandate COVID-19 Vaccinations.

The [[HYPERLINK "https://www.latimes.com/california/story/2021-09-08/new-vaccine-mandates-falter-legislature-protesters-undeterred"](https://www.latimes.com/california/story/2021-09-08/new-vaccine-mandates-falter-legislature-protesters-undeterred)] (9/8, Gutierrez, 3.37M) reports, "A drumbeat of chants criticizing vaccine and mask mandates carried into the unusually sparse hallways on Wednesday at the state Capitol, where lawmakers were wrapping up the final week of their legislative session without the bills that hundreds came to Sacramento to protest." A late push "by Assemblyman Evan Low (D-Campbell) to create a new workplace vaccine requirement failed to come together before a legislative deadline Tuesday evening." Another proposal "by Assemblywoman Buffy Wicks (D-Oakland), which fell apart last week, would have created sweeping new vaccine mandates in the workplace and to enter businesses."

Colleges In 12 Conservative-Leaning States Grapple With Mask, Vaccine Mandates.

[[HYPERLINK "https://khn.org/news/article/colleges-mandate-covid-vaccination-masks-even-in-conservative-states/"](https://khn.org/news/article/colleges-mandate-covid-vaccination-masks-even-in-conservative-states/)] (9/9, Andrews) reports that "12 conservative-leaning states prohibit vaccine mandates at higher education institutions, according to an analysis by the National Academy for State Health Policy." Red states with Republican leadership "are hardly the only ones where colleges and universities are facing restrictions on their ability to put public health protections in place." But for teachers, "whose professions are rooted in encouraging the pursuit of learning and knowledge, prohibitions that fly in the face of science and jeopardize public health can be tough to swallow."

Baltimore County To Require Government Employees To Provide Proof Of COVID-19 Vaccination.

The [[HYPERLINK "https://www.baltimoresun.com/maryland/baltimore-county/bs-md-co-mandatory-county-vaccines-20210909-fzv25yuiurdzthz6gnufycgxv4-story.html#ed=rss_www.baltimoresun.com/arcio/rss/category/latest/"](https://www.baltimoresun.com/maryland/baltimore-county/bs-md-co-mandatory-county-vaccines-20210909-fzv25yuiurdzthz6gnufycgxv4-story.html#ed=rss_www.baltimoresun.com/arcio/rss/category/latest/)] (9/9, DeVille, 629K) reports that Baltimore County "will require government employees to prove they are vaccinated or submit to weekly testing, County Executive Johnny Olszewski Jr. announced Thursday." The state's

"third-largest jurisdiction will require its 9,000 employees to prove by Oct. 15 that they've received at least one dose of the Pfizer/BioNTech or Moderna vaccines or the single-dose Johnson & Johnson vaccine." Employees "will have to prove they've received all vaccination doses by Nov. 15."

FDA IN THE NEWS

FDA Announces Delay In Decisions On E-Cigarette Products, Including Juul.

The [[HYPERLINK "https://www.wsj.com/articles/fda-to-see-more-time-to-decide-fate-of-juul-11631196029"](https://www.wsj.com/articles/fda-to-see-more-time-to-decide-fate-of-juul-11631196029)] (9/9, Maloney, Subscription Publication, 8.41M) reports the FDA has announced it needs to extend its deadline for deciding whether or not e-cigarette products can stay on the US market. The original deadline was Thursday, Sept. 9. The agency reported it had acted on 93% of the received applications.

The [[HYPERLINK "https://apnews.com/article/business-science-health-tobacco-industry-regulation-26c9e4b2d7368a08443d432dae1266dd"](https://apnews.com/article/business-science-health-tobacco-industry-regulation-26c9e4b2d7368a08443d432dae1266dd)] (9/9, Perrone) reports the FDA "said it rejected applications for nearly 950,000 e-cigarettes and related products, mainly due to their potential appeal to underage teens," but has yet to rule on Juul, the most popular brand. In a statement, the agency said, "There's more work to be done to complete our remaining reviews and ensure that we continue taking appropriate action to protect our nation's youth from the dangers of all tobacco products, including e-cigarettes."

[[HYPERLINK "https://www.reuters.com/world/us/us-fda-delays-decision-juul-e-cigarettes-2021-09-09/"](https://www.reuters.com/world/us/us-fda-delays-decision-juul-e-cigarettes-2021-09-09/)] (9/9, Banerjee, Kirkham) reports the FDA "continues to 'work expeditiously on the remaining applications,' adding many are 'in the final stages of review.'"

[[HYPERLINK "https://www.usatoday.com/story/news/health/2021/09/09/fda-delays-juul-decision-vaping-products/8256400002/"](https://www.usatoday.com/story/news/health/2021/09/09/fda-delays-juul-decision-vaping-products/8256400002/)] (9/9, Rodriguez, 12.7M) reports Theodore Wagener, Director of the Center for Tobacco Research and co-leader of the Cancer Control Program at the Ohio State University Comprehensive Cancer Center, said, "(The FDA) is doing this the right way. ... Their slow walk of these things is a measured approach where they're trying to review what the manufacturers supply while also recalling the science and having a longer-term view of what the science continues to suggest." Meanwhile, Acting FDA Commissioner Janet Woodcock and Mitch Zeller, Director of the FDA's Center for Tobacco Products, said in a joint statement, "We are committed to working as quickly as possible to transition the current marketplace for deemed new tobacco products to one in which all products available for sale have undergone a careful, science-based review by the FDA and met the statutory standard."

[HYPERLINK "https://www.npr.org/2021/09/09/1035288252/fda-vaping-ecigarettes-smoking"] (9/9, Sullivan, 3.69M) reports, "The news comes after years in which critics have urged tighter federal controls on vaping to combat a new surge of nicotine use by minors." The agency "has issued marketing denial orders for more than 946,000 flavored vaping products because their applications 'lacked sufficient evidence that they have a benefit to adult smokers sufficient to overcome the public health threat posed by the well-documented, alarming levels of youth use of such products.'"

[HYPERLINK "https://us.cnn.com/2021/09/09/health/fda-vaping-ecigs-market-decision-deadline/index.html"] (9/9, Howard, 89.21M) reports "the long-term safety of vaping products is not yet known, said Marielle Brinkman, a senior research scientist at The Ohio State University Comprehensive Cancer Center." Brinkman said, "At the Center for Tobacco Research, we have measured short-term effects in lung and heart health; we have measured harmful and potentially harmful constituents in the vapor that [users] inhale from these products."

The [HYPERLINK "https://tobaccoreporter.com/2021/09/09/more-marketing-denials-overnight-fda-request-more-time-for-juul/"] (9/9) reports, "Many companies reportedly received marketing denial orders (MDO) from the U.S. Food and Drug Administration overnight" before the announcement Thursday.

Among other news outlets reporting on the story are the [HYPERLINK "https://www.washingtonpost.com/health/2021/09/09/fda-decision-juul-ban-delayed/"] (9/9, McGinley, 10.52M), the [HYPERLINK "https://www.nytimes.com/2021/09/09/health/fda-e-cigarettes-vaping.html"] (9/9, Richtel, 20.6M), [HYPERLINK "https://abcnews.go.com/Health/fda-delays-decision-juul-banning-popular-cigarette-products/story?id=79912388"] (9/9, Salzman, Wu, 2.44M), [HYPERLINK "https://www.cnn.com/2021/09/09/fda-will-reportedly-seek-more-time-before-deciding-if-juul-can-keep-selling-its-e-cigarettes.html"] (9/9, Lucas, 7.34M), [HYPERLINK "https://thehill.com/policy/healthcare/571571-fda-postpones-its-decision-on-juul-e-cigarettes"] (9/9, Coleman, 5.69M), [HYPERLINK "https://www.newsweek.com/fda-kicks-multiple-vape-companies-out-us-market-juul-could-follow-1627536"] (9/9, Rouhandeh, 2.67M), [HYPERLINK "https://www.politico.com/news/2021/09/09/fda-electronic-cigarettes-off-market-510967"] (9/9, Foley, 6.73M), [HYPERLINK "https://news.bloomberglaw.com/pharma-and-life-sciences/almost-1-million-flavored-vaping-products-to-see-market-removal"] (9/9, Subscription Publication, 4K), [HYPERLINK "https://www.statnews.com/2021/09/09/fda-

misses-deadline-for-deciding-which-ecigarette-products-should-be-removed-from-market/"] (9/9, Florko, 262K), the [HYPERLINK

"https://www.washingtontimes.com/news/2021/sep/9/fda-rejects-almost-1-million-flavored-e-cigarette/"] (9/9, Tan, 626K), the [HYPERLINK "https://nypost.com/2021/09/09/fda-reportedly-taking-more-time-to-rule-on-pulling-juul/"] (9/9, Feuer, 7.45M), the [HYPERLINK "https://www.nydailynews.com/news/national/ny-fda-regulation-e-cigarette-vaping-ban-20210909-bby2cfyq5bx7emxjdggnjf4t4-story.html"] (9/9, Sapienza, 2.51M), [HYPERLINK "https://www.axios.com/fda-asks-for-more-time-juul-e-cig-ban-a1a19648-391c-4c42-a892-fa63b4d41cf3.html"] (9/9, Fernandez, 1.26M), the [HYPERLINK "https://journalnow.com/business/local/fda-doesnt-meet-federal-deadline-on-new-e-cigarette-regulations/article_31558c8a-1168-11ec-bfc8-f7ead4570c58.html"] (9/9, Craver, 226K), [HYPERLINK "https://www.cspdailynews.com/tobacco/fda-needs-more-time-review-pmtas"] (9/9, Prokop), [HYPERLINK "https://www.newsweek.com/fda-rejects-950k-applications-flavored-e-cigarettes-delays-decision-juul-1627634"] (9/9, Klapper, 2.67M), [HYPERLINK "https://filtermag.org/fda-deadline-fail-vapes/"] (9/9, Norcia), [HYPERLINK "https://csnews.com/fda-reportedly-seeking-more-time-decision-juul"] (9/9, 278K), [HYPERLINK "https://vaping360.com/vape-news/111124/pmta-decision-day-fda-leaves-vape-industry-twisting-in-the-wind/"] (9/9, McDonald), the [HYPERLINK "https://tobaccoreporter.com/2021/09/09/fda-rejects-nearly-1-million-ends-fails-to-rule-on-market-leaders/"] (9/9), [HYPERLINK "https://www.theverge.com/2021/9/9/22665333/fda-vaping-flavors-authorization-juul"] (9/9, Wetsman, 1.54M), and [HYPERLINK "https://vaporvoice.net/2021/09/09/many-firms-received-marketing-denial-from-fda-overnight/"] (9/9).

Biden Announces New COVID-19 Vaccination Mandates For Businesses, Healthcare Facilities.

The [HYPERLINK "https://www.washingtonpost.com/politics/vaccine-mandate-federal-employees/2021/09/09/1c1ce9dc-116b-11ec-882f-2dd15a067dc4_story.html"] (9/9, Linskey, Abutaleb, Min Kim, Rein, 10.52M) reports "President Biden announced sweeping new coronavirus vaccine mandates Thursday designed to affect tens of millions of Americans, ordering all businesses with more than 100 employees to require their workers to be immunized or face weekly testing." The President "also said that he would require most health-care facilities that accept Medicare or Medicaid funding to vaccinate their employees, which the White House believes will cover 50,000 locations." Furthermore, Biden "signed an executive order compelling all

federal employees to get vaccinated – without an option for those who prefer to be regularly tested instead – in an effort to create a model he hopes state governments will embrace.” Biden “is also ordering all staffers in Head Start programs, along with Defense Department and federally operated schools for Native Americans, to be vaccinated.” NIAID Director Anthony Fauci said, “This was not just superficial stuff. This is going to have a significant impact.”

The [HYPERLINK "https://www.nytimes.com/2021/09/09/us/politics/biden-mandates-vaccines.html"] (9/9, A1, Rogers, Stolberg, 20.6M) reports Biden “moved to mandate shots for health care workers, federal contractors and the vast majority of federal workers, who could face disciplinary measures if they refuse.” Biden said, “We’ve been patient. ... But our patience is wearing thin. And your refusal has cost all of us.” The vaccine “mandate for health care workers will apply to those employed by most institutions that accept Medicare and Medicaid reimbursement, including hospitals, dialysis centers and nursing homes, according to” officials. The mandate “will be enforced by” CMS.

The [HYPERLINK "https://www.nytimes.com/live/2021/09/09/world/covid-delta-variant-vaccine/osha-vaccine-biden-mandate"] (9/9, Hirsch, 20.6M) reports in a separate piece that the proposed rules “will require workers to be inoculated or face weekly testing and will mandate that the businesses offer employees paid time off to get vaccinated.” The rules “will be imposed by the Department of Labor and its Occupational Safety and Health Administration, which is drafting an emergency temporary standard to carry out the mandate, according to the White House.”

The [HYPERLINK "https://www.latimes.com/politics/story/2021-09-09/biden-require-covid-vaccination-federal-workers"] (9/9, Megerian, 3.37M) reports “firms that don’t comply could face \$14,000 fines from the Department of Labor.” The President “is also tightening rules for federal employees and contractors.” Even though “they were previously allowed to choose weekly testing instead of vaccination, that will no longer be an option,” and “anyone who isn’t fully vaccinated within three months could face disciplinary action, with limited exceptions.”

[HYPERLINK "https://www.bloomberg.com/news/articles/2021-09-09/biden-to-sign-order-requiring-vaccines-for-federal-workers?srd=premium"] (9/9, Wingrove, Leonard, 3.57M) reports “Biden also delivered some of his harshest criticism yet of the 25% of U.S. adults who’ve so far not been inoculated, saying that they’re dragging out the pandemic.” He stated, “My message to unvaccinated Americans is this: What more is there to wait for? What more do you need to see?” Bloomberg adds that the Indian Health Service and the

National Institutes of Health are “implementing previously announced vaccination requirements that cover 2.5 million workers, according to the administration.”

The [HYPERLINK "https://apnews.com/article/joe-biden-business-health-coronavirus-pandemic-executive-branch-18fb12993f05be13bf760946a6fb89be"] (9/9, Miller) reports “Republican leaders – and some union chiefs, too – said Biden was going too far in trying to muscle private companies and workers, a certain sign of legal challenges to come.” However, “there were strong words of praise for Biden’s efforts to get the nation vaccinated from the American Medical Association, the National Association of Manufacturers and the Business Roundtable – though no direct mention of his mandate for private companies.”

The [HYPERLINK "https://apnews.com/article/technology-joe-biden-health-business-coronavirus-pandemic-6ab9a65961289d5415984e2c52c7bb83"] (9/10, Ortutay) reports in a separate piece that “larger U.S. businesses now won’t have to decide whether to require their employees to get vaccinated against COVID-19,” because “doing so is now federal policy.”

[HYPERLINK "https://www.reuters.com/world/us/biden-deliver-six-step-plan-covid-19-pandemic-2021-09-09/"] (9/9, Hunnicutt, Mason, Aboulenein) reports “infectious disease and health policy experts said the mandates are unlikely to significantly change infection rates quickly.” However, the mandates “would help against potential future waves of the virus, reducing deaths and hospitalizations and alleviating the stress on the healthcare system, said Georgetown University’s Dr. Jesse Goodman, a former chief scientist at the U.S. Food and Drug Administration.”

[HYPERLINK "https://www.cnn.com/2021/09/09/biden-to-detail-new-six-pronged-plan-to-increase-us-covid-vaccination-rates-fight-virus.html"] (9/9, Lovelace, Mendez, Towey, 7.34M) reports “the Biden administration has also encouraged private businesses to implement vaccine mandates, though CDC Director Dr. Rochelle Walensky previously told CNBC that federal officials would not issue a nationwide mandate.”

[HYPERLINK "https://thehill.com/policy/healthcare/571593-biden-blames-unvaccinated-for-covid-19s-slog"] (9/9, Chalfant, 5.69M) reports Biden “admonished unvaccinated Americans and some elected officials for exacerbating the coronavirus pandemic.” He stated, “We have the tools to combat COVID-19, and a distinct minority of Americans, supported by a distinct minority of elected officials, are keeping us from turning the corner. These pandemic politics, as I refer to it, are making people sick, causing unvaccinated people to die.”

[HYPERLINK "https://www.rollcall.com/2021/09/09/biden-to-announce-sweeping-federal-workforce-vaccine-mandate/"] (9/9,

McIntire, Lesniewski, 130K) reports "the president's plan will also double fines for people not in compliance with federal masking requirements, such as on airplanes."

Among other news outlets reporting on the story are [[HYPERLINK](#)

"https://playvideo.bulletinintelligence.com/fcf94b7376e44ace8acfc7fccf35e74a?pubid=fda"] (9/9, 6:32 p.m. EST, lead story, 6:00, Muir, 6.32M), the [[HYPERLINK](#) "https://playvideo.bulletinintelligence.com/02ae11a459914b99af457414ad4d448c?pubid=fda"] (9/9, 6:31 p.m. EST, lead story, 3:50, O'Donnell, 3.48M), [[HYPERLINK](#) "https://playvideo.bulletinintelligence.com/fc297be21a394379b21e51f44e1fadb1?pubid=fda"] (9/9, 6:31 p.m. EST, lead story, 3:00, Holt, 4.78M), the [[HYPERLINK](#) "https://www.wsj.com/articles/biden-to-require-all-federal-workers-government-contractors-to-be-vaccinated-against-covid-19-11631195011"] (9/9, A1, Armour, Siddiqui, Subscription Publication, 8.41M), [[HYPERLINK](#) "https://abcnews.go.com/Politics/biden-order-federal-workers-vaccinated-strategy-combat-delta/story?id=79905030"] (9/9, Nagle, Kolinovsky, Gomez, 2.44M), [[HYPERLINK](#) "https://www.cnn.com/2021/09/09/politics/joe-biden-covid-speech/index.html"] (9/9, Collins, Liptak, 89.21M), [[HYPERLINK](#)

"https://www.forbes.com/sites/edwardsegal/2021/09/09/biden-unveils-new-action-plan-to-combat-surging-covid-crisis/"] (9/9, Segal, 10.33M), [[HYPERLINK](#) "https://www.newsweek.com/biden-does-u-turn-vaccine-mandates-two-months-after-declaring-independence-covid-19-1627695"] (9/9, Villarreal, 2.67M), [[HYPERLINK](#) "https://www.npr.org/2021/09/09/1035149651/biden-will-require-vaccines-for-federal-workers-as-part-of-a-new-covid-strategy"] (9/9, Wise, Keith, 3.69M), [[HYPERLINK](#) "https://www.politico.com/news/2021/09/09/biden-federal-workers-vaccines-510851"] (9/9, Cancryn, Lim, 6.73M), [[HYPERLINK](#) "https://www.foxbusiness.com/politics/biden-administration-covid-vaccine-mandate-all-federal-workers-contractors"] (9/9, Singman, 3.06M), the [[HYPERLINK](#) "https://nypost.com/2021/09/09/biden-to-require-all-federal-workers-be-vaccinated-for-covid-19/"] (9/9, Moore, Nelson, Patteson, 7.45M), the [[HYPERLINK](#) "https://www.nydailynews.com/coronavirus/ny-biden-covid-vaccine-mandate-20210909-3xnerbu2cvebnpei3c36jrtaqa-story.html"] (9/9, Goldiner, Balk, 2.51M), the [[HYPERLINK](#) "https://www.freep.com/story/news/health/2021/09/09/what-bidens-sweeping-covid-19-vaccine-mandate-means-michigan/8263546002/"] (9/9, Shamus, Boucher, Roberts, 2.16M), the [[HYPERLINK](#) "https://www.bostonherald.com/2021/09/09/joe-biden-outlines-sweeping-vaccine-requirements-for-american-workers-urges-governors-mandate-teacher-vaccinations/"] (9/9, Tiernan, 327K), and [[HYPERLINK](#) "https://www.govexec.com/management/2021/09/federal-

contractors-will-have-vaccine-mandate-similar-federal-employees/185237/"] (9/9, Bubl , 29K).

Biden Requires In-Home Nurses To Receive COVID-19 Vaccine Under New Plan. [[HYPERLINK](#)

"https://www.bloomberg.com/news/articles/2021-09-09/home-nurses-caring-for-fragile-children-must-now-be-vaccinated?srnd=premium"] (9/9, Edney, 3.57M) reports "parents of children with severe health conditions won a victory Thursday when President Joseph Biden required that in-home nurses be vaccinated against Covid-19." A plan the President "revealed to boost vaccine requirements across the U.S. includes home health workers among 17 million medical staff who must be inoculated, according to a White House summary."

Amazon, Kroger, Walmart Playing Key Role In Biden's Most Recent COVID-19 Plan. [[HYPERLINK](#)

"https://www.foxbusiness.com/politics/walmart-amazon-kroger-biden-covid-19-plan"] (9/9, Barrabi, 3.06M) reports "Walmart, Amazon, and Kroger will play a key role in President Biden's latest plan to curb the COVID-19 pandemic, the White House revealed on Thursday." The three retailers "have agreed to sell at-home rapid COVID-19 tests to the public at cost beginning later this week, a senior administration official said." The COVID-19 "tests will be available for purchase online or in-stores at a discount of up to 35% for the next three months." Meanwhile, HHS' "free COVID-19 testing program will expand to provide services at 10,000 pharmacies nationwide."

[[HYPERLINK](#) "https://www.reuters.com/business/healthcare-pharmaceuticals/amazoncom-cut-cost-covid-19-pcr-test-kits-2021-09-09/"] (9/9, Shepardson) reports Amazon.com "said it will cut the cost of its direct-to-consumer COVID-19 PCR Test Collection kit to \$36.99, a \$3 price reduction." Amazon "said the price cut reflects its costs for selling the FDA-approved kit and is the result of a public-private partnership with the Biden administration."

Companies Say Federal Government Must Give More Details About COVID-19 Vaccination Mandate. [[HYPERLINK](#)

"https://www.bloomberg.com/news/articles/2021-09-10/businesses-question-logistics-cost-of-biden-vaccine-plan?srnd=premium"] (9/9, Roeder, 3.57M) reports "U.S. companies grappling with the thorny issue of whether to require worker vaccinations against Covid-19 say the U.S. government needs to provide more details about" the "new, far-reaching mandate announced Thursday." Businesses are "digesting" Biden's "order, but there are concerns over what the mandate may entail." For example, the Consumer Brands Association said, "President Biden's announcement prompts critical questions that require immediate clarification. ... As with other mandates, the devil is in the details. Without

additional clarification for the business community, employee anxieties and questions will multiply."

RNC Promises To Sue Biden Administration Over COVID-19 Vaccine, Testing Requirements. [[HYPERLINK "https://thehill.com/homenews/administration/571623-rnc-vows-to-sue-over-biden-vaccine-testing-mandate"](https://thehill.com/homenews/administration/571623-rnc-vows-to-sue-over-biden-vaccine-testing-mandate)] (9/9, Oshin, 5.69M) reports "the Republican National Committee (RNC) has vowed to sue the Biden administration over its COVID-19 vaccine and testing mandate." RNC Chairwoman Ronna McDaniel said, "Many small businesses and workers do not have the money or legal resources to fight Biden's unconstitutional actions and authoritarian decrees." She continued, "But when his decree goes into effect, the RNC will sue the administration to protect Americans and their liberties."

Unions Push Back Against Biden's Proposal To Require Federal Employees To Receive COVID-19 Vaccine. The [[HYPERLINK "https://www.washingtontimes.com/news/2021/sep/9/federal-employee-unions-push-back-against-bidens-v/"](https://www.washingtontimes.com/news/2021/sep/9/federal-employee-unions-push-back-against-bidens-v/)] (9/9, Mordock, 626K) reports "unions that represent federal workers pushed back Thursday against President Biden's plan to require all federal employees and contractors to be vaccinated against COVID-19." The American Federation of Government Employees "said COVID-19 vaccine requirements should be negotiated with the unions during collective bargaining, not mandated through an executive order." Meanwhile, the National Treasury Employees Union "said the administration must accommodate workers who decide against the vaccine for religious or medical reasons."

Biden's Executive Order Forcing COVID-19 Vaccinations Reportedly Does Not Include Postal Service Employees. The [[HYPERLINK "https://www.washingtontimes.com/news/2021/sep/9/us-postal-service-exempt-biden-vaccine-mandate-aft/"](https://www.washingtontimes.com/news/2021/sep/9/us-postal-service-exempt-biden-vaccine-mandate-aft/)] (9/9, Morton, 626K) says "the Washington Post reported Thursday night that [Biden's] executive order forcing federal workers to take a COVID-19 vaccine does not include U.S. Postal Service workers." Citing one White House official, "the Post reported that those workers merely would be strongly encouraged to follow the mandate." Since "the Postal Service is an independent agency of the executive branch, the Post explained, an executive order on federal employees' labor issues does not apply to the agency, unless a president specifies that it does."

Arizona Governor Vows To "Push Back" On Biden's COVID-19 Vaccination Plan For Large Employers. The [[HYPERLINK "https://www.azcentral.com/story/news/politics/arizona/2021/09/09/arizona-leaders-criticize-biden-vaccine-rules-doug-ducey-push-back/8263811002/"](https://www.azcentral.com/story/news/politics/arizona/2021/09/09/arizona-leaders-criticize-biden-vaccine-rules-doug-ducey-push-back/8263811002/)] (9/9, Barchenger, 1.05M) reports Arizona Gov. Doug Ducey (R) "has promised to 'push back' on President Joe Biden's plan to require large

employers to mandate vaccines or weekly COVID-19 tests for their workers." The governor "did not say what form a pushback would take, only promising 'more to follow.'" Ducey said to reporters, "What the Biden administration is doing is government overreach, pure and simple. And people don't want them hammering down on them to get this vaccine."

NYTimes Examines Who Will Pay For COVID-19 Tests Under Biden Administration's New Rule For Large Companies. The [[HYPERLINK "https://www.nytimes.com/article/unvaccinated-covid-tests.html"](https://www.nytimes.com/article/unvaccinated-covid-tests.html)] (9/9, Kliff, 20.6M) reports the Biden Administration's intent "to mandate that workers at large companies get vaccinated" against COVID-19 "or submit to regular testing" leads to "a thorny question: Who pays for those coronavirus tests?" So far, employers have "considered a range of approaches, from fully covering the costs to having unvaccinated workers pay full freight." A number of "employers have had workers chip in for the costs of coronavirus testing," and "many states and cities still have free coronavirus testing sites that they started earlier in the pandemic." These different "approaches could provide a menu of options for many workplaces that fall under the new federal rule."

Analysis: Biden's Move To Expand Number Of Vaccinated Americans Is Attempt To Counter COVID-19's Threat To Economy. In an analysis, the [[HYPERLINK "https://www.nytimes.com/2021/09/09/us/politics/biden-vaccines-economy.html"](https://www.nytimes.com/2021/09/09/us/politics/biden-vaccines-economy.html)] (9/9, A1, Tankersley, 20.6M) says "President Biden's aggressive move to expand the number of vaccinated Americans and halt the spread of the Delta variant is not just an effort to save lives." The move "is also an attempt to counter the continuing and evolving threat that the virus poses to the economy." The rise of the Delta variant "has been fueled in part by the inability of Mr. Biden and his administration to persuade millions of vaccine-refusing Americans to inoculate themselves against the virus," which "has created another problem: a drag on the economic recovery." According to the Times, "real-time gauges of restaurant visits, airline travel and other services show consumers pulled back on some face-to-face spending in recent weeks."

Columnist: Biden's New COVID-19 Strategy Does Not Go Far Enough To Boost Vaccinations. [[HYPERLINK "https://www.washingtonpost.com/opinions/2021/09/09/biden-s-six-step-covid-strategy-does-not-go-far-enough-compel-vaccinations/"](https://www.washingtonpost.com/opinions/2021/09/09/biden-s-six-step-covid-strategy-does-not-go-far-enough-compel-vaccinations/)] (9/9, 10.52M) columnist Leana Wen says "President Biden's much-hyped new strategy for fighting covid-19 is a tepid half-measure that falls short of the dramatic reset the country needs." Wen asserts that "the six-pronged strategy announced on Thursday can be summarized as 'more of the same' – these are good steps in the right direction, but they're not enough to get the job done." She writes that "the biggest problem with Biden's plan is that

it does not go nearly far enough toward compelling vaccinations."

FDA, FTC Warn 10 Companies Against Making "Fraudulent Claims" That Unapproved Dietary Supplements Can Help With Diabetes.

[HYPERLINK

"<https://www.cardiovascularbusiness.com/topics/prevention-risk-reduction/fda-ftc-fraudulent-claims-unapproved-diabetes-supplements>"] (9/9, Walter) reports, "The FDA and FTC have warned 10 companies for illegally selling dietary supplements that claim to 'cure, treat, mitigate or prevent diabetes,'" which the agencies said violates the Federal Food, Drug and Cosmetic Act. Acting FDA Center for Food Safety and Applied Nutrition Office of Dietary Supplement Programs Director Cara Welch, PhD, stated, "More than 34 million Americans...are living with diabetes. ... Dietary supplements that make fraudulent claims to treat diabetes are unapproved new drugs that could potentially harm consumers who use these products instead of seeking safe and effective FDA-approved treatments."

The [HYPERLINK
"<https://www.miamiherald.com/news/health-care/article254114263.html>"] (9/9, Neal, 647K) also reports on the story.

FDA Authorizes Additional Batch Of J&J COVID-19 Vaccine From Troubled Baltimore Plant.

The [HYPERLINK
"https://www.baltimoresun.com/coronavirus/bs-md-fda-johnson-johnson-baltimore-batch-20210909-pttd7ypd3rhznmhfbj7fujesy-story.html#ed=rss_www.baltimoresun.com/arcio/rss/category/latest/"] (9/9, Miller, 629K) reports that the FDA "has authorized an additional batch of COVID-19 vaccine produced by Johnson & Johnson at an East Baltimore plant where regulators previously determined several million doses of vaccine were unusable." The Sun adds that "the vaccine being produced at the facility, owned and operated by the Gaithersburg-based Emergent BioSolutions, still has not been authorized fully under the FDA's emergency use authorization, said Marion Gruber, director of the office of vaccines research and review center for biologics evaluation and research."

MEDICAL PRODUCT SAFETY

Biden Administration Releases Proposal To Lower Prescription Drug Prices.

The [HYPERLINK
"<https://www.wsj.com/articles/white-house-unveils-plan-to-cut-prescription-drug-prices-11631181600>"] (9/9, Armour,

Subscription Publication, 8.41M) reports on Thursday, the Biden Administration unveiled a plan which seeks to reduce prescription drug prices. HHS Secretary Xavier Becerra said, "The Biden-Harris administration remains committed to making health care more affordable for American families, and this plan outlines one key way we will do that. ... By promoting negotiation, competition, and innovation in the health-care industry, we will ensure cost fairness and protect access to care." The article mentions the FDA.

The [HYPERLINK
"<https://www.nytimes.com/2021/09/09/upshot/biden-drug-prices-analysis.html>"] (9/9, Sanger-Katz, 20.6M) reports the initiative was an endorsement of "an aggressive proposal to limit prices for prescription drugs, a signal to congressional Democrats as they consider options for their large legislative package." The plan "was published as a 29-page white paper from the Department of Health and Human Services. It included a range of recommendations to foster more competition among drugmakers and improve the affordability of drugs for patients enrolled in Medicare." However, "its primary recommendation was a big one: that the department should negotiate with drug companies on their product prices, and that those prices should apply to all drug purchasers in the country, not just Medicare, the federal program primarily for people 65 and older."

[HYPERLINK "<https://news.bloomberglaw.com/health-law-and-business/hhs-calls-for-authority-to-negotiate-medicare-drug-prices>"] (9/9, Stein, Subscription Publication, 4K) reports HHS "attributed the high cost of prescription drug prices in the U.S. to 'lack of competition,' and said Americans pay more than \$1,500 per person a year for medications." The department "said the pharmaceutical industry is 'characterized by multiple market failures,' attributable to several factors, including 'monopolistic or oligopolistic behavior' and 'legal abuses.'" Becerra stated, "Life-saving prescription medication should not cost anyone their life savings. Yet too often, many low-income families cannot take their prescription medications because of cost concerns."

In a separate article, [HYPERLINK
"<https://news.bloomberglaw.com/health-law-and-business/hhs-chief-calls-on-patent-office-to-help-curb-rising-drug-prices>"] (9/9, Lopez, Subscription Publication, 4K) reports Becerra "is calling on the U.S. Patent and Trademark Office to help rein in skyrocketing drug prices as part of the government's push to curb practices that allegedly keep lower-cost medicines from hitting the market." He stated in the proposal that the patent office and the FDA "will work together to develop solutions' to facilitate faster market entry of cheaper, copycat versions of name brand drugs."

[HYPERLINK "<https://www.reuters.com/world/us/us-government-lays-out-plans-curb-medicare-drugs-costs-2021-09-09/>"] (9/9, Heavey, O'Donnell) reports although "the administration has lauded drugmakers for their efforts amid

the COVID-19 pandemic, it also has highlighted the high costs of medications in the United States compared to other nations. Pharmaceutical manufacturers have raised prices on more than 500 medicines, according to an analysis by healthcare research firm 46brooklyn in January."

Among other news outlets covering the story are [HYPERLINK "<https://www.cnn.com/2021/09/09/politics/medicare-drug-prices-white-house-plan/index.html>"] (9/9, Lovelace, 7.34M), [HYPERLINK "<https://www.politico.com/news/2021/09/09/biden-drug-price-negotiations-510828>"] (9/9, Cancryn, 6.73M), [HYPERLINK "<https://thehill.com/policy/healthcare/571478-white-house-plan-backs-medicare-drug-price-negotiation>"] (9/9, Weixel, 5.69M), [HYPERLINK "<https://fortune.com/2021/09/09/health-care-biden-lower-prescription-drug-prices-big-pharma-american-medicine/>"] (9/9, Morris, 3.68M), [HYPERLINK "<https://www.newsweek.com/biden-looks-slash-drug-prices-going-after-big-pharmas-monopolies-1627504>"] (9/9, Rouhandeh, 2.67M), [HYPERLINK "<https://www.statnews.com/2021/09/09/new-biden-plan-to-lower-drug-prices-drawn-from-familiar-democratic-playbook/>"] (9/9, Facher, 262K), [HYPERLINK "<https://www.modernhealthcare.com/politics-policy/hhs-suggests-medicare-part-b-payment-changes-promote-biosimilars>"] (9/9, Hellmann, Subscription Publication, 215K), [HYPERLINK "<https://www.fiercehealthcare.com/payer/hhs-aims-to-use-value-based-care-payment-models-to-lower-drug-prices>"] (9/9, King, 150K), [HYPERLINK "<https://insidehealthpolicy.com/inside-drug-pricing-daily-news/hhs%E2%80%99s-drug-pricing-plan-noncommittal-march-omits-intl-prices>"] (9/9, Wanneh, Subscription Publication), a separate piece by [HYPERLINK "<https://insidehealthpolicy.com/becerra-releases-proposal-lower-prescription-drug-costs>"] (9/9, Subscription Publication), and the [HYPERLINK "<https://www.thefiscaltimes.com/2021/09/09/Biden-Administration-Backs-Medicare-Negotiations-Plan-Lower-Drug-Prices>"] (9/9, Rosenberg).

Endo, New York Settle Opioid Suit. The [HYPERLINK "<https://apnews.com/article/business-health-new-york-lawsuits-opioids-f533831773c6ca58c357f1680d75bfb1>"] (9/9) reports, "The state of New York and two large counties agreed Thursday [to] a \$50 million deal to end their lawsuits with drugmaker Endo International, in the latest of a progression of settlements of government claims over the opioid addiction and overdose crisis." Under the deal, "the Dublin-based drugmaker and its subsidiary Par are to pay \$22.3 million to

the state attorney general's office and \$13.85 million to both Suffolk and Nassau counties."

[HYPERLINK "<https://www.reuters.com/business/healthcare-pharmaceuticals/drugmaker-endo-settles-opioid-claims-by-new-york-counties-50-mln-2021-09-10/>"] (9/10) also covers the story.

Study: Black People Died From Opioid Overdoses At Higher Rates Than White, Hispanic People In 2019.

The [HYPERLINK "<https://www.bostonglobe.com/2021/09/09/metro/new-study-affirms-rising-opioid-overdoses-among-black-people/>"] (9/9, Freyer, 1.04M) reports "the opioid epidemic has long been portrayed as a crisis among white Americans, but increasingly, Black people are dying of overdoses at alarming rates." One "study led by Boston Medical Center researchers, published Thursday, shows that in 2019, for the first time, Black people died of overdoses at higher rates than white and Hispanic people." The [HYPERLINK "<https://ajph.aphapublications.org/doi/10.2105/AJPH.2021.306431>"], which was "published in the American Journal of Public Health, is among the first research papers to emerge from the HEALing Communities study, a \$350 million project that started in 2019." As part of the agency's "Helping to End Addiction Long-term Initiative, the National Institutes of Health project is testing possible solutions to the opioid crisis in hard-hit communities in Kentucky, Massachusetts, New York, and Ohio."

The [HYPERLINK "<https://r.bulletinintelligence.com/41552891f8d14d40b4a4ac94627559eb>"] (9/10) reports "non-Hispanic Black individuals in four U.S. states experienced a 38% increase in the rate of opioid overdose deaths from 2018 to 2019, while the rates for other [races] and ethnicity groups held steady or decreased, according to" the study. The HEALing Communities Study "is the largest addiction implementation study ever conducted and is administered in partnership by NIH's National Institute on Drug Abuse (NIDA) and the Substance Abuse and Mental Health Services Administration." NIDA Director Nora D. Volkow said, "Systemic racism fuels the opioid crisis, just as it contributes mightily to other areas of health disparities and inequity, especially for Black people."

[HYPERLINK "<https://r.bulletinintelligence.com/4c85c95752c14937b79c233d0e1c7db3>"] (9/9, Lopez, Baumann, Subscription Publication, 4K) also reports on the story.

President Biden Orders FDA To Finish Draft Rules On OTC Hearing Aids By November.

[HYPERLINK "<https://consumer.healthday.com/8-18-why-is-fda-stalling-on-sale-of-cheaper-sleeker-hearing-aids>"]

2654599025.html"] (9/9, 11K) reports, "Until now, folks suffering from hearing loss typically have had to fork out thousands of dollars for a device that could be adjusted only by a professional audiologist." It is "no wonder that only one-quarter of the nearly 29 million U.S. adults who could benefit from a hearing aid have actually tried one, according to the U.S. National Institutes of Health." A 2017 law "requires the U.S. Food and Drug Administration to establish a category of over-the-counter hearing aids, as well as the standards for them." The FDA "missed its August 2020 deadline to propose those new rules, blaming the COVID-19 pandemic for the delay," but the "wait for consumers might be over soon: President Joe Biden has ordered the agency to finish its draft rules by November."

Apellis Still Plans To File For FDA Approval For Eye Disorder Drug Despite Mixed Phase 3 Results. [[HYPERLINK](https://www.fiercepharma.com/pharma/despite-mixed-phase-3-results-apellis-believes-it-can-push-eye-disorder-drug-over-fda-finish)

"https://www.fiercepharma.com/pharma/despite-mixed-phase-3-results-apellis-believes-it-can-push-eye-disorder-drug-over-fda-finish"] (9/9, Higgins-Dunn, 12K) reports, "Apellis Pharmaceuticals' pegcetacoplan posted mixed results in two late-stage trials studying the C3 inhibitor in patients living with geographic atrophy (GA)." However, "despite the mixed showing, CEO Cedric Francois thinks the med's performance was strong enough to warrant an FDA nod." Apellis "plans to file the med with the FDA in the first half of 2022."

ICU Medical Voluntarily Recalling Amino Acid IV After Hair, Other Foreign Substances Found In Batch. [[HYPERLINK](https://r.bulletinintelligence.com/770e9abf7e4c490eb977016b794e11ae)

"https://r.bulletinintelligence.com/770e9abf7e4c490eb977016b794e11ae"] (9/9, Sullivan) reports that California-based ICU Medical "is voluntarily recalling one lot of an amino acid injection made by Pfizer spinout company Hospira after reports of fibers, hair and other particles of foreign materials were found in the solution." The infusion therapy "recalled 2,112 units of Aminosyn II [(amino acid Injection with electrolytes) 15%, after it became aware of the issue while inspecting samples as part of a routine process, the FDA said."

Court Says AbbVie Cannot Force Takeda To Supply Drug Amid Shortage. [[HYPERLINK](https://www.fiercepharma.com/pharma/no-dice-court-tells-abbvie-it-can-t-force-takeda-to-supply-a-drug-s-short-supply-worldwide)

"https://www.fiercepharma.com/pharma/no-dice-court-tells-abbvie-it-can-t-force-takeda-to-supply-a-drug-s-short-supply-worldwide"] (9/9, Sagonowsky, 12K) reports that this week, "Delaware Chancery Court Vice Chancellor Sam Glasscock III denied AbbVie's request to compel Takeda to fill supply orders" of Lupron (leuporelin), "used to treat pain associated with cancer," from its plant in Hikari, Japan. AbbVie had taken

Takeda "to court over alleged failures to live up to supply agreements" amid shortages of the medication "after the FDA and Takeda in 2019 found 'protocol violations' at the plant." Glasscock wrote that "remediation efforts" have continued to this day and that an order forcing Takeda to supply the treatment would be "unworkable" because it would force the court to oversee Takeda's operations and "inevitably lead to contempt hearings."

Jazz, PharmaMar To Run Another Phase 3 Confirmatory Trial For Small Cell Lung Cancer Drug After Previous Failure. [[HYPERLINK](https://www.fiercepharma.com/marketing/wclc-jazz-charts-new-path-for-small-cell-lung-cancer-drug-zepzelca-after-late-stage-flop)

"https://www.fiercepharma.com/marketing/wclc-jazz-charts-new-path-for-small-cell-lung-cancer-drug-zepzelca-after-late-stage-flop"] (9/9, Liu, 12K) reports, "Jazz Pharmaceuticals wasn't able to ride a breakthrough FDA nod to a pivotal trial win for conditionally approved small cell lung cancer (SCLC) drug Zepzelca" (lurbinectedin), but "after talking with the FDA, the company and partner PharmaMar see a new path forward." According to FiercePharma, "In previously treated SCLC, the combination of Zepzelca and doxorubicin failed to extend patients' lives over physician's choice of doxorubicin-based chemo or Hycamtin [topotecan], according to data presented at the 2021 World Conference on Lung Cancer virtual meeting." Now, "Jazz and PharmaMar have agreed 'in principle' with the FDA to run another phase 3 as the confirmatory trial."

Mammoth Biosciences Raises \$195M In Financing Round. [[HYPERLINK](https://www.forbes.com/sites/katiejennings/2021/09/09/mammoth-biosciences-raises-195-million-vaulting-its-crispr-tech-to-unicorn-status/)

"https://www.forbes.com/sites/katiejennings/2021/09/09/mammoth-biosciences-raises-195-million-vaulting-its-crispr-tech-to-unicorn-status/"] (9/9, Jennings, 10.33M) reports that Mammoth Biosciences on Thursday "announced the company raised \$195 million of financing at a valuation of more than \$1 billion." The funding "includes a \$150 million Series D led by Redmile Group and a \$45 million Series C, also led by Redmile Group and Foresite Capital, with participation from Sixth Street, Decheng Capital, Mayfield and Amazon, among others." A Mammoth COVID-19 diagnostic solution "was selected for the RADx program, a 'shark-tank' style challenge led by the National Institutes of Health, as well as a contract with the Department of Defense." Mammoth "received an emergency use authorization for its DETECTR diagnostic platform for Covid-19 from the FDA last summer."

Also reporting are [[HYPERLINK](https://techcrunch.com/2021/09/09/breakout-crispr-platform-company-mammoth-biosciences-is-officially-a-unicorn/) "https://techcrunch.com/2021/09/09/breakout-crispr-platform-company-mammoth-biosciences-is-officially-a-unicorn/"] (9/9, 502K), [[HYPERLINK](https://medcitynews.com/2021/09/mammoth-bios-massive-195m-financing-makes-the-crispr-company-a-unicorn/) "https://medcitynews.com/2021/09/mammoth-bios-massive-195m-financing-makes-the-crispr-company-a-unicorn/"] (9/9), and the [[HYPERLINK](https://www.ft.com/content/cca7dad4-) "https://www.ft.com/content/cca7dad4-

25e9-4bd5-9fde-
d6140c9fa364?FTCamp=engage/CAP/webapp/Channel_Bulletin/B2B"] (9/9, Kuchler, Subscription Publication, 1.48M).

Recro Acquires San Diego-Based Opioid Addiction Drugmaker For \$50M. [[HYPERLINK "https://r.bulletinintelligence.com/00112435d23146f38c8fff765bec4acd"](https://r.bulletinintelligence.com/00112435d23146f38c8fff765bec4acd)]

(9/9, Sullivan) reports that CDMO Recro Pharma three weeks ago "acquired San Diego-based Irisys for \$50 million cash." According to Endpoint News, "Recro will serve as both an advisor to BioCorRx in its small molecule development for its opiate addiction treatment drug, dubbed Bicx104, in addition to manufacturing it." The FDA has approved the subcutaneous pellet of naltrexone "to start in-person trials, largely funded by the National Institute on Drug Abuse and the NIH."

TOBACCO

Tax On E-Cigarettes Runs Risk Of Pushing Teens Toward Regular Cigarettes, Economists Warn. The [[HYPERLINK "https://www.washingtonexaminer.com/news/taxing-e-cigarettes-could-have-opposite-effect-in-trying-to-end-the-epidemic-of-teen-tobacco-use"](https://www.washingtonexaminer.com/news/taxing-e-cigarettes-could-have-opposite-effect-in-trying-to-end-the-epidemic-of-teen-tobacco-use)]

(9/9, Morrison, 888K) reports "health economists warned that taxes on e-cigarettes and vape products alone would not be enough to keep teenagers from using other risky tobacco products such as regular cigarettes." The authors of a "[[HYPERLINK "https://www.nber.org/system/files/working_papers/w29216/w29216.pdf"](https://www.nber.org/system/files/working_papers/w29216/w29216.pdf)] on whether the United States should put a blanket tax on" vapes "reported that a tax on electronic nicotine delivery systems reduces the likelihood that the youth will buy the products in retail stores." But, "hiking up the prices of vaping products runs the risk of pushing young people toward cheaper alternatives, such as regular cigarettes." The economists who wrote "the report used extensive data collection from two national studies – Monitoring the Future, funded by the National Institute on Drug Abuse, and the Youth Risk Behavior Surveillance System supported by the" CDC.

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TO: THE COMMISSIONER AND SENIOR OFFICIALS

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COVID-19

Biden Calls On Governors To Mandate COVID-19 Vaccinations For School Workers.

The New York Times (9/9, Green, 20.6M) reports "President Biden on Thursday amplified calls for states to take more aggressive measures to keep children in school amid surging coronavirus cases, calling on governors to require vaccinations for all school employees and for districts to implement more regular testing." In the most recent "iteration of the White House's plan to combat the coronavirus pandemic, the administration will also require teachers and other employees of schools run by federal agencies to be vaccinated as part of his broad push to get the federal work force protected." That rule "would apply to those who teach in Head Start programs, Department of Defense Schools, and schools operated by the Bureau of Indian Education."

US News & World Report (9/9, Camera, 1.91M) says "the pressure from Biden...signals a new strategy for the administration, which has been trying to push states and school districts to adopt on their own the suite of safety measures recommended by the" CDC.

Biden Says He Will Take On GOP Governors Attempting To Stop COVID-19 Measures In Schools. The Hill (9/9, Gangitano, 5.69M) reports "President Biden said

Thursday he will take on Republican governors who are trying to prevent COVID-19 protocols in schools, vowing to 'get them out of the way.'" Biden said, "Right now, local school officials are trying to keep children safe in a pandemic while their governor picks a fight with them and even threatens their salaries or their jobs. Talk about bullying in schools." He added, "If they'll not help, if these governors won't help us beat the pandemic, I'll use my power as president to get them out of the way."

Los Angeles School District Makes Student COVID-19 Vaccinations Mandatory.

The Los Angeles Times (9/9, Gomez, 3.37M) reports that all children 12 and older in Los Angeles public schools "must be fully vaccinated against COVID-19 by January to enter campus." LAUSD interim Superintendent Megan K. Reilly "said the student mandate was the next logical step to keep children, staff and community members safer from a COVID-19 pandemic that still poses significant risks."

The New York Times (9/9, Goldstein, 20.6M) reports the district's Board of Education "voted, 6-0, to pass the measure on Thursday afternoon," which "would eventually apply to more than 460,000 students, including some enrolled at independent charter schools located in district buildings."

The Washington Post (9/9, Meckler, Knowles, 10.52M) reports the new regulations require "students 12 and older

who participate in in-person extracurricular programs to receive their first shot of the Pfizer-BioNTech vaccine by Oct. 3 and their second by the end of that month." All other eligible "students must get their first dose by Nov. 21 and their second by mid-December."

The Wall Street Journal (9/9, Koh, Subscription Publication, 8.41M) reports students who are not vaccinated by January 10 will not be allowed on campus and must enroll in virtual learning.

Among other outlets covering the story are the AP (9/9, Taxin, Antczak), the CBS Evening News (9/9, 6:35 p.m. EST, story 2, 2:30, O'Donnell, 3.48M), NBC Nightly News (9/9, 6:34 p.m. EST, story 2, 1:50, Holt, 4.78M), Reuters (9/9, Allen, O'Brien), CNN (9/9, Andone, Chan, Mossburg, 89.21M), The Hill (9/9, Choi, 5.69M), Fox News (9/9, 23.99M), Forbes (9/9, Speakman, 10.33M), and The Hill (9/9, Vakil, 5.69M).

FDA Rejects Humanigen's Monoclonal Antibody As COVID-19 Treatment. Reuters (9/9, Bhalla) reports the FDA "declined Humanigen Inc's request for emergency use authorization (EUA) of its lenzilumab drug to treat newly hospitalized COVID-19 patients, the company said on Thursday." In a statement, Humanigen said, "In its letter, FDA stated that it was unable to conclude that the known and potential benefits of lenzilumab outweigh the known and potential risks of its use as a treatment for COVID-19." The company "expects an ongoing study to provide additional safety and efficacy data to support its new EUA request."

Among other sources covering the story are The Hill (9/9, Schnell, 5.69M), FierceBiotech (9/9, Adams, 4K), STAT (9/9, Herper, Subscription Publication, 262K), and Endpoints News (9/9, Brennan).

US COVID-19 Cases More Than 10 Times Higher Than What Is Needed To End Pandemic, Fauci Says. The New York Times (9/9, Anthes, Slotnik, 20.6M) reports, "Coronavirus infections are more than ten times higher than they need to be in order to end the pandemic, Dr. Anthony S. Fauci, the nation's top infectious disease doctor, told the political news site Axios." He said, "In a country of our size, you can't be hanging around and having 100,000 infections a day. ... That's not even modestly good control." Fauci added, "You've got to get well below 10,000 before you start feeling comfortable."

The Hill (9/9, Choi, 5.69M) and Axios (9/9, Drage O'Reilly, 1.26M) also cover the story.

TSA Doubles Fines For People Who Refuse To Wear Masks On Public Transit. The AP (9/9) reports the Transportation Security Administration "is doubling the

fine for people who break the rule requiring masks on planes, trains and other forms of public transit to slow the spread of COVID-19, with President Joe Biden warning Thursday that violators should 'be prepared to pay.'"

The Washington Post (9/9, Aratani, 10.52M) says, "Beginning Friday, the fine for refusing to wear a mask will increase to a range of \$500 to \$1,000 for first offenders," and "penalties for a second offense will range from \$1,000 to \$3,000." Meanwhile, the Department of Homeland Security extended the mask mandate until January 18.

Among other outlets covering the story are Bloomberg (9/9, Laing, 3.57M), Reuters (9/9, Shepardson), CNN (9/9, Wallace, Sullivan, 89.21M), and Forbes (9/9, 10.33M).

Government Counts Of COVID-19 Deaths In Nursing Homes Likely Missed More Than 16,000 Fatalities, Study Indicates. USA Today (9/9, Stein, 12.7M) reports, "Government counts of the devastation from coronavirus among the most vulnerable elderly likely missed more than 16,000 COVID-19 deaths in U.S. nursing homes during the early months of the pandemic, an academic study" has found. Additionally, researchers "estimated that 68,000 additional coronavirus cases – representing nearly 12% of last year's total nursing home cases – were omitted before a federal reporting requirement took effect in late May 2020."

The Hill (9/9, Weixel, 5.69M) reports the Trump Administration "first imposed a requirement for nursing homes to report weekly cases and deaths on May 24, 2020, through the Centers for Disease Control and Prevention's National Healthcare Safety Network (NHSN) system" but "nursing homes were not required to report retroactive data, and it's not known how many facilities chose to do so."

Forbes (9/9, McEvoy, 10.33M) also reports on the story.

WHO Warns COVID-19 Vaccine Supply In Africa Will Drop By 25% Partly Due To Booster Shots For Developed Countries. Axios (9/9, Knutson, 1.26M) reports, "The World Health Organization warned on Thursday that Africa will receive 25% fewer doses of the coronavirus vaccine than expected by the end of the year, in part because of the approval of booster shots in developed countries." The WHO's regional director for Africa, Matshidiso Moeti, "criticized developed countries for wasting vaccine doses and specifically referenced the more than 15 million doses that have been discarded in the U.S. since March." According to data from the Africa CDC, "just over 3% of the continent's population has been fully vaccinated against the virus."

The AP (9/9, Anna) reports, "The Africa CDC says 145 million vaccine doses have been procured across the

continent of 1.3 billion people, and 111 million of them, or 77%, have been administered.”

Forbes (9/9, Beer, 10.33M) reports, “Responding to Wednesday’s announcement that the United Nations-backed COVAX program has again been forced to reduce its delivery projections, Matshidiso Moeti, the WHO’s Africa Director, said the goal of vaccinating 10% of people in Africa by the end of September is no longer a realistic target.”

UK’s MHRA Approves COVID-19 Boosters, But Government Will Make Final Decision On Implementing Program. Reuters (9/9, Smout) reports Britain’s MHRA “on Thursday gave the go-ahead for Pfizer and AstraZeneca’s COVID-19 vaccine to be used as booster shots.” While “the final decision on whether there should be booster shots is for the government,” the agency’s “decision clears one hurdle along that path.” Meanwhile, the UK’s Joint Committee for Vaccination and Immunisation (JCVI) is also “discussing whether booster shots for the elderly and vulnerable are needed, with planning underway for a booster programme that could begin this month.”

Among other sources covering the story are Bloomberg (9/9, Ring, 3.57M), The Hill (9/9, Lonas, 5.69M), the Daily Mail (UK) (9/9, Craig, 4.11M), The Guardian (UK) (9/9, Walker, Davis, 5.53M), and BBC News (9/9, 876K).

Microsoft Employees’ Return To Offices In US Delayed Indefinitely As COVID-19 Cases Surge. The AP (9/9) reports, “Microsoft told employees Thursday that it has indefinitely delayed their return to U.S. offices until it’s safer to do so.” The software maker “had already postponed its planned return to the workplace from September to no earlier than Oct. 4, but now says the re-opening won’t be next month.”

Bloomberg (9/9, Bass, 3.57M) reports, “‘Given the uncertainty of Covid-19, we’ve decided against attempting to forecast a new date for a full reopening of our U.S. work sites in favor of opening U.S. work sites as soon as we’re able to do so safely based on public health guidance,’ the Redmond, Washington-based software maker said in a blog post Thursday. ‘From there, we’ll communicate a 30-day transition period that provides time for employees to prepare.’”

Among other news outlets covering the story are the Wall Street Journal (9/9, Tilley, Subscription Publication, 8.41M), the New York Daily News (9/10, Sapienza, 2.51M) and the Financial Times (9/9, Wells, Subscription Publication, 1.48M).

US Could Easily Reach Some Level Of Normalcy If All Residents Did Their Part, Expert Says. CNN (9/9, Caldwell, 89.21M) reports COVID-19 “hospitalizations for those 17 and under peaked in late

August at a rate approaching twice that of this year’s early winter surge, according to CDC data.” The rise “in pediatric cases is alarming some school officials, although one health expert said Wednesday that the best way to turn the corner was to keep taking the pandemic seriously.” Dimitri Christakis, Director of the Center for Child Health, Behavior and Development at Seattle Children’s Hospital, said, “People in this country have no appreciation of how fortunate we are and how easily we could return to some level of normalcy if everybody would just do their part, and I think it is unfortunately going to take sticks because we’re all out of carrots.” To end the pandemic, “preemptive vaccination remains the key, US Surgeon General Dr. Vivek Murthy told CNN’s Jim Acosta.”

Analysis: Pandemic Aid Provides Framework For Ending Hunger In The US. In an approximately 3,300-word article, Politico (9/9, Bottemiller Evich, 6.73M) writes that “the pandemic triggered a country-wide policy experiment aimed at keeping families fed and financially afloat. There have been big increases in food stamps and unemployment benefits. Three rounds of stimulus checks. Universal free meals at schools and new grocery benefits for kids who are learning virtually, or out of school during the summer. Hundreds of millions of food boxes flooded into churches and other nonprofits.” Politico continues by saying the “latest tranche of aid may carry the biggest bang yet: six monthly child tax credit payments that will be dispersed through the end of the year. The first two rounds of payments that went out in July and August fueled a dramatic reduction in the rate of American households with kids who report sometimes or often not having enough to eat in the past week, according to the Census Bureau.”

Scientific Guidance On Protecting Babies From Coronavirus Variants Highlighted. NPR (9/9, Simmons-Duffin, 3.69M) reports, “Babies can’t get vaccinated against COVID-19 yet – and the youngest age included in current vaccine clinical studies is six-months-old.” NPR highlights the latest scientific guidance “on how to protect a new baby from all variants of the coronavirus.” The NIH is mentioned.

Analysis: Vaccine Mandates Are “American Tradition,” But So Is Public Anger Over Them. In an analysis, the New York Times (9/9, Astor, 20.6M) says that from 1898 to 1903, as smallpox “and death reigned around them, some Americans declared that they would never get vaccinated and raged at government efforts to compel them.” If that “sounds familiar, well, there is nothing new under the sun: not years that feel like centuries, not the wailing and gnashing of teeth over masks, and not vaccine

mandates either.” As COVID-19 spreads, “the same pattern is emerging.” According to the Times, vaccine mandates “are a time-honored American tradition,” but “so is public fury over them.”

NIH Director Discusses Biden Administration’s COVID-19 Vaccination Effort, Disease’s Impact On Children During Interview.

During an interview on MSNBC’s All In (9/9, 251K), NIH Director Francis Collins discussed the Biden Administration’s COVID-19 vaccination effort and how the disease is impacting children. Collins said, “If you’re unvaccinated and you are therefore much more likely to get infected with Delta and be asymptomatic quite likely for a couple of days, you are spreading that around to others around you, including kids under 12 who can’t be vaccinated. So it’s not just about you.” He added, “If we really want to get through this, we’ve got to figure out how those 80 million people who still haven’t rolled up their sleeves can see this as an opportunity and a responsibility, not just about them.” Collins also stated that “if you want kids to be safe right now, the best thing to do is surround them with other vaccinated people.”

Fauci Discusses Biden’s Six-Point Plan To Combat COVID-19 During Interviews.

In an interview on CNN’s Anderson Cooper 360 (9/9, 840K), NIAID Director Anthony Fauci discussed President Biden’s new six-point program to combat COVID-19. Fauci said, “The data are overwhelming to show why it is so important if we want to get this outbreak under control in this country to get vaccinated. And the President is understandably frustrated, and that’s the reason why he came out with his six-point plan.” During the interview, Fauci was asked, “The President outlined an aggressive new testing plan, but just yesterday your colleague Dr. Francis Collins, Director of the NIH, said there’s still a problem distributing tests. How can that be?” He responded, “No, what the President was saying is that he’s using the Defense Production Act to get literally millions and millions of tests, many of them free for individuals. So the distribution will come.”

PBS NewsHour (9/9, 792K) reports on a different interview in which Fauci stated, “We have tried everything we can...to get people vaccinated. We have the solution within our grasp, within our power. We just need to implement it. And that is what the president has said tonight in his speech, that we are going to implement that. And if that means more mandates, so be it.” He added that he “would respectfully object to that particular appreciation or interpretation of this as being too late. It isn’t. It’s something that is important. And I’m very glad that the president did it when he did it.”

Biden Promises To Fill Gaps In School Funding That Result From Districts Opposing States To Bolster COVID-19 Measures.

NPR (9/9, Kamenetz, 3.69M) reports President Biden “announced new steps to encourage K-12 schools to mandate masks for all, require vaccines for employees and step up testing for COVID-19.” Some school “districts are opposing their states to strengthen COVID-19 safety measures.” The President “promises to fill in any gaps in funding that result.” According to the White House, “Local school districts will be able to apply to the Department of Education in the coming weeks to restore funding withheld by state leaders...when a school district implemented strategies to help prevent the spread of COVID-19 in schools.” NPR adds that “teachers and staff directly employed or funded by the federal government – including those at... Head Start and Early Head Start preschool programs for children in poverty – are now subject to a vaccine requirement.”

Biden Rails Against “Pandemic Politics.”

ABC World News Tonight (9/9, 6:38 p.m. EST, story 2, 1:10, Muir, 6.32M) reported President Biden “railed against what he called pandemic politics – the fights against masks and vaccinations.” Biden took “aim at local officials that he said are actively working to undermine the fight against COVID by opposing masks and vaccination mandates.” Biden stated, “I’ll get them out of the way.”

Biden Urges Large Entertainment Venues To Require COVID-19 Vaccination As Condition Of Entry.

USA Today (9/9, Della Cava, Ruggieri, 12.7M) reports President “Biden called upon the nation’s entertainment-industry executives Thursday to do their part to help beat back the raging COVID-19 pandemic by adopting admission requirements that could cut into their bottom line and spark partisan pushback.” Biden said during a speech, “To those of you running large entertainment venues, from sports arenas to concert venues to movie theaters, please require folks to get vaccinated or show a negative test as a condition of entry.” However, “it remains to be seen if Biden’s words will turn into action at those venues that have stood their ground in declining to require audiences to be vaccinated, with most simply suggesting that masks be worn during the show.”

Democratic Representative Proposes Proof Of COVID-19 Vaccination Or Negative Test Mandate For Airplane, Train Passengers.

The Washington Post (9/9, Flynn, Aratani, 10.52M) reports that on Thursday, Rep. Don Beyer (D-VA) introduced legislation “to require all domestic travelers to show proof of vaccination or a negative coronavirus test at airports or for Amtrak trips,

seeking to revive largely stagnant discussions among federal officials about more restrictive pandemic travel requirements.”

The Hill (9/9, Castronuovo, 5.69M) reports the legislation, named the Safe Travel Act, also applies to “all employees, contractors and subcontractors of Amtrak and commercial airlines.”

Department Of Education To Launch Program To Help Schools Penalized For COVID-19 Mitigation Policies.

Bloomberg (9/9, Querolo, Moran, 3.57M) reports, “The U.S. Department of Education will launch a program aimed at protecting school districts that have been penalized by their state over Covid-19 mitigation policies like mask mandates.” It “will allow local school districts to apply for grants to restore funding withheld as punishment for implementing strategies to prevent the spread of the coronavirus that are consistent with guidance from the Centers for Disease Control and Prevention.”

Opinion: Democrats Need To Focus On GOP’s “Extremist And Destructive” Behavior During COVID-19 Surge.

In an opinion piece for the Washington Post’s (9/9, 10.52M) The Plum Line, Greg Sargent writes, “Judging by the GOP’s continuing slide into extremist and destructive behavior in the face of a surging covid-19, electing more Republicans to positions of responsibility right now would likely mean more economic malaise, sickness, misery and death.” He adds, “A confluence of new factors is making it obvious that Democrats need to take on this argument much more forcefully, not just for the good of the party, but for the good of the country.” Sargent concludes, “Democrats should engage this forcefully...because the majorities who favor sensible collective action on behalf of public health need to hear officials giving voice to their aspirations for recovery.”

Psaki Defends Fauci, Denies Allegations He Lied About Wuhan Research Support.

Forbes (9/9, McEvoy, 10.33M) reports that on Thursday, White House Press Secretary Jen Psaki defended NIAID Director Dr. Anthony Fauci against new “attacks inflamed by a report published” Monday by The Intercept, which reveals “two previously unpublished grant proposals from the U.S.-based health organization EcoHealth Alliance that were funded by the NIAID to conduct bat coronavirus research at the Wuhan Institute of Virology in China.” Critics now “accuse Fauci of lying when he previously told Congress the NIAID had not funded ‘gain-of-function’ research at the Chinese lab, referencing a controversial method that involves researchers modifying a virus to predict its evolutionary trajectory.”

Fox News (9/9, Flood, 23.99M) correspondent Peter Doocy questioned “Psaki about the report during Thursday’s

press briefing.” She responded, “NIH has never approved any research that would make a coronavirus more dangerous to humans, a reminder that there are previous and different coronaviruses than the existing one we’re battling.”

Rep. Mike Gallagher Calls On Fauci To Resign.

Fox News (9/9, Nelson, 23.99M) reports that during “America’s Newsroom” on Thursday, Rep. Mike Gallagher (R-WI) “called on Dr. Anthony Fauci to resign after newly released documents showing the nation’s top infectious disease expert lied about the U.S. support for gain-of-function research.”

Major News Outlets Not Reporting On Recent Allegations Made About Fauci.

Fox News (9/9, Wulfsohn, 23.99M) reports recent “bombshell revelations” about NIAID Director Anthony Fauci “have received zero coverage on CNN, MSNBC, ABC, CBS and NBC throughout Tuesday and Wednesday, according to Grabien transcripts.” In addition, “The New York Times, The Washington Post, USA Today, and The Associated Press also appeared to skip the major development.”

CDC Changes Definitions Of “Vaccine” And “Vaccination.”

McClatchy (9/9, Camero, 29K) reports, “Social media is calling bluff on the Centers for Disease Control and Prevention for modifying its definition of the words ‘vaccine’ and ‘vaccination’ on its website.” Vaccination was defined as “the act of introducing a vaccine into the body to produce immunity to a specific disease,” but the CDC changed the word “immunity” to “protection.” Originally, a vaccine was “a product that stimulates a person’s immune system to produce immunity to a specific disease,” but it is now “a preparation that is used to stimulate the body’s immune response against diseases.” People are claiming the alterations are the “CDC’s attempt to hide the fact COVID-19 vaccines are not 100% effective at preventing coronavirus infection,” but “a CDC spokesperson told McClatchy News the ‘slight changes in wording over time...haven’t impacted the overall definition.’”

Some Democrats Believe COVID-19 Could Be Winning Issue In Upcoming Elections.

Politico (9/9, Korecki, Siders, 6.73M) reports, “The resurgence of Covid-19 is rapidly dominating Joe Biden’s administration, putting into question the future of his broader legislative agenda and increasingly steering Democrats into treacherous political territory” as midterm elections approach. However, “Democrats in some state races still believe Covid could prove a winning issue, painting their Republican challengers as dangerous because of their opposition to vaccinations and mask mandates – which polling shows a majority of Americans favor.”

Biden's Coronavirus Response Coordinator Discusses President's New COVID-19 Plan.

NPR (9/9, Chang, Handel, Yu, 3.69M) reports hosts Ailsa Chang and Scott Detrow spoke with White House Coronavirus Response Coordinator Jeff Zients on Thursday after President Biden "announced a variety of new rules, including several mandating vaccinations in many workplaces." Zients walked them "through the administration's strategy."

Fauci Says Government May Need To Eventually Put Pressure On People To Receive COVID-19 Vaccine.

NBC Nightly News (9/9, 6:36 p.m. EST, story 3, 2:15, Holt, 4.78M) interviewed NIAID Director Dr. Anthony Fauci about President Biden's "sweeping new [COVID-19] requirements." He hopes people will start getting vaccinated against COVID-19 willingly, but said "there will have to be things that will essentially have to put pressure" on those who refuse. He mentioned the inability to work at certain places or attend certain schools without vaccination as a way to motivate people.

Surgeon General, Education Secretary Discuss COVID-19's Effects On Children.

Fox News (9/9, Halon, 23.99M) reports that on Thursday, US Surgeon General Vivek Murthy and Education Secretary Miguel Cardona joined "Special Report" to discuss "some of the looming questions surrounding the [COVID-19] vaccine and President Biden's updated COVID-19 guidance." They addressed the effects COVID-19 has on children, natural immunity, and the science behind masks, among other topics.

CNN's Jake Tapper Says Biden Is Not Addressing COVID-19 Misinformation Problem.

Fox News (9/9, Wulfsohn, 23.99M) reports, "CNN anchor Jake Tapper expressed his frustration with President Biden following a White House address laying out his administration's six-pronged plan to tackle the coronavirus pandemic." He "seemed to suggest that the actions that the Biden administration is taking don't solve the real 'problem.'" Tapper said, "One of the biggest problems that the United States is having right now getting past this pandemic is that tens of millions of Americans are being lied to, are being misinformed, are being told a whole bunch of things that are contrary to what the medical and scientific establishment say is true in terms of what's the best way to combat this virus."

CDC Predicts COVID-19 Deaths Likely To Increase Over Next Four Weeks.

Newsweek (9/9, Khaled, 2.67M) reports, "The Centers for Disease Control and Prevention (CDC) on Wednesday said that COVID-19 deaths

in the U.S. are likely to increase over the next four weeks to reach between 683,000 and 710,000 by October 2." The agency "also said new daily COVID-19 hospitalizations are expected to remain stable or have an uncertain trend over the upcoming four weeks with 6,400 to 19,500 newly confirmed hospitalizations likely to be reported on October 4."

North Carolina Passes 15K COVID-19 Deaths.

The Raleigh (NC) News & Observer (9/9, Sessoms, 396K) reports, "North Carolina added 110 deaths due to COVID-19 to the state's tally on Thursday, bringing the total over the pandemic to 15,004."

Louisiana COVID-19 Cases "Exorbitantly High," State Health Officer Says.

The Baton Rouge (LA) Advocate (9/9, Sentell, 255K) reports, "While COVID-19 cases in Louisiana remain 'exorbitantly high,' the state appears to have peaked in its fourth surge of the deadly disease, state health officer Dr. Joseph Kanter said Thursday." He added, "The challenge here is given the disruption that [Hurricane Ida] presented us there is no guarantee we continue on the downward trajectory."

Washington State Requires Masks At Large Outdoor Events.

The AP (9/9, Corte) reports that on Thursday, Washington Gov. Jay Inslee (D) announced "that starting next week, the state's indoor mask mandate will be expanded to include outdoor events with 500 or more attendees, regardless of vaccination status."

Maryland School System Tightens COVID-19 Vaccination Requirements For Employees.

The Washington Post (9/9, St. George, 10.52M) reports, "Maryland's largest school system tightened coronavirus vaccine requirements for its 24,000 employees, deciding on Thursday to fully mandate the shots rather than allow the option of weekly testing." Employees must receive the first dose of the vaccine by the end of September and the second dose by October 29.

Iowa Judge Considers Whether To Stop School Mask Mandate Ban.

The AP (9/9, Pitt) reports that on Thursday, a lawyer representing a mother and her sons argued "Iowa Republican legislators and the governor prohibited mask mandates in schools for purely political reasons to appease complaining constituents." The hearing focused on whether the judge "should issue a temporary injunction to prevent the state from enforcing the law while the case proceeds." Frances Parr, the mother, "wants a court to order the state to issue a universal mask mandate for all students and school personnel until a voluntary plan can be implemented that separates mask-wearing students and staff from those who refuse."

West Virginia Democratic Senators Oppose Special Session On COVID-19 Regulations.

The AP (9/9) reports that on Thursday, West Virginia Democratic Senate leaders announced “they’re against a special session to consider bills that would bar schools and businesses from requiring COVID-19 vaccinations or other measures.”

New Mexico’s \$100 COVID-19 Vaccine Incentive’s Deadline Is Friday.

The AP (9/9) reports, “The New Mexico Department of Health says people who got a COVID-19 vaccine shot between Aug. 2-31 must sign up by Friday if they want to claim the \$100 incentive being offered to entice people to get inoculated.”

Democratic Lawmakers Question Amazon’s Algorithms Promoting COVID-19 Misinformation.

NPR (9/9, Bond, 3.69M) reports, “Amazon is under pressure from Democrats in Congress over how its algorithms promote hoax COVID-19 cures, including the livestock dewormer ivermectin, as well as anti-vaccination claims and other medical misinformation.” This week, Rep. Adam Schiff (D-CA) and Sen. Elizabeth Warren (D-MA) sent letters “to CEO Andy Jassy pressing for information on Amazon’s misinformation policies and what the company is doing to stop its systems from recommending books and other products linked to falsehoods about the pandemic and vaccines.” Amazon spokesperson Tina Pelkey said, “We are constantly evaluating the books we list to ensure they comply with our content guidelines, and as an additional service to customers, at the top of relevant search results pages we link to the CDC advice on COVID and protection measures.”

Mississippi Records 72 Stillbirths In Pregnant Women Unvaccinated Against COVID-19.

The Washington Post (9/9, Shamas, 10.52M) reports, “Mississippi has recorded 72 fetal deaths in unvaccinated pregnant women infected with the coronavirus, state health officials announced Wednesday, sounding the alarm on the virus’s danger in pregnancy.” Mississippi State Health Officer Thomas Dobbs and other state health leaders are urging pregnant women to receive the vaccine. CDC Director Rochelle Walensky said, “The vaccines are safe and effective, and it has never been more urgent to increase vaccinations as we face the highly transmissible Delta variant and see severe outcomes from covid-19 among unvaccinated pregnant people.”

Forbes (9/9, Porterfield, 10.33M) also covers the story.

CNN Crew Tells Story Of Spending Time In Rural Missouri County With 27% COVID-19 Vaccination Rate.

CNN (9/9, Reeve, Guff, Brunswick,

89.21M) chronicles the story of a CNN crew that spent five days in Carter County, Missouri. The county has around 6,000 people and a COVID-19 vaccination rate of 27%. Brandon Helvey said, “Everybody’s scared. Everybody’s coming down with it. And it’s almost like a plague.” Yet neither he, nor the majority of residents, will get vaccinated because things are still “unproven.” The crew spoke with other locals to understand why they refuse to get vaccinated when the virus is prevalent in the community.

GOP Senator Recommends Sanctions, Immigration Control Over COVID-19 Spread.

Newsweek (9/9, Ellington, 2.67M) reports that on Thursday, Senator Roger Marshall (R-KS) “released an eight-point strategy regarding the COVID-19 pandemic” and “recommended sanctions and immigration control due to ‘substantial evidence that COVID-19 was spreading throughout China in September or October of 2019.’”

Columnist: Masks Are More About Politics Than Pandemic.

In his column for the New York Post (9/9, 7.45M), Glenn Reynolds writes, “Masks, as Twitter wags have noted, are by now the equivalent of MAGA hats for Blue America.” He adds, “We’re so politically tribalized that even our response to the pandemic says more about politics than about anything else. That’s especially true when it comes to masking.” Reynolds concludes, “A more mature society, with a more responsible media and more sensible politicians, would address matters more rationally and less tribally. We do not live in such a society.”

Former HHS Emergency Preparedness Chiefs Suggest Integrating Public Health System Into Medical Care Delivery.

Behind a paywall, Bloomberg Law (9/9, Baumann, Subscription Publication, 4K) reports, “The U.S. must better integrate the public health system into the delivery of medical care as the nation looks to overhaul its response to future outbreaks in light of the Covid-19 pandemic, former HHS emergency preparedness chiefs said.”

Researchers At Harvard, BU Receive NIH Award To Study Links Between COVID-19 Vaccines, Changes In Menstruation.

The Boston Globe (9/9, Finucane, 1.04M) reports Harvard Medical School and the Boston University School of Public Health are among “five institutions...awarded a total of \$1.67 million by the National Institutes of Health to look into...anecdotal reports from women earlier this year that after they were vaccinated [against COVID-19], they saw changes, including earlier, heavier, and more painful periods.” NIH Institute of Child Health and Human Development Director Diana Bianchi said,

"Our goal is to provide menstruating people with information, mainly as to what to expect, because I think that was the biggest issue: Nobody expected it to affect the menstrual system because the information wasn't being collected in the early vaccine studies." The National Institute of Child Health and Human Development and the NIH's Office of Research on Women's Health are funding the research.

Pandemic Causing Disability Service Providers In Many States To Close, Survey Finds. Disability Scoop (9/10, Diament) reports, "Half of states are reporting that Medicaid home and community-based services providers have closed since the start of the pandemic, with many seeing multiple types of services shut their doors." A survey, conducted through mid-July, of Medicaid home and community-based services programs in 41 states found "that the pandemic exacerbated existing workforce shortages at group homes and for services provided in individuals' homes, but there were also impacts on providers from people with disabilities and their families declining to allow workers into their homes due to coronavirus concerns."

Maryland Permits Seniors In Some Group Homes To Receive COVID-19 Booster Shot. Disability Scoop (9/10, Wood) reports, "Maryland is moving forward with allowing COVID-19 vaccine booster shots for older adults and others living in nursing homes and other group settings, even though the federal government has not yet issued formal rules for when most Americans should get them." Gov. Larry Hogan (R) announced everyone aged 65 or older living "in nursing homes, assisted living centers, residential treatment centers and group homes for people with disabilities" is automatically eligible for the booster shot.

US Officials Reauthorize Use Of Lilly's COVID-19 Antibody Combination Therapy. Endpoints News (9/9, Brennan) reports in continuing coverage that US officials have reauthorized Lilly's COVID-19 antibody combination therapy bamlanivimab and etesevimab nationwide. According to the article, "Based on the most currently available data, bamlanivimab and etesevimab are now authorized in all U.S. states, territories, and jurisdictions," the FDA said yesterday, noting in a fact sheet that the combo retained activity against the Delta variant."

EMA Approves German Sites In Boost To Pfizer COVID-19 Vaccine Supply. Reuters (9/9, Aripaka) reports that the European Medicines Agency "on Thursday approved an increase in manufacturing capacity for the COVID-19 vaccine from Pfizer/BioNTech, which would help produce up to 50 million additional doses this year." Both of the sites approved by the EMA are located in Germany.

Sinovac To Trial COVID-19 Vaccine In South African Children Aged 6 Months To 17 Years As Part Of Global Study. Reuters (9/9, Winning) reports, "China's Sinovac Biotech...will trial its COVID-19 vaccine in children and adolescents" aged 6 months to 17 years "in South Africa as part of a global Phase III study, Sinovac and local partner Numolux Group said on Thursday."

AstraZeneca Amends COVID-19 Vaccine License Deal With Chinese Biotech. Endpoints News (9/9, Sullivan) reports, "AstraZeneca has amended its license agreement with" Chinese biotech Biokangtai to commercialize its AAV Covid-19 vaccine "in mainland China, Pakistan and Indonesia, Biokangtai announced Wednesday." According to the article, "Biokangtai will market, manufacture and distribute the drug to ease the shortened supply and make the vaccine more accessible."

Physician's Group Suing Over FDA's Revoked EUA For Hydroxychloroquine Loses Appeal. Bloomberg Law (9/9, Barker, Subscription Publication, 4K) reports the US Court of Appeals for the Sixth Circuit has ruled that the Association of American Physicians & Surgeons cannot sue over the scope of the FDA's "now-revoked emergency use authorization for hydroxychloroquine." According to Bloomberg Law, "The association's claims that members couldn't prescribe, and that patients were unable to obtain, the drug for use as a prophylaxis or as an initial treatment after a Covid-19 diagnosis don't plausibly plead any injury to its members, the court said."

EMA Flags AstraZeneca COVID-19 Vaccine For Rare Guillain-Barre Syndrome. FiercePharma (9/9, Dunleavy, 12K) reports that the European Medicines Agency has flagged the AstraZeneca COVID-19 vaccine for the rare nerve disorder Guillain-Barre syndrome (GBS). The EMA "will now list GBS as a possible and 'very rare' side effect of Vaxzevria, which is administered as a two-dose regimen." According to FiercePharma, "Of the 592 million doses of the vaccine administered worldwide through the end of July, 833 cases of the nerve-damaging condition were reported."

CMS Expands COVID-19 Vaccine Mandate To All Facilities Participating In Medicare And Medicaid. Fierce Healthcare (9/9, King, 150K) reports, "The Centers for Medicare & Medicaid Services announced Thursday its intention to release an interim final rule in October to require hospitals, dialysis facilities, ambulatory surgical centers and other facilities to vaccinate their staff" against COVID-19 "as a condition for participating in Medicare and Medicaid." The announcement comes as "more

than 100 hospitals and health systems have already implemented vaccination mandates for staff" but "lower vaccination rates in several other facilities prompted the agency to expand the mandate."

Southeastern Missouri Now "Epicenter" Of State's COVID-19 Outbreak. The AP (9/9) reports, "The epicenter of Missouri's COVID-19 outbreak has shifted to the state's southeast, and no place is getting hit harder than Sikeston." State tracking showed "eight of the nine hardest-hit counties over the past week are in the southeastern corner of the state."

Researchers Examine The First COVID-19 Deaths In The US. The New York Times (9/9, Mueller, 20.6M) reports that last year, the CDC kicked off a "quiet, yearlong campaign at the agency to check and recheck the country's first suspected Covid-related deaths in the uncertain days of early 2020." Research shows "at least four possible Covid-19 deaths from January 2020 have survived" the vetting and they "have begun drawing more attention as scientists and intelligence officials try to unravel how the pandemic began."

Younger Californians Who Are Unvaccinated Are Increasingly Getting Hospitalized With COVID-19. The Los Angeles Times (9/9, Lin, Money, 3.37M) reports that in California, "younger people are being vaccinated" against COVID-19 at lower rates and the "age gap is causing growing concern among healthcare professionals and bringing new calls to get younger people vaccinated." These "unvaccinated people who are younger and otherwise healthier" are "filling hospital beds." While some "demographic groups that were hit hard earlier in the pandemic" like Native Americans are "now reporting higher vaccination rates," younger people are still proving more hesitant.

Kentucky's COVID-19 Surge Is Pushing Hospitals To Their Limits. CNN (9/9, Vera, Marquez, Kapp, 89.21M) reports that because of vaccine hesitancy, "Covid-19 has run rampant in Kentucky, where nearly all of the state's 120 counties are in the 'red' Covid-19 zone and hospitals" are "on the brink of being overwhelmed, according to Gov. Andy Beshear." State lawmakers voted to extend Kentucky's state of emergency until January due to the surge. On Thursday, the governor "said 60 of the state's 96 hospitals are reporting staff shortages, and there are only 90 available adult ICU beds statewide, the fewest amount available throughout the pandemic." The surge "illustrates that big, urban hospitals aren't the only ones being bombarded by patients."

The AP (9/9, Blackburn) reports the governor "added the limited number of ICU beds also will put those in non-virus related emergencies in danger of not receiving care, such as people in bad car accidents."

North Carolina Health Officials Say Middle And High School Sports Are Leading To COVID-19 Clusters. CNN (9/9, Simon, 89.21M) reports, "Forty-five percent of all Covid-19 clusters in North Carolina's middle and high schools since July have been among sports teams, according to the North Carolina Department of Health and Human Services (NCDHHS)." The department "said the numbers underscored an urgent need for vaccination among school children as soon as possible." The announcement comes as the "Centers for Disease Control and Prevention and the American Academy of Pediatrics (AAP) have advised that sports can play a role in the spread of Covid-19 among students, especially those who are unvaccinated."

Idaho Hospitals May Be Forced To Ration Healthcare As Students Return To School. The AP (9/9, Boone) reports, "When Idaho public health officials announced this week that northern hospitals were so crowded with coronavirus patients that they would be allowed to ration health care, roughly 11,000 kids" were preparing for their first day of school. Vaccination rates in Idaho "remain among the lowest in the U.S., and coronavirus cases have grown by 44% in the last two weeks as the highly contagious delta variant burns through the population." State officials expressed concern that there will not be enough beds for the growing surge.

San Francisco Schools "Defy National Trends," See No COVID-19 Outbreaks. The San Francisco Chronicle (9/9, Tucker, 2.44M) reports, "No COVID-19 outbreaks have occurred in San Francisco schools since they reopened to in-person learning in mid-August and case rates have remained steady among young children in recent months, even as the highly contagious delta variant has spread, according to data released Thursday by the Department of Public Health." The numbers "defy national trends that have shown large rises in cases and hospitalizations among school-aged children overall during the delta surge."

Colorado Sees Rise In Children's COVID-19 Cases Since Beginning Of School Year. The Denver Post (9/9, Seaman, 660K) reports, "COVID-19 cases in Colorado's school-aged children more than doubled in the past three weeks as a new school year began, with the increase most 'notable among children ages 6 to 11 – a group that is not yet eligible for a COVID-19 vaccine.'" Even

though “most children don’t experience severe COVID cases, there is a concern more infections will mean more hospitalizations at a time when hospitals are already ‘under significant strain’ with an uptick in other respiratory viruses that don’t normally circulate this time of year.”

Pediatric Hospitalizations Increase Across US, Overwhelm Children’s Hospitals. The New York Times (9/9, Leatherby, Walker, 20.6M) reports, “Just as millions of families around the United States navigate sending their children back to school at an uncertain moment in the pandemic, the number of children admitted to the hospital with Covid-19 has risen to the highest levels reported to date.” An increase in infections has driven pediatric hospitalizations “across the country, overwhelming children’s hospitals and intensive care units in states like Louisiana and Texas.” This strain on resources “has prompted doctors and hospital executives to plead with adults to get vaccinated and return to mask wearing and social distancing to protect children, especially those under 12, who cannot yet be vaccinated.”

Rep. Schiff Calls On Facebook, Amazon To Provide Details About Efforts To Curb COVID-19 Vaccine Misinformation. Reuters (9/9, Shepardson, Heavey) reports, “U.S. Democratic Representative Adam Schiff called on Facebook Inc and Amazon.com Inc on Thursday to provide a more thorough explanation of their efforts to mitigate the spread of COVID-19 vaccine misinformation.” Some “other companies, including Twitter Inc and Alphabet Inc’s Google and YouTube, have also faced criticism for allowing false information on COVID-19, including vaccines, to proliferate.”

Battle Over COVID-19 Policies Brewing At UT Austin. The Wall Street Journal (9/9, Belkin, Subscription Publication, 8.41M) reports that because of prohibition by Republican state policy makers, the University of Texas at Austin does not require masking, surveillance testing, or vaccines. Professors and graduate students are unhappy about the situation. Hundreds of them signed a petition demanding that the school impose tougher COVID-19 protection measures.

Qantas To Require International Passengers Be Vaccinated Against COVID-19. The New York Times (9/9, Murphy, 20.6M) reports, “Qantas, Australia’s largest airline, will require that all passengers on international flights are vaccinated against the coronavirus when it restarts worldwide operations in December, its chief executive said Wednesday, making it one of the first airlines in the world to require proof of vaccination for everyone on board.” The

airline, “which is headquartered in Sydney, suspended international operations during the pandemic – but did resume flights to New Zealand in April this year before suspending them again on July 31.” Qantas “plans to restart flights abroad in December.”

“Remarkable” Ability Of Coronavirus Delta Variant To Replicate Tossing New “Twists” Into The Pandemic. STAT (9/9, Joseph, 262K) reports, “One of the key reasons the Delta variant has ignited new surges of COVID-19 infections across the United States is its remarkable ability to make copies of itself.” That remarkable ability “has helped make Delta far more transmissible than any other iteration of the coronavirus seen thus far.” The ability to replicate “could also be at the heart of the other twists Delta has thrown into the pandemic, including the increase in breakthrough infections with the variant and why it potentially causes severe COVID-19 more often.”

“Religious” Exemptions Add Potential Legal Obstacles To Looming COVID-19 Vaccine Mandates. Kaiser Health News (9/9, Kreidler) reports, “With workplace vaccine mandates in the offing, opponents are turning to a tried-and-true recourse for avoiding a COVID-19 shot: the claim that vaccination interferes with religious beliefs.” Legal experts say if “a person claims their privately held religious beliefs forbid vaccination, that defense is unlikely to hold up in court if challenged.” But the EEOC “grants broad leeway to what constitutes a sincerely held religious belief,” meaning “some experts predict most employers and administrators won’t want to challenge such objections from their employees.”

Analysis: Average COVID-19 Hospitalization Costs Medicare 150 Times More Than It Does To Vaccinate One Individual. CNN (9/9, McPhillips, Sealy, Andone, 89.21M) reports, “A Medicare beneficiary hospitalized with Covid-19 costs the health insurance program about 150 times more than it does to fully vaccinate that individual, a CNN analysis shows.” The “vaccines are provided at no direct cost to residents of the United States, but Medicare reimburses health care providers up to \$150 to fully vaccinate beneficiaries with both doses of the two-dose regimen vaccines by Pfizer/BioNtech and Moderna, per billing documents published by the US Centers for Medicare and Medicaid Services. That’s \$40 for each dose administered and \$35 to administer the shots at the individual’s home or group living setting.” On the other hand, “the average cost to hospitalize a Medicare beneficiary with COVID-19 is \$21,752 over an average stay of 9.2 days, according to research published in August in the *Annals of Internal Medicine*.”

Trump Urges Americans To Consider Getting COVID-19 Vaccines.

Fox News (9/9, Creitz, 23.99M) reports during an interview with Fox News host Greg Gutfeld, former President Trump “urged Americans to consider getting vaccinated, after Gutfeld told him his wife remains hesitant as many others in the country do.” Trump said, “I got vaccinated and I feel very good, and I think we saved millions and millions of people worldwide [with the vaccine development]...comparing the COVID-19 death toll to the 1918 Spanish Flu pandemic, which he said killed 100 million people.”

More Patients With COVID-19 Report Suffering From Long-Term Symptoms, Study Indicates.

The Hill (9/9, Coleman, 5.69M) says a CDC study indicates “people who tested positive for COVID-19 reported having long-term symptoms 1.5 times more frequently than those who tested negative.” Almost “66 percent of people who tested positive for the virus reported at least one of their COVID-19-related symptoms lasted longer than four weeks, compared to 42.9 percent of those who always tested negative,” said a survey conducted by Porter Novelli Public Services from April 9-23.

Job Postings With COVID-19 Vaccination Requirements Doubled In August, Indeed Says.

The Hill (9/9, Lane, 5.69M) reports, “Job postings that require applicants to be vaccinated against COVID-19 skyrocketed in August amid a sharp increase in coronavirus cases, according to data released Thursday by hiring website Indeed.” In the week “ending Aug. 30, the share of job postings on Indeed that required COVID-19 vaccinations rose 119 percent from the same period in July. Job postings that mentioned a general vaccination requirement, but not COVID-19 specifically, rose by 242 percent over the same period.”

US News & World Report (9/9, 1.91M) says, “According to Indeed, a small batch of job postings recommend vaccinations but stop short of requiring them, while other postings ask potential employees to verify their vaccination status by uploading their vaccination card, and others have offered vaccine bonuses to new hires.”

Workers Slow To Return To In-Person Jobs, Bureau of Labor Statistics Data Show.

The Stateline (9/9, 2K) reports, “Hospitality workers and others who can’t telework are key to job recovery,” however, “despite a sudden spike in demand for their services in a reopened economy, many are reluctant to return to the workplace.” Jobs are “down about 9% in Connecticut and Vermont from pre-pandemic levels as of July, and down significantly in Hawaii (8.1%), Illinois (6.9%), Maryland

(6.7%), New Jersey (6.1%) and Rhode Island (6.1%), according to a Stateline analysis of Bureau of Labor Statistics data.” In The US, “hospitality job growth halted in August and is still down 1.7 million jobs from before the pandemic, according to a federal jobs report released Sept. 3.”

New Mexico Health Officials Investigating Possible Fatal Poisoning From Ivermectin Used To Treat COVID-19.

The Las Cruces (NM) Sun-News (9/8, D’Amassa, 85K) reports, “New Mexico’s acting Health Secretary came down hard Wednesday on unfounded claims that the drug Ivermectin is a suitable treatment for COVID-19 disease.” State Human Services Secretary Dr. David Scrase, “temporarily heading the state health department as well, said” during a weekly update on COVID-19 cases and response that “clinicians were investigating what was likely the state’s first fatal case of an individual dosing themselves with the drug, while a suspected second case was in critical condition.” Scrase “stressed that it might take weeks to confirm that ivermectin was the cause of the individual’s death, but his expectation was that it would be confirmed, while another severely ill patient was suspected of taking the drug.”

Pair Of Penn Scientists Wins \$3M Breakthrough Prize For RNA Research That Led To Creation Of COVID-19 Vaccines.

The Philadelphia Inquirer (9/9, Avril) reports, “Sixteen years after their research at the University of Pennsylvania paved the way for billions to be vaccinated against COVID-19, two scientists have been honored with a \$3 million Breakthrough Prize.” The prize “for Katalin Karikó and Drew Weissman, one of five such honors announced Thursday for achievements in science and math, recognizes their success in modifying the genetic molecule called messenger RNA (mRNA) so it can instruct human cells to make customized proteins.”

Forbes (9/9, Knapp, 10.33M) reports “Weissman isn’t resting on his accomplishments.” The “team is working to understand how the coronavirus that causes Covid-19 is changing so that pharmaceutical companies are better able to vaccinate against future variants of the disease,” and “is also focused on developing a vaccine that could help prevent future coronavirus pandemics.”

Latinos Have Higher COVID-19 Vaccination Rates In Major Metropolitan Areas.

Axios (9/9, Gonzalez, 1.26M) reports, “The number of Latinos getting vaccinated against the coronavirus in some of the most populated cities in the U.S. is slowly increasing.” In New York City, “Latinos have surpassed whites in the percentage of fully vaccinated adults.” In Houston, “the Houston Health

Department has administered approximately 32% of its vaccines to Latinos and 28% to whites, as of Wednesday."

Review Gives Prisons In Arizona An F For COVID-19 Pandemic Response. The Arizona Republic (9/9, Jenkins, 1.05M) reports, "Arizona gets an F for its response to the COVID-19 pandemic in state prisons." At least, "according to a recent nationwide review conducted by the Prison Policy Initiative, a nonpartisan advocacy group." Wanda Bertram, a Prison Policy Initiative spokesperson, "says Arizona was one of 13 states that did not adopt any policies aimed at reducing prison populations, such as accelerating release dates for those near the end of their sentence or expanding compassionate and medical releases."

Parents Of Children Who Need Home Care Have Tough Time Finding Nurses Vaccinated Against COVID-19. Bloomberg (9/9, Edney, 3.57M) reports, "A shortage of vaccinated home-care nurses has some parents of children with conditions like cerebral palsy or severe epilepsy giving up sleep and their own mental health to ensure their kids keep breathing at night." An industry group "estimates a vaccination rate as low as 40% among the industry's 2 million nurses, who work in a broad variety of specialties." A small number "of states require in-home nurses to be vaccinated against COVID-19, and the Biden administration hasn't created a federal mandate."

As COVID-19 Surges, NFL Opens Regular Season To Full Stadiums. The AP (9/9, Maaddi) reports, "When Tom Brady runs out from the tunnel, screaming 'Let's Go!' and leading the defending Super Bowl champion Buccaneers onto the field Thursday night against the Dallas Cowboys, a full stadium will greet players for the first time since COVID-19 upended the world and changed the way sports were viewed." Fans are returning "as COVID-19 surges, with about 150,000 new cases daily." The coronavirus "delta variant is filling hospitals, children are getting sick, and some schools are abruptly switching back to remote learning because of outbreaks."

Commentary Criticizes News Outlets For Running Phony Story About Ivermectin Overdoses In Oklahoma. Erik Wemple writes in the Washington Post (9/9, 10.52M) that "a number of prominent news outlets couldn't resist a report last week from Oklahoma City-based TV station KFOR with the headline: 'Patients overdosing on ivermectin backing up rural Oklahoma hospitals, ambulances.'" Media outlets such as The Hill, the Guardian, Rolling Stone, DailyMail.com, the New York Daily News and MSNBC's Rachel Maddow "bit hard." But "after the

story went viral, two hospitals in eastern Oklahoma – Northeastern Health System-Sequoyah and Integris Grove Hospital – issued statements denying that they had been overrun by ivermectin users." Wemple says while Fox News "dominates the playing field of false, racist and damaging news coverage in the United States," there "should be no minimizing the two-step horror of what...Maddow-Daily News-Rolling Stone and others did in this case."

Connecticut Officials Express Concern About Inconsistent Mask Wearing After Latest COVID-19 Infections. The AP (9/9) reports, "Connecticut officials expressed concerns Thursday about inconsistent mask wearing and people shunning coronavirus vaccines after 37 people became infected in outbreaks at a summer camp, group home and a party." Acting Health Commissioner Deidre Gifford and Gov. Ned Lamont "said most of the people who became infected were not vaccinated and some of them spread the virus to relatives who did not go to those locations." Each of the outbreaks occurred in August.

Hospital Leaders In Michigan Renew Plea For People To Get Vaccinated Amid Staff Shortages, COVID-19 Deaths. The AP (9/9, Eggert) reports, "Michigan health and business officials on Thursday renewed their plea for people to get vaccinated against COVID-19, citing hospital workforce shortages, unnecessary deaths and concerns that end-of-summer travel and the return to school could fuel a surge in cases." Some "59% of Michigan residents ages 12 and older have been fully vaccinated and 65% have gotten at least one dose." These figures "trail behind the national rates of 62% and 73%," respectively.

The Detroit Free Press (9/9, 2.16M) reports, "Michigan's hospitals face a critical staffing shortage that has neared a crisis point as they also struggle to manage a growing number of patients with COVID-19, the CEO of the state's hospital association said Thursday."

Some Students In Pennsylvania Using Loophole To Avoid Masking. The AP (9/9, Rubinkam, Scolforo) reports, "An apparent loophole in Pennsylvania's mask mandate for schools is making it easier for some students to go to class without having to cover their faces, even as state education regulators sought to make an example of one openly defiant school board." The order by the state health secretary "requiring masks to be worn inside K-12 school and child care facilities, which took effect Tuesday, includes an exemption for students who claim it would cause or worsen a medical condition."

Iowa To Use Federal COVID-19 Relief Funds For \$3.7M Ad Campaign To Promote The State.

The AP (9/9) reports, "Iowa will use federal coronavirus relief funds to pay for a \$3.7 million national ad campaign that promotes the state as a destination for visitors and workers, an agency spokeswoman said Thursday." The advertisement "is part of a larger 'This Is Iowa' promotional campaign and will air on cable television, online on social media sites and through streaming services, said Staci Hupp Ballard, spokeswoman for Iowa Economic Development Authority." Ballard said "funding for the campaign will come from federal American Rescue Plan funding."

Kentucky Legislators Move Closer To Scrapping Statewide Mask Mandate In Public Schools.

The AP (9/9, Schreiner) reports GOP "lawmakers in Kentucky moved closer Thursday to scrapping a statewide mask mandate in public schools, advancing a measure to shift masking decisions to local school boards as a surging COVID-19 outbreak threatens to overwhelm hospitals."

Editorial Criticizes Florida Governor's Comments About Vaccines.

The Orlando (FL) Sentinel (9/9, 599K) editorializes, "For a guy who claims to follow the science, Gov. Ron DeSantis has a shaky grasp of how vaccines work. Consider his recent boneheaded statement about vaccines at a news conference last week: 'It's about your health and whether you want that protection or not. It really doesn't impact me or anyone else.'" The paper adds that NIAID Director Dr. Anthony Fauci, "a real scientist who[m] DeSantis mocks on campaign merchandise, had this to say in response: 'Yeah, that's not true at all.'"

Republicans On Michigan House Oversight Committee Say State Unemployment Agency Failed Residents Amid Pandemic.

The AP (9/9, Nichols) reports, "Republicans in the Michigan House Oversight committee criticized the state's Unemployment Insurance Agency acting director on Thursday, asking her to address how the agency has failed residents during the COVID-19 pandemic." Rep. Steve Johnson, the committee chair, "citing what he's heard from his constituents, went back and forth with Estlund Olson throughout the meeting to ask her to address what he calls 'failures' by the agency." The reported failures "include slow payment of benefits and the nearly 700,000 residents who received notifications that they may have to repay their Pandemic Unemployment Assistance from the past 18 months."

Japan Extends COVID-19 State Of Emergency Until End Of September.

The AP (9/9, Yamaguchi) reports, "Japanese Prime Minister Yoshihide Suga on Thursday announced an extension of a coronavirus state of emergency in Tokyo and 18 other areas until the end of September, saying healthcare systems remain under severe strain, and that the continuing challenges of fighting the virus had led to his decision not to seek another term." Originally scheduled to end Sunday, "the state of emergency...was issued first in Okinawa in May and gradually expanded and extended as the country prepared to host the Olympics."

The Hill (9/9, Castronuovo, 5.69M) reports the "pace of vaccinations in Japan has ramped up in recent months, with about 56 percent of the country's total population fully vaccinated."

Italian Anti-Terrorism Police Raid Homes Of People Advocating Violence Over Government COVID-19 Vaccine Requirements.

The AP (9/9, D'Emilio) reports, "Italian anti-terrorism police on Thursday raided the homes of eight people who allegedly advocated violence, particularly against journalists, in upcoming protests in Rome and elsewhere against the government's COVID-19 vaccine requirements, officials said." These people "had formed a group called 'the warriors' on the Telegram messaging app, police said," adding that "they are under investigation but have not been arrested or formally charged."

COVID-19 Incidence Drops Below "High Risk" Level In Spain, Data Show.

Reuters (9/9) reports, "Spain's coronavirus incidence slipped below 150 cases per 100,000 people – a threshold the Health Ministry considers a 'high risk' of contagion – for the first time in more than two months on Thursday." Health ministry data show that "the indicator, measured over the past 14 days, fell to around 140 cases from 150 the previous day, a dramatic change from a record 900 cases per 100,000 people at the end of January." ICU occupancy rates "also slipped to 13.7% compared to 14.36% reported on Wednesday and 21.98% just a month ago."

Experts Suggest "Bolder" COVID-19 Strategy As Singapore Halts Reopening.

Reuters (9/9, Aravindan) reports, "Singapore is ramping up COVID-19 testing and has warned it would not rule out reimposing tighter curbs as infections rise – moves some experts see as too cautious for the vaccine frontrunner that is preparing to live with the virus as an endemic disease." Upset "by the fast pace of transmission, the government has said it would hold off on reopening measures and urged people to reduce social activities, sparking a debate among experts on if Singapore

could 'be bolder' given it has one of the highest vaccination rates in the world."

Italy Adds More Job Groups For Which COVID-19 Vaccination Will Be Required.

Reuters (9/9) reports, "The Italian government ruled on Thursday that catering and cleaning staff in schools and nursing homes can only work if they have proof of COVID-19 immunity, extending mandatory vaccination and the use of the so-called 'Green Pass' document." The document "was already required for teachers in Italy, while mandatory vaccination for health workers was introduced in March." The Italian "government said on Thursday that under the new rules people working in schools in any capacity must have the health document, and that all nursing home staff will have to be vaccinated."

COVID-19 Variants In Africa Could Worsen Pandemic, Scientists Warn.

Bloomberg (9/9, Sguazzin, 3.57M) reports, "The proliferation of COVID-19 variants in Africa, partly attributed to the low rates of vaccination on the continent, could lead to vaccine-evading mutations that complicate attempts to end the pandemic, a group of 112 African and 25 international organizations said." Published in the journal *Science* on Thursday was "a study of genomes from 33 African nations and two 'overseas territories'" that "tracks the evolution of the pandemic across the continent and the emergence of a number of so-called Variants of Concern and Variants of Interest."

Analysis Examines Why COVAX Has Fallen Behind In Its Efforts To Distribute COVID-19 Vaccines To Poorer Countries.

TIME (9/9, Ducharme, 18.1M) reports, "In March 2020, as the COVID-19 virus continued to spread across the planet, Dr. Richard Hatchett wrote a white paper laying the groundwork for a solution: a global initiative to support the development of COVID-19 vaccines through advance agreements with numerous pharmaceutical companies, and to distribute shots to countries around the world, whether or not those nations could pay for them." But Hatchett's concept had flaws. "The very issues that Hatchett feared – vaccine nationalism, self-interest, unequal access to limited supply – soon scarred COVAX's idealistic facade." Earlier this week, COVAX announced it would miss its COVID-19 vaccine goal for this year by about 500 million doses.

Australia's Victoria State Records Highest Single-Day Rise In COVID-19 Cases As Regions Exit Lockdown.

Reuters (9/10, Jose) says, "Australia's Victoria state reported its biggest single-day rise in COVID-19 cases this year, the majority in Melbourne, as most other regions in the state exited lockdown on Friday."

Plans were also announced "to bring Melbourne and Sydney out of extended lockdowns in coming weeks, despite infections continuing to rise in both of the country's two biggest cities." The strategy shift "of living with, rather than suppressing, the virus after hitting national vaccine coverage of 70-80% is part of a four-stage national reopening plan unveiled by the federal government in July."

Attorney Demands Kansas School District Change Its COVID-19 Policies.

The AP (9/9) reports that a lawyer "who has filed lawsuits challenging mask mandates in two Kansas counties is demanding that a Kansas City-area school district revise its COVID-19 policies, arguing that they violate state and federal laws." Attorney Ryan Kriegshauser "said continuing to enforce the policies would be 'evidence of willful illegal conduct' against students."

Florida Governor's Lawyers File Emergency Appeal On School Mask Mandates.

CNN (9/9, Simon, 89.21M) reports that lawyers for Gov. Ron DeSantis (R-FL) "have filed an emergency appeal with the First District Court of Appeals in Florida in hopes of halting school mask mandates while the case goes through the court system." DeSantis' lawyers "argue they 'have a high likelihood of success on appeal. Therefore, the trial court abused its discretion in vacating the automatic stay, and this Court should reinstate the stay pending review,' according to a court document."

Also reporting is USA Today (9/9, Saunders).

California Lawmakers Fail To Introduce Measures To Mandate COVID-19 Vaccinations.

The Los Angeles Times (9/8, Gutierrez, 3.37M) reports, "A drumbeat of chants criticizing vaccine and mask mandates carried into the unusually sparse hallways on Wednesday at the state Capitol, where lawmakers were wrapping up the final week of their legislative session without the bills that hundreds came to Sacramento to protest." A late push "by Assemblyman Evan Low (D-Campbell) to create a new workplace vaccine requirement failed to come together before a legislative deadline Tuesday evening." Another proposal "by Assemblywoman Buffy Wicks (D-Oakland), which fell apart last week, would have created sweeping new vaccine mandates in the workplace and to enter businesses."

Colleges In 12 Conservative-Leaning States Grapple With Mask, Vaccine Mandates.

Kaiser Health News (9/9, Andrews) reports that "12 conservative-leaning states prohibit vaccine mandates at higher education institutions, according to an analysis by the National Academy for State Health Policy." Red states with Republican leadership "are hardly the only ones where colleges and

universities are facing restrictions on their ability to put public health protections in place.” But for teachers, “whose professions are rooted in encouraging the pursuit of learning and knowledge, prohibitions that fly in the face of science and jeopardize public health can be tough to swallow.”

Baltimore County To Require Government Employees To Provide Proof Of COVID-19 Vaccination.

The Baltimore Sun (9/9, DeVille, 629K) reports that Baltimore County “will require government employees to prove they are vaccinated or submit to weekly testing, County Executive Johnny Olszewski Jr. announced Thursday.” The state’s “third-largest jurisdiction will require its 9,000 employees to prove by Oct. 15 that they’ve received at least one dose of the Pfizer/BioNTech or Moderna vaccines or the single-dose Johnson & Johnson vaccine.” Employees “will have to prove they’ve received all vaccination doses by Nov. 15.”

FDA IN THE NEWS

FDA Announces Delay In Decisions On E-Cigarette Products, Including Juul.

The Wall Street Journal (9/9, Maloney, Subscription Publication, 8.41M) reports the FDA has announced it needs to extend its deadline for deciding whether or not e-cigarette products can stay on the US market. The original deadline was Thursday, Sept. 9. The agency reported it had acted on 93% of the received applications.

The AP (9/9, Perrone) reports the FDA “said it rejected applications for nearly 950,000 e-cigarettes and related products, mainly due to their potential appeal to underage teens,” but has yet to rule on Juul, the most popular brand. In a statement, the agency said, “There’s more work to be done to complete our remaining reviews and ensure that we continue taking appropriate action to protect our nation’s youth from the dangers of all tobacco products, including e-cigarettes.”

Reuters (9/9, Banerjee, Kirkham) reports the FDA “continues to ‘work expeditiously on the remaining applications,’ adding many are ‘in the final stages of review.’”

USA Today (9/9, Rodriguez, 12.7M) reports Theodore Wagener, Director of the Center for Tobacco Research and co-leader of the Cancer Control Program at the Ohio State University Comprehensive Cancer Center, said, “(The FDA) is doing this the right way. ... Their slow walk of these things is a measured approach where they’re trying to review what the manufacturers supply while also recalling the science and having a longer-term view of what the science continues to suggest.” Meanwhile, Acting FDA Commissioner Janet Woodcock and Mitch Zeller, Director of the FDA’s Center for Tobacco Products, said in a joint statement, “We are

committed to working as quickly as possible to transition the current marketplace for deemed new tobacco products to one in which all products available for sale have undergone a careful, science-based review by the FDA and met the statutory standard.”

NPR (9/9, Sullivan, 3.69M) reports, “The news comes after years in which critics have urged tighter federal controls on vaping to combat a new surge of nicotine use by minors.” The agency “has issued marketing denial orders for more than 946,000 flavored vaping products because their applications ‘lacked sufficient evidence that they have a benefit to adult smokers sufficient to overcome the public health threat posed by the well-documented, alarming levels of youth use of such products.’”

CNN (9/9, Howard, 89.21M) reports “the long-term safety of vaping products is not yet known, said Marielle Brinkman, a senior research scientist at The Ohio State University Comprehensive Cancer Center.” Brinkman said, “At the Center for Tobacco Research, we have measured short-term effects in lung and heart health; we have measured harmful and potentially harmful constituents in the vapor that [users] inhale from these products.”

The Tobacco Reporter (9/9) reports, “Many companies reportedly received marketing denial orders (MDO) from the U.S. Food and Drug Administration overnight” before the announcement Thursday.

Among other news outlets reporting on the story are the Washington Post (9/9, McGinley, 10.52M), the New York Times (9/9, Richtel, 20.6M), ABC News (9/9, Salzman, Wu, 2.44M), CNBC (9/9, Lucas, 7.34M), The Hill (9/9, Coleman, 5.69M), Newsweek (9/9, Rouhandeh, 2.67M), Politico (9/9, Foley, 6.73M), Bloomberg Law (9/9, Subscription Publication, 4K), STAT (9/9, Florko, 262K), the Washington Times (9/9, Tan, 626K), the New York Post (9/9, Feuer, 7.45M), the New York Daily News (9/9, Sapienza, 2.51M), Axios (9/9, Fernandez, 1.26M), the Winston-Salem (NC) Journal (9/9, Craver, 226K), CSP Magazine (9/9, Prokop), Newsweek (9/9, Klapper, 2.67M), Filter (NY) (9/9, Norcia), Convenience Store News (9/9, 278K), Vaping360 (9/9, McDonald), the Tobacco Reporter (9/9), The Verge (9/9, Wetsman, 1.54M), and Vapor Voice (9/9).

Biden Announces New COVID-19 Vaccination Mandates For Businesses, Healthcare Facilities.

The Washington Post (9/9, Linskey, Abutaleb, Min Kim, Rein, 10.52M) reports “President Biden announced sweeping new coronavirus vaccine mandates Thursday designed to affect tens of millions of Americans, ordering all businesses with more than 100 employees to require their workers to be immunized or face weekly testing.” The President “also said that he would require most health-care facilities that accept Medicare or Medicaid funding to

vaccinate their employees, which the White House believes will cover 50,000 locations.” Furthermore, Biden “signed an executive order compelling all federal employees to get vaccinated – without an option for those who prefer to be regularly tested instead – in an effort to create a model he hopes state governments will embrace.” Biden “is also ordering all staffers in Head Start programs, along with Defense Department and federally operated schools for Native Americans, to be vaccinated.” NIAID Director Anthony Fauci said, “This was not just superficial stuff. This is going to have a significant impact.”

The New York Times (9/9, A1, Rogers, Stolberg, 20.6M) reports Biden “moved to mandate shots for health care workers, federal contractors and the vast majority of federal workers, who could face disciplinary measures if they refuse.” Biden said, “We’ve been patient. ... But our patience is wearing thin. And your refusal has cost all of us.” The vaccine “mandate for health care workers will apply to those employed by most institutions that accept Medicare and Medicaid reimbursement, including hospitals, dialysis centers and nursing homes, according to” officials. The mandate “will be enforced by” CMS.

The New York Times (9/9, Hirsch, 20.6M) reports in a separate piece that the proposed rules “will require workers to be inoculated or face weekly testing and will mandate that the businesses offer employees paid time off to get vaccinated.” The rules “will be imposed by the Department of Labor and its Occupational Safety and Health Administration, which is drafting an emergency temporary standard to carry out the mandate, according to the White House.”

The Los Angeles Times (9/9, Megerian, 3.37M) reports “firms that don’t comply could face \$14,000 fines from the Department of Labor.” The President “is also tightening rules for federal employees and contractors.” Even though “they were previously allowed to choose weekly testing instead of vaccination, that will no longer be an option,” and “anyone who isn’t fully vaccinated within three months could face disciplinary action, with limited exceptions.”

Bloomberg (9/9, Wingrove, Leonard, 3.57M) reports “Biden also delivered some of his harshest criticism yet of the 25% of U.S. adults who’ve so far not been inoculated, saying that they’re dragging out the pandemic.” He stated, “My message to unvaccinated Americans is this: What more is there to wait for? What more do you need to see?” Bloomberg adds that the Indian Health Service and the National Institutes of Health are “implementing previously announced vaccination requirements that cover 2.5 million workers, according to the administration.”

The AP (9/9, Miller) reports “Republican leaders – and some union chiefs, too – said Biden was going too far in trying to muscle private companies and workers, a certain sign of legal challenges to come.” However, “there were strong words of praise for Biden’s efforts to get the nation

vaccinated from the American Medical Association, the National Association of Manufacturers and the Business Roundtable – though no direct mention of his mandate for private companies.”

The AP (9/10, Ortutay) reports in a separate piece that “larger U.S. businesses now won’t have to decide whether to require their employees to get vaccinated against COVID-19,” because “doing so is now federal policy.”

Reuters (9/9, Hunnicutt, Mason, Aboulenein) reports “infectious disease and health policy experts said the mandates are unlikely to significantly change infection rates quickly.” However, the mandates “would help against potential future waves of the virus, reducing deaths and hospitalizations and alleviating the stress on the healthcare system, said Georgetown University’s Dr. Jesse Goodman, a former chief scientist at the U.S. Food and Drug Administration.”

CNBC (9/9, Lovelace, Mendez, Towey, 7.34M) reports “the Biden administration has also encouraged private businesses to implement vaccine mandates, though CDC Director Dr. Rochelle Walensky previously told CNBC that federal officials would not issue a nationwide mandate.”

The Hill (9/9, Chalfant, 5.69M) reports Biden “admonished unvaccinated Americans and some elected officials for exacerbating the coronavirus pandemic.” He stated, “We have the tools to combat COVID-19, and a distinct minority of Americans, supported by a distinct minority of elected officials, are keeping us from turning the corner. These pandemic politics, as I refer to it, are making people sick, causing unvaccinated people to die.”

Roll Call (9/9, McIntire, Lesniewski, 130K) reports “the president’s plan will also double fines for people not in compliance with federal masking requirements, such as on airplanes.”

Among other news outlets reporting on the story are ABC World News Tonight (9/9, 6:32 p.m. EST, lead story, 6:00, Muir, 6.32M), the CBS Evening News (9/9, 6:31 p.m. EST, lead story, 3:50, O’Donnell, 3.48M), NBC Nightly News (9/9, 6:31 p.m. EST, lead story, 3:00, Holt, 4.78M), the Wall Street Journal (9/9, A1, Armour, Siddiqui, Subscription Publication, 8.41M), ABC News (9/9, Nagle, Kolinovsky, Gomez, 2.44M), CNN (9/9, Collins, Liptak, 89.21M), Forbes (9/9, Segal, 10.33M), Newsweek (9/9, Villarreal, 2.67M), NPR (9/9, Wise, Keith, 3.69M), Politico (9/9, Cancryn, Lim, 6.73M), Fox Business (9/9, Singman, 3.06M), the New York Post (9/9, Moore, Nelson, Patteson, 7.45M), the New York Daily News (9/9, Goldiner, Balk, 2.51M), the Detroit Free Press (9/9, Shamus, Boucher, Roberts, 2.16M), the Boston Herald (9/9, Tiernan, 327K), and Government Executive (9/9, Bubl , 29K).

Biden Requires In-Home Nurses To Receive COVID-19 Vaccine Under New Plan. Bloomberg (9/9, Edney, 3.57M) reports “parents of children with severe health conditions won a victory Thursday when President Joseph

Biden required that in-home nurses be vaccinated against Covid-19." A plan the President "revealed to boost vaccine requirements across the U.S. includes home health workers among 17 million medical staff who must be inoculated, according to a White House summary."

Amazon, Kroger, Walmart Playing Key Role In Biden's Most Recent COVID-19 Plan. Fox Business (9/9, Barrabi, 3.06M) reports "Walmart, Amazon, and Kroger will play a key role in President Biden's latest plan to curb the COVID-19 pandemic, the White House revealed on Thursday." The three retailers "have agreed to sell at-home rapid COVID-19 tests to the public at cost beginning later this week, a senior administration official said." The COVID-19 "tests will be available for purchase online or in-stores at a discount of up to 35% for the next three months." Meanwhile, HHS' "free COVID-19 testing program will expand to provide services at 10,000 pharmacies nationwide."

Reuters (9/9, Shepardson) reports Amazon.com "said it will cut the cost of its direct-to-consumer COVID-19 PCR Test Collection kit to \$36.99, a \$3 price reduction." Amazon "said the price cut reflects its costs for selling the FDA-approved kit and is the result of a public-private partnership with the Biden administration."

Companies Say Federal Government Must Give More Details About COVID-19 Vaccination Mandate. Bloomberg (9/9, Roeder, 3.57M) reports "U.S. companies grappling with the thorny issue of whether to require worker vaccinations against Covid-19 say the U.S. government needs to provide more details about" the "new, far-reaching mandate announced Thursday." Businesses are "digesting" Biden's "order, but there are concerns over what the mandate may entail." For example, the Consumer Brands Association said, "President Biden's announcement prompts critical questions that require immediate clarification. ... As with other mandates, the devil is in the details. Without additional clarification for the business community, employee anxieties and questions will multiply."

RNC Promises To Sue Biden Administration Over COVID-19 Vaccine, Testing Requirements. The Hill (9/9, Oshin, 5.69M) reports "the Republican National Committee (RNC) has vowed to sue the Biden administration over its COVID-19 vaccine and testing mandate." RNC Chairwoman Ronna McDaniel said, "Many small businesses and workers do not have the money or legal resources to fight Biden's unconstitutional actions and authoritarian decrees." She continued, "But when his decree goes into effect, the RNC will sue the administration to protect Americans and their liberties."

Unions Push Back Against Biden's Proposal To Require Federal Employees To Receive COVID-19 Vaccine. The Washington Times (9/9, Mordock, 626K) reports "unions that represent federal workers pushed back Thursday against President Biden's plan to require all federal

employees and contractors to be vaccinated against COVID-19." The American Federation of Government Employees "said COVID-19 vaccine requirements should be negotiated with the unions during collective bargaining, not mandated through an executive order." Meanwhile, the National Treasury Employees Union "said the administration must accommodate workers who decide against the vaccine for religious or medical reasons."

Biden's Executive Order Forcing COVID-19 Vaccinations Reportedly Does Not Include Postal Service Employees. The Washington Times (9/9, Morton, 626K) says "the Washington Post reported Thursday night that [Biden's] executive order forcing federal workers to take a COVID-19 vaccine does not include U.S. Postal Service workers." Citing one White House official, "the Post reported that those workers merely would be strongly encouraged to follow the mandate." Since "the Postal Service is an independent agency of the executive branch, the Post explained, an executive order on federal employees' labor issues does not apply to the agency, unless a president specifies that it does."

Arizona Governor Vows To "Push Back" On Biden's COVID-19 Vaccination Plan For Large Employers. The Arizona Republic (9/9, Barchenger, 1.05M) reports Arizona Gov. Doug Ducey (R) "has promised to 'push back' on President Joe Biden's plan to require large employers to mandate vaccines or weekly COVID-19 tests for their workers." The governor "did not say what form a pushback would take, only promising 'more to follow.'" Ducey said to reporters, "What the Biden administration is doing is government overreach, pure and simple. And people don't want them hammering down on them to get this vaccine."

NYTimes Examines Who Will Pay For COVID-19 Tests Under Biden Administration's New Rule For Large Companies. The New York Times (9/9, Kliff, 20.6M) reports the Biden Administration's intent "to mandate that workers at large companies get vaccinated" against COVID-19 "or submit to regular testing" leads to "a thorny question: Who pays for those coronavirus tests?" So far, employers have "considered a range of approaches, from fully covering the costs to having unvaccinated workers pay full freight." A number of "employers have had workers chip in for the costs of coronavirus testing," and "many states and cities still have free coronavirus testing sites that they started earlier in the pandemic." These different "approaches could provide a menu of options for many workplaces that fall under the new federal rule."

Analysis: Biden's Move To Expand Number Of Vaccinated Americans Is Attempt To Counter COVID-19's Threat To Economy. In an analysis, the New York Times (9/9, A1, Tankersley, 20.6M) says "President Biden's aggressive move to expand the number of vaccinated Americans and halt the spread of the Delta variant is not just

an effort to save lives.” The move “is also an attempt to counter the continuing and evolving threat that the virus poses to the economy.” The rise of the Delta variant “has been fueled in part by the inability of Mr. Biden and his administration to persuade millions of vaccine-refusing Americans to inoculate themselves against the virus,” which “has created another problem: a drag on the economic recovery.” According to the Times, “real-time gauges of restaurant visits, airline travel and other services show consumers pulled back on some face-to-face spending in recent weeks.”

Columnist: Biden’s New COVID-19 Strategy Does Not Go Far Enough To Boost Vaccinations. Washington Post (9/9, 10.52M) columnist Leana Wen says “President Biden’s much-hyped new strategy for fighting covid-19 is a tepid half-measure that falls short of the dramatic reset the country needs.” Wen asserts that “the six-pronged strategy announced on Thursday can be summarized as ‘more of the same’ – these are good steps in the right direction, but they’re not enough to get the job done.” She writes that “the biggest problem with Biden’s plan is that it does not go nearly far enough toward compelling vaccinations.”

FDA, FTC Warn 10 Companies Against Making “Fraudulent Claims” That Unapproved Dietary Supplements Can Help With Diabetes. Cardiovascular Business (9/9, Walter) reports, “The FDA and FTC have warned 10 companies for illegally selling dietary supplements that claim to ‘cure, treat, mitigate or prevent diabetes,” which the agencies said violates the Federal Food, Drug and Cosmetic Act. Acting FDA Center for Food Safety and Applied Nutrition Office of Dietary Supplement Programs Director Cara Welch, PhD, stated, “More than 34 million Americans...are living with diabetes. ... Dietary supplements that make fraudulent claims to treat diabetes are unapproved new drugs that could potentially harm consumers who use these products instead of seeking safe and effective FDA-approved treatments.”

The Miami Herald (9/9, Neal, 647K) also reports on the story.

FDA Authorizes Additional Batch Of J&J COVID-19 Vaccine From Troubled Baltimore Plant. The Baltimore Sun (9/9, Miller, 629K) reports that the FDA “has authorized an additional batch of COVID-19 vaccine produced by Johnson & Johnson at an East Baltimore plant where regulators previously determined several million doses of vaccine were unusable.” The Sun adds that “the vaccine being produced at the facility, owned and operated by the Gaithersburg-based Emergent BioSolutions, still has not been authorized fully under the FDA’s emergency use authorization, said Marion Gruber,

director of the office of vaccines research and review center for biologics evaluation and research.”

MEDICAL PRODUCT SAFETY

Biden Administration Releases Proposal To Lower Prescription Drug Prices. The Wall Street Journal (9/9, Armour, Subscription Publication, 8.41M) reports on Thursday, the Biden Administration unveiled a plan which seeks to reduce prescription drug prices. HHS Secretary Xavier Becerra said, “The Biden-Harris administration remains committed to making health care more affordable for American families, and this plan outlines one key way we will do that. ... By promoting negotiation, competition, and innovation in the health-care industry, we will ensure cost fairness and protect access to care.” The article mentions the FDA.

The New York Times (9/9, Sanger-Katz, 20.6M) reports the initiative was an endorsement of “an aggressive proposal to limit prices for prescription drugs, a signal to congressional Democrats as they consider options for their large legislative package.” The plan “was published as a 29-page white paper from the Department of Health and Human Services. It included a range of recommendations to foster more competition among drugmakers and improve the affordability of drugs for patients enrolled in Medicare.” However, “its primary recommendation was a big one: that the department should negotiate with drug companies on their product prices, and that those prices should apply to all drug purchasers in the country, not just Medicare, the federal program primarily for people 65 and older.”

Bloomberg Law (9/9, Stein, Subscription Publication, 4K) reports HHS “attributed the high cost of prescription drug prices in the U.S. to ‘lack of competition,’ and said Americans pay more than \$1,500 per person a year for medications.” The department “said the pharmaceutical industry is ‘characterized by multiple market failures,’ attributable to several factors, including ‘monopolistic or oligopolistic behavior’ and ‘legal abuses.’” Becerra stated, “Life-saving prescription medication should not cost anyone their life savings. Yet too often, many low-income families cannot take their prescription medications because of cost concerns.”

In a separate article, Bloomberg Law (9/9, Lopez, Subscription Publication, 4K) reports Becerra “is calling on the U.S. Patent and Trademark Office to help rein in skyrocketing drug prices as part of the government’s push to curb practices that allegedly keep lower-cost medicines from hitting the market.” He stated in the proposal that the patent office and the FDA “will work together to develop solutions’ to facilitate faster market entry of cheaper, copycat versions of name brand drugs.”

Reuters (9/9, Heavey, O'Donnell) reports although "the administration has lauded drugmakers for their efforts amid the COVID-19 pandemic, it also has highlighted the high costs of medications in the United States compared to other nations. Pharmaceutical manufacturers have raised prices on more than 500 medicines, according to an analysis by healthcare research firm 46brooklyn in January."

Among other news outlets covering the story are CNBC (9/9, Lovelace, 7.34M), CNN (9/9, Luhby, 89.21M), Politico (9/9, Cancryn, 6.73M), The Hill (9/9, Weixel, 5.69M), Fortune (9/9, Morris, 3.68M), Newsweek (9/9, Rouhandeh, 2.67M), STAT (9/9, Facher, 262K), Modern Healthcare (9/9, Hellmann, Subscription Publication, 215K), Fierce Healthcare (9/9, King, 150K), Inside Health Policy (9/9, Wanneh, Subscription Publication), a separate piece by Inside Health Policy (9/9, Subscription Publication), and the Fiscal Times (9/9, Rosenberg).

Endo, New York Settle Opioid Suit. The AP (9/9) reports, "The state of New York and two large counties agreed Thursday [to] a \$50 million deal to end their lawsuits with drugmaker Endo International, in the latest of a progression of settlements of government claims over the opioid addiction and overdose crisis." Under the deal, "the Dublin-based drugmaker and its subsidiary Par are to pay \$22.3 million to the state attorney general's office and \$13.85 million to both Suffolk and Nassau counties."

Reuters (9/10) also covers the story.

Study: Black People Died From Opioid Overdoses At Higher Rates Than White, Hispanic People In 2019. The Boston Globe (9/9, Freyer, 1.04M) reports "the opioid epidemic has long been portrayed as a crisis among white Americans, but increasingly, Black people are dying of overdoses at alarming rates." One "study led by Boston Medical Center researchers, published Thursday, shows that in 2019, for the first time, Black people died of overdoses at higher rates than white and Hispanic people." The report, which was "published in the American Journal of Public Health, is among the first research papers to emerge from the HEALing Communities study, a \$350 million project that started in 2019." As part of the agency's "Helping to End Addiction Long-term Initiative, the National Institutes of Health project is testing possible solutions to the opioid crisis in hard-hit communities in Kentucky, Massachusetts, New York, and Ohio."

The Eurasia Review (9/10) reports "non-Hispanic Black individuals in four U.S. states experienced a 38% increase in the rate of opioid overdose deaths from 2018 to 2019, while the rates for other [races] and ethnicity groups held steady or decreased, according to" the study. The HEALing Communities Study "is the largest addiction implementation

study ever conducted and is administered in partnership by NIH's National Institute on Drug Abuse (NIDA) and the Substance Abuse and Mental Health Services Administration." NIDA Director Nora D. Volkow said, "Systemic racism fuels the opioid crisis, just as it contributes mightily to other areas of health disparities and inequity, especially for Black people."

Bloomberg Law (9/9, Lopez, Baumann, Subscription Publication, 4K) also reports on the story.

President Biden Orders FDA To Finish Draft Rules On OTC Hearing Aids By November.

HealthDay (9/9, 11K) reports, "Until now, folks suffering from hearing loss typically have had to fork out thousands of dollars for a device that could be adjusted only by a professional audiologist." It is "no wonder that only one-quarter of the nearly 29 million U.S. adults who could benefit from a hearing aid have actually tried one, according to the U.S. National Institutes of Health." A 2017 law "requires the U.S. Food and Drug Administration to establish a category of over-the-counter hearing aids, as well as the standards for them." The FDA "missed its August 2020 deadline to propose those new rules, blaming the COVID-19 pandemic for the delay," but the "wait for consumers might be over soon: President Joe Biden has ordered the agency to finish its draft rules by November."

Apellis Still Plans To File For FDA Approval For Eye Disorder Drug Despite Mixed Phase 3 Results.

FiercePharma (9/9, Higgins-Dunn, 12K) reports, "Apellis Pharmaceuticals' pegcetacoplan posted mixed results in two late-stage trials studying the C3 inhibitor in patients living with geographic atrophy (GA)." However, "despite the mixed showing, CEO Cedric Francois thinks the med's performance was strong enough to warrant an FDA nod." Apellis "plans to file the med with the FDA in the first half of 2022."

ICU Medical Voluntarily Recalling Amino Acid IV After Hair, Other Foreign Substances Found In Batch.

Endpoints News (9/9, Sullivan) reports that California-based ICU Medical "is voluntarily recalling one lot of an amino acid injection made by Pfizer spinout company Hospira after reports of fibers, hair and other particles of foreign materials were found in the solution." The infusion therapy "recalled 2,112 units of Aminosyn II [(amino acid Injection with electrolytes) 15%, after it became aware of the issue while inspecting samples as part of a routine process, the FDA said."

Court Says AbbVie Cannot Force Takeda To Supply Drug Amid Shortage.

FiercePharma (9/9,

Sagonowsky, 12K) reports that this week, “Delaware Chancery Court Vice Chancellor Sam Glasscock III denied AbbVie’s request to compel Takeda to fill supply orders” of Lupron (leuporelin), “used to treat pain associated with cancer,” from its plant in Hikari, Japan. AbbVie had taken Takeda “to court over alleged failures to live up to supply agreements” amid shortages of the medication “after the FDA and Takeda in 2019 found ‘protocol violations’ at the plant.” Glasscock wrote that “remediation efforts” have continued to this day and that an order forcing Takeda to supply the treatment would be “unworkable” because it would force the court to oversee Takeda’s operations and “inevitably lead to contempt hearings.”

Jazz, PharmaMar To Run Another Phase 3 Confirmatory Trial For Small Cell Lung Cancer Drug After Previous Failure.

FiercePharma (9/9, Liu, 12K) reports, “Jazz Pharmaceuticals wasn’t able to ride a breakthrough FDA nod to a pivotal trial win for conditionally approved small cell lung cancer (SCLC) drug Zepzelca” (lurbinectedin), but “after talking with the FDA, the company and partner PharmaMar see a new path forward.” According to FiercePharma, “In previously treated SCLC, the combination of Zepzelca and doxorubicin failed to extend patients’ lives over physician’s choice of doxorubicin-based chemo or Hycamtin [topotecan], according to data presented at the 2021 World Conference on Lung Cancer virtual meeting.” Now, “Jazz and PharmaMar have agreed ‘in principle’ with the FDA to run another phase 3 as the confirmatory trial.”

Mammoth Biosciences Raises \$195M In Financing Round.

Forbes (9/9, Jennings, 10.33M) reports that Mammoth Biosciences on Thursday “announced the company raised \$195 million of financing at a valuation of more than \$1 billion.” The funding “includes a \$150 million Series D led by Redmile Group and a \$45 million Series C, also led by Redmile Group and Foresite Capital, with participation from Sixth Street, Decheng Capital, Mayfield and Amazon, among others.” A Mammoth COVID-19 diagnostic solution “was selected for the RADx program, a ‘shark-tank’ style challenge led by the National Institutes of Health, as well as a contract with the Department of Defense.” Mammoth “received an emergency use authorization for its DETECTR diagnostic platform for Covid-19 from the FDA last summer.”

Also reporting are TechCrunch (9/9, 502K), MedCity News (9/9), and the Financial Times (9/9, Kuchler, Subscription Publication, 1.48M).

Recro Acquires San Diego-Based Opioid Addiction Drugmaker For \$50M.

Endpoints News (9/9, Sullivan) reports that CDMO Recro Pharma three weeks

ago “acquired San Diego-based Irisys for \$50 million cash.” According to Endpoint News, “Recro will serve as both an advisor to BioCorRx in its small molecule development for its opiate addiction treatment drug, dubbed Bicx104, in addition to manufacturing it.” The FDA has approved the subcutaneous pellet of naltrexone “to start in-person trials, largely funded by the National Institute on Drug Abuse and the NIH.”

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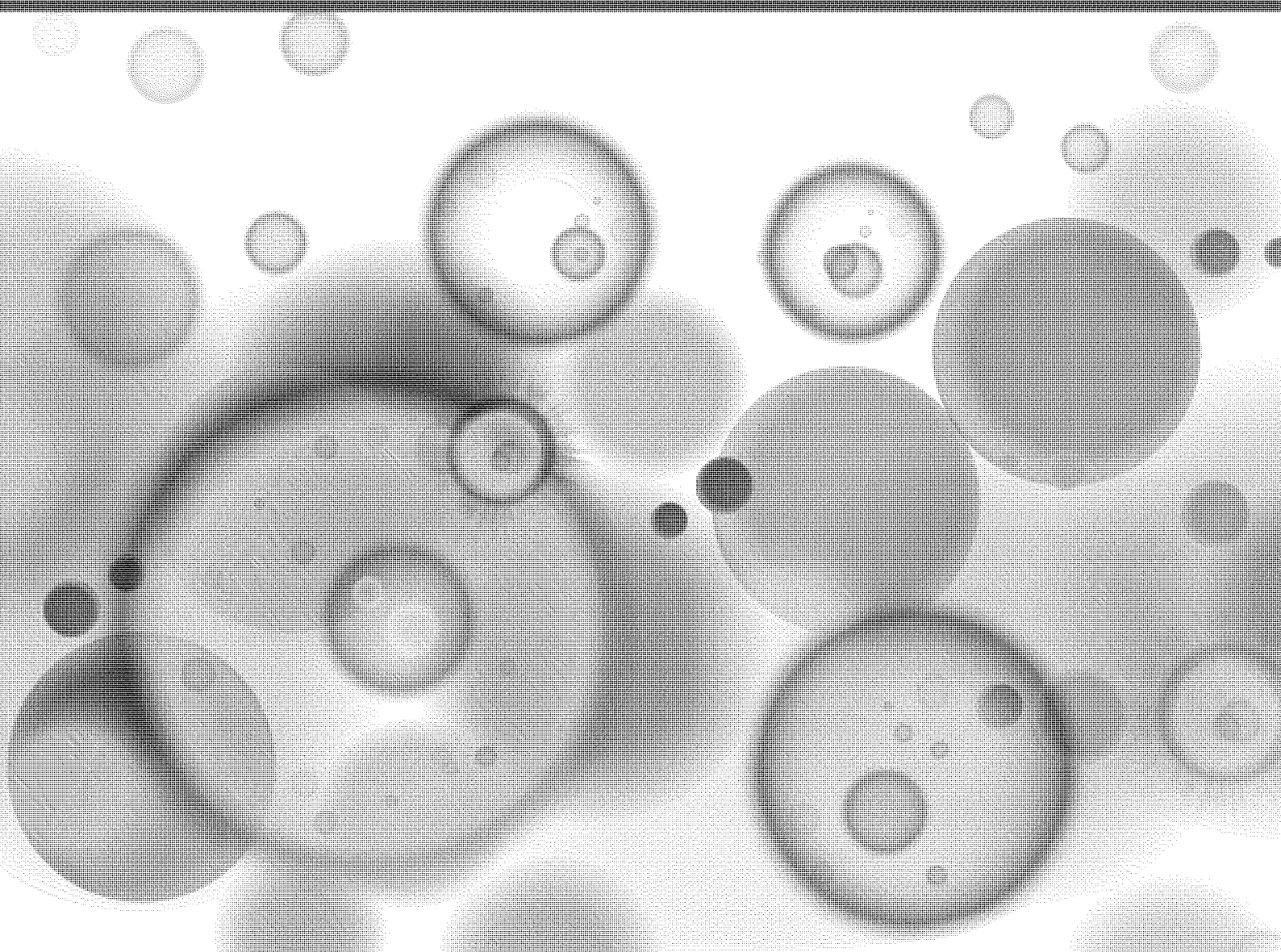
Tax On E-Cigarettes Runs Risk Of Pushing Teens Toward Regular Cigarettes, Economists Warn.

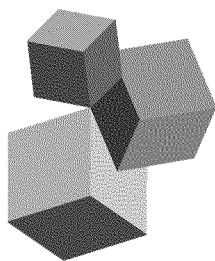
The Washington Examiner (9/9, Morrison, 888K) reports “health economists warned that taxes on e-cigarettes and vape products alone would not be enough to keep teenagers from using other risky tobacco products such as regular cigarettes.” The authors of a “report on whether the United States should put a blanket tax on” vapes “reported that a tax on electronic nicotine delivery systems reduces the likelihood that the youth will buy the products in retail stores.” But, “hiking up the prices of vaping products runs the risk of pushing young people toward cheaper alternatives, such as regular cigarettes.” The economists who wrote “the report used extensive data collection from two national studies – Monitoring the Future, funded by the National Institute on Drug Abuse, and the Youth Risk Behavior Surveillance System supported by the” CDC.

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IMMUNOGENICITY OF PROTEINS USED AS THERAPEUTICS

EDITED BY: Amy Rosenberg, Zuben E. Sauna, Bernard Maillere,
Susan Richards and Elizabeth C. Jury
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IMMUNOGENICITY OF PROTEINS USED AS THERAPEUTICS

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Editorial: Immunogenicity of Proteins Used as Therapeutics

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Editorial on the Research Topic

Immunogenicity of Proteins Used as Therapeutics

“And a little child shall lead them”

Nowhere is immunogenicity of therapeutic proteins of greater concern or impact than in children who potentially face a lifetime of treatment for chronic disease. This is brought into focus by three publications [Gress et al.; Desai et al.; Scott and Pratt].

Gress et al. make clear that the outcome for children with Inflammatory Bowel Disease (IBD) who lose efficacy due to Anti-Drug Antibodies (ADA) requires recurrent surgeries and eventually bowel resection and colostomy creation. While this outcome is hard for adults with IBD, it is devastating to children. To allow better Tumor Necrosis Factor (TNF)-inhibitor mediated control of IBD, this article suggests: 1) Keeping levels of TNF inhibitors over a critical level (which appears to preclude ADA in most patients) using therapeutic drug monitoring (TDM). 2) Accessibility to tests detecting ADA at the earliest time point allowing consideration of therapeutics that will prevent further escalation of ADA. 3) Development of immune tolerance induction protocols. The addition of co-administered immune suppressant agents (e.g. methotrexate, azathioprine) at the introduction of the biological therapy has been shown in numerous studies to reduce ADA development and potentially induce tolerance to the biological therapy. Whether patients co-administered these agents are then truly tolerant to the TNF antagonist has not been formally evaluated. Moreover, the duration of the dual immune suppressive therapy necessary for insuring tolerance to the biological component should be studied. Key to these approaches is the ability to readily test, at no or low cost, patient samples for levels of the TNF antagonist as well as ADA.

Desai et al. present an elegant approach to addressing ADA that neutralize a life-saving enzyme replacement therapy (ERT), recombinant human alpha glucosidase (rhGAA). In a subpopulation of Infantile Pompe Disease (IPD) patients that lack endogenous GAA, immune tolerance induction (ITI) is essential to a favorable patient outcome. This protocol, in which a short course of methotrexate, rituximab, and IVIG are given concurrently with initial doses of ERT proved highly tolerogenic. In the absence of this protocol these IPD patients would lose motor milestones and die of cardiopulmonary failure. Patients treated with ITI protocol recovered their B cell populations over several months and mounted vaccine responses, demonstrating the transient

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nature of the safety concerns. A case study by Gupta et al. demonstrated the effectiveness of early ITI in a prenatally diagnosed patient with IPD who started treatment immediately after birth. This is the first report of successful ITI at such an early age. Critically, if patients were not treated concurrently with initial dosing of the ERT and did develop life threatening ADA, the prophylactic regimen failed to eliminate antibody producing plasma cells. Thus, adding a proteasome inhibitor (bortezomib) to the regimen, resulted in dramatic diminution and elimination of the ADA, facilitating immune tolerance to ERT and allowing patients to recover their motor milestones (1). Finally, Scott and Pratt provide a description of the onerous ITI protocol following immune response to coagulation factor replacement therapy which hits children the hardest.

Anticipating an ADA response to therapeutic proteins remains the holy grail. In the ideal world, reliable non-clinical methods would predict immune responses early in clinical development. There is an unmet need for two classes of tools that assess the likelihood that a drug will generate a significant immune response. The first is a suite of *in silico* and *in vitro* methods that can assess immunogenicity during drug development prior to the clinical use of a protein drug. However, as most approved therapeutic proteins have some degree of ADA formation, there is also a need for assays that can be used in the clinic so that physicians can make informed decisions for individual patients with respect to either tempering the immune response or seeking out alternative treatment options. The report by Baker et al. on Alemtuzumab illustrates these key points. Alemtuzumab is a humanized monoclonal antibody that is paradoxically among the most immunogenic of monoclonal antibody products; humanization does not render a protein fully human and even fully human proteins may elicit ADA. The experience with Alemtuzumab is not surprising because although immune responses are broadly predicated on the concept of “self” and “non-self”, immuno-biology is nuanced and complex (as evidenced by the challenge posed by autoimmune diseases). In this issue, two reviews from Vaisman-Mentesh et al. and Nabhan et al. provide an excellent introduction to the molecular and cellular mechanisms involved in the generation of ADAs. Additionally, Fitzpatrick et al. provide a fascinating overview of the role of recombinant Fc multimers in immune tolerance induction and suggest that monoclonal antibodies used for treatment of cancer and autoimmunity, may mediate tolerogenic effects. This could be mediated by immune complex formation or by antibodies coating target cells, triggering multiple mechanisms including antibody dependent cell-mediated cytotoxicity, complement activation and regulatory T cell expansion, that reset immune homeostasis in an antigen independent manner. Thus, an understanding of the underlying biology is essential for designing tools to assess and circumvent immunogenicity.

Following up on the concept that immunogenicity goes beyond strict self/non self-discrimination, two comprehensive surveys of the current state of the art with respect to non-clinical immunogenicity assessments are provided by Jawa et al. and Meunier et al.. Jawa et al. list technical approaches to assess immunogenic potential and provide a very useful discussion of

integrating immunogenicity data in regulatory submissions. These two articles also provide mechanistic context for immunogenicity and of the assays being discussed. The identification of T cell epitopes in biopharmaceuticals reveals multiple mechanisms leading to T cell activation, ADA response or regulation (Meunier et al.). Variability, diversity, and joining (VDJ) recombination of antibodies and somatic mutations resulting from affinity maturation processes contribute to making non germline sequences, which could be recognized as new T cell epitopes (neoepitopes). However, though not mutated, therapeutic protein counterparts of endogenous proteins such as recombinant hormones, growth factors and cytokines can elicit CD4 T cell responses and elicit ADAs. The basis for their immunogenicity may pertain to the insufficient expression of their endogenous counterpart in the body and thus failure to induce central tolerance. Functional T cells therefore escape from negative thymic selection by insufficient levels or affinity for self-antigens leading to failure of deletion or induction of anergy, although they are specific for self-sequences and not neoepitopes. These escaped T cells may be activated in the periphery by infusion of the therapeutic proteins (Meunier et al.). Both articles (Jawa et al.; Meunier et al.) also provide an important caveat to the use of T-cell proliferation assays to assess immunogenic potential. Although T cell activation is essential for high titer, class switched, affinity matured antibody response, they do not always promote ADA responses and might differentiate instead into regulatory IL-10 secreting T cells (Tr1), with IL-10 being able to dampen activation of effector T cells (Jawa et al.; Meunier et al.).

In addition to these broad surveys of the literature, Karle provides an in-depth critical discussion of an important emerging technology. The MHC-associated peptide proteomics (MAPPs) assay is technically challenging but allows the direct identification of the naturally processed peptides derived from biopharmaceuticals displayed by HLA class II molecules on DCs. MHC class II molecules are immunopurified and the bound peptides are eluted by acidic treatment and sequenced by liquid chromatography-mass spectrometry. Karle describes how MAPPs is used to investigate immunogenicity risk of biopharmaceuticals but also to evaluate their capture by antigen presenting cells and the impact of post-translational modifications, folding, and aggregation on peptide presentation. The article provides a useful summary of all studies that have applied the MAPPs technology to therapeutic proteins, and by comparing multiple studies using the same therapeutic, shows that the technique is reliable and renders consistent results. Unfortunately, as with all non-clinical estimates of therapeutic protein immunogenicity, the clinical significance of the results remains the critical unknown.

Data sets of peptides eluted from MHC molecules in MAPPs assays are rapidly growing and are being leveraged to improve *in silico* algorithms used in immunogenicity assessments. Peptide-MHC engagement is a necessary step in the initiation of an immune response to therapeutic proteins. Thus, algorithms that predict peptide-MHC binding affinities have become an initial rapid and inexpensive screen for potential immunogenicity. One

reason why such algorithms overestimate immunogenicity risk is that not all potential high-affinity peptides are actually generated by the protease machinery of antigen presenting cells. By directly identifying therapeutic protein-derived peptides on MHC molecules, the results of MAPPs assays report on both peptide processing and presentation. Thus, in this Research Topic, Barra et al. report an artificial neural network (ANN) model, trained to predict T cell epitopes. The algorithm presented by Barra et al. joins multiple *in silico* methods that are freely available to estimate immunogenicity risk based either on HLA binding affinity or on MAPPs data.

The profusion of freely available tools is a double-edged sword for those not familiar with the computational and statistical methods used. For end users of such algorithms, Paul et al. introduce the need of rigorous benchmarking to compare the different methods of identification of T cell epitopes and provide quantitative data to point out the benefits and insufficiencies of each method (Paul et al.). While some algorithms have become increasingly accurate at specific tasks, e.g. estimating peptide-MHC affinity or which peptides will be presented by MHC molecules, the ability to predict clinical immunogenicity remains a key challenge. Predicting immunogenicity for biotherapies using patient and drug-related factors is attractive but no robust method has as yet been developed. With the growing ability to collect massive amounts of data, machine learning algorithms could identify predictive variables. Two studies applied machine learning models to predict ADA status (Duhazé et al. and Waddington et al.) utilized clinical data collected in the multi-cohort of autoimmune diseases treated with biotherapies from the ABIRISK consortium. Duhazé et al. evaluated the predictive power of a custom-built machine learning model, the random survival forest model (2), for predicting the occurrence of anti-drug antibodies. The approach provided a good predictive accuracy and outperformed current methods, although validation in larger cohorts is needed.

In the discussions thus far, the MHC repertoire is as an important parameter in assessing immunogenicity because foreign-peptide-MHC binding is a necessary (albeit not sufficient) step in the immune response leading to ADAs. However, the MHC genes are the most polymorphic in the human genome and occur at different frequencies in various human subpopulations. This creates a problem in putting together a suitable real or virtual cohort for immunogenicity assessments that is representative of the population of interest with respect to distribution of MHC variants. McGill et al. have addressed this challenge by developing an algorithm, SampPick, that permits the selection of a cohort of subjects that matches a population MHC distribution.

As discussed earlier, assessment of the potential for immunogenicity represents an unmet need not only for biomolecules in the early stages of development but also in the clinic. Most approved therapeutic proteins do exhibit various levels of immunogenicity and their clinical use would benefit from accurate, reproducible and clinically meaningful measurements of ADAs and TDM. Atiqi et al., Mehta and

Manson, and Gress et al. describe the scope of the problem using the example of TNF-Inhibitors. These medications have revolutionized the management of rheumatoid arthritis and other diseases but only a small proportion of patients maintain long-term clinical response (3, 4). Boyer-Suavet et al. describe how the presence of neutralizing anti-rituximab antibodies is similarly associated with disease relapse. Selection of, and switching between, biologics is mainly empirical, experiential, and not evidence-based. While it is broadly acknowledged that immunogenicity is one of the main reasons for loss of therapeutic efficacy (secondary failure) the field is beset by challenges. ADA identification is technically difficult and not standardized, making interpretation of immunogenicity data and application in the clinic almost impossible (Mehta and Manson). However, Lallemand et al. reported on the potential of using a highly sensitive reporter gene assay to quantify both an anti-VEGF ADA and the therapeutic drug activity to monitor responder vs. non-responder patients. Overall, longer term information is needed to determine the utility of these approaches. This Research Topic, however, does also offer potential strategies to overcome some of these problems (Kharlamova et al.) and novel assays that may be more reliable (Kharlamova et al.).

In keeping with our increased understanding of the complexity and diversity of immune responses four papers provide a glimpse into the application of new but rapidly emerging fields to therapeutic protein immunogenicity. Fu et al. address biotherapeutic immunogenicity in the context of the orchestrated function of highly differentiated T and B cells, including follicular helper CD4 T cells and germinal center B cells, for the optimal generation of antibody responses. They suggest that understanding the cellular and molecular mechanisms mediating the antibody responses against therapeutics could lead to novel strategies to reduce their immunogenicity. Kishimoto describes a promising approach to antigen targeted immune tolerance induction to prevent the formation of ADAs across a wide variety of biologics. Antigen-targeted tolerance is induced in several experimental animal models (haemophilia A, inflammatory arthritis, and Pompe disease) by the incorporation of rapamycin in nanoparticles in the presence of therapeutic antigen at the time of administration (ImmTOR). The problematic immunogenicity issues pertaining to recombinant immunotoxins (chimeric proteins consisting of a targeting element such as a Fv antibody region bound to a toxin) are reviewed by Mazor and Pastan. These therapies have potential for use in a wide variety of diseases and have been tested and approved in cancer. This review outlines the strategies used to mitigate the immunogenicity of immunotoxins which has a major impact on the efficacy of these promising drugs. Finally, Waddington et al. have presented original research that applies the rapidly expanding field of serum metabolomics to therapeutic protein immunogenicity. Their study shows that serum metabolites are a promising biomarker for early identification of ADA development in MS patients treated with IFN β and could provide novel insights into mechanisms of immunogenicity.

Significant effort is spent during clinical development in commissioning robust, specific and sensitive ADA assays that are validated to support clinical studies for product approval. However, longer term evaluation is needed to facilitate translatability to routine clinical practice that can impact patient care. ADA status (ADA-positive vs. ADA-negative) is just the first tier for assessment. Clinical relevance of ADA on PK, PD biomarkers and safety (e.g. infusion associated reactions, hypersensitivity) is more evident when assessed in the context of either quartile or tertile ADA titer groups as well as evaluation of neutralizing activity. Determining relevance of ADA on efficacy parameters is complex as the measured clinical outcomes are often distal from the site of drug action and more time is needed to observe the consequences of diminished drug efficacy due to ADA. This outcome is now seen with the class of TNF- α inhibitor drugs and enzyme replacement therapies for some lysosomal storage diseases. Determining a clinically relevant ADA titer can be challenging and therefore, TDM is an alternative approach as data indicate that keeping levels of some biological therapeutics above a threshold value diminishes the probability of ADA generation and improves patient outcome. Post marketing commitments and requirements are mechanisms for obtaining long term data to address practical questions. However, there are challenges with this approach. After approval or licensure, such testing is usually done in a CLIA or other regulated clinical diagnostic lab. The lack of standardized assays to assess drug concentrations and ADA further complicates

interpretation of results and identification of the reason for the loss of response. Companies, in collaboration with health care providers and insurers, have a responsibility to address in clinical practice the key questions regarding ADA and therapeutic drug monitoring, provide actionable answers, and establish means to make such testing readily available to ensure patients receive efficacious drug dosing. Robust analyses of the economic impact of ADA may motivate payers to support these efforts.

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All authors contributed to the article and approved the submitted version.

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Molecular Landscape of Anti-Drug Antibodies Reveals the Mechanism of the Immune Response Following Treatment With TNF α Antagonists

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Drugs formulated from monoclonal antibodies (mAbs) are clinically effective in various diseases. Repeated administration of mAbs, however, elicits an immune response in the form of anti-drug-antibodies (ADA), thereby reducing the drug's efficacy. Notwithstanding their importance, the molecular landscape of ADA and the mechanisms involved in their formation are not fully understood. Using a newly developed quantitative bio-immunoassay, we found that ADA concentrations specific to TNF α antagonists can exceed extreme concentrations of 1 mg/ml with a wide range of neutralization capacity. Our data further suggest a preferential use of the λ light chain in a subset of neutralizing ADA. Moreover, we show that administration of TNF α antagonists result in a vaccine-like response whereby ADA formation is governed by the extrafollicular T cell-independent immune response. Our bio-immunoassay coupled with insights on the nature of the immune response can be leveraged to improve mAb immunogenicity assessment and facilitate improvement in therapeutic intervention strategies.

Keywords: immunogenicity, anti-drug antibodies, next generation sequencing, antibody repertoire, proteomics, high-throughput sequencing, monoclonal antibody, biologics

INTRODUCTION

More than 30 years since the approval of the first therapeutic monoclonal antibody (mAb) for clinical use, the therapeutic mAb market has expanded exponentially, establishing mAbs as one of the leading biopharmaceutical therapeutic modalities (1). Although mAbs hold significant promise for improving human health, their repeated administration is often highly immunogenic and can elicit an undesirable anti-drug antibody (ADA) response (2). The formation of an ADA response interferes with the effect of the drug or neutralizes it thereby altering the drug's pharmacokinetic (PK) and pharmacodynamic (PD) properties and reducing its efficacy (3), and eventually may lead to a severe adverse immune reaction in humans (4).

Immunogenicity of mAbs and the formation of an ADA response has been suggested to be dependent on the interplay between factors related to the drug itself (e.g., non-human sequence, glycosylation, impurities, aggregation), to the patient (e.g., disease type, genetic factors,

concomitant immunomodulators), or to the drug's route and frequency of administration (5, 6). However, the molecular mechanisms that lead to the induction of ADA are not well-understood and were initially thought to be related to the murine origin of the mAbs because they were recognized as "non-self" by the human immune system. This idea propelled the mAb discovery field to focus on engineering refined mAbs by reducing the non-human portions and developing chimeric, humanized, and fully human mAbs by using human libraries or humanized mice at the mAb discovery phase (7).

Unfortunately, this strategy did not abolish the immunogenicity potential of mAbs and the associated induction of ADA. The question of why and how ADA develop is further complicated by data indicating that some patients develop ADA, and some do not, and by the observation that the extent of immunogenicity may differ among patients receiving the same mAb (8). ADA that develop in patients treated with an mAb can be stratified into two main categories: (1) neutralizing ADA (*ntADA*) that directly block and interfere with the drug's ability to bind its target, and (2) non-neutralizing ADA (i.e., binding ADA *bADA*) that recognize other epitopes on the drug while still retaining the mAb binding activity (9). *ntADA* are generally considered to be more important in the clinical setting than *bADA* because they directly reduce a drug's efficacy. However, *bADA* may indirectly reduce the therapeutic efficacy of an mAb by compromising bioavailability or accelerating drug clearance from the circulation. In both cases, *ntADA* and *bADA* substantially alter the PK and PD of the mAb being administered (10).

Originator and biosimilar tumor necrosis factor alpha (TNF α) antagonistic mAbs are used extensively in clinical settings to treat inflammatory bowel disease (IBD; e.g., Crohn's disease and ulcerative colitis), rheumatoid arthritis, and other chronic inflammatory associated disorders such as psoriasis, psoriatic arthritis, and ankylosing spondylitis (11). TNF α antagonists help reduce inflammatory responses by targeting both membrane-bound and soluble TNF α . Neutralizing soluble TNF α prevents its binding to its receptor and impedes the secretion and upregulation of the signal cascade, thereby inhibiting its biological activity. The binding of TNF α antagonists to transmembrane TNF α on immune effector cells causes their destruction by inducing cell apoptosis or cell lysis through reverse signaling (12).

Currently, five TNF α antagonists have been approved by both the U.S. Food and Drug Administration and the European Medicines Agency: infliximab (IFX), adalimumab (ADL), etanercept, golimumab, and certolizumab pegol (2). Additionally, several biosimilars have already been approved or are in various stages of development (13). Both IFX and ADL belong to the group of TNF α antagonists and are routinely used in clinical settings to treat inflammatory diseases. IFX is a chimeric mAb (75% human and 25% murine), whereas ADL is fully human. The reported immunogenicity extent of these drugs is inconsistent. Whereas, pharmaceutical companies report 10–15 and 2.6–26% immunogenicity for IFX and ADL, respectively (14), clinical data suggest higher immunogenicity rates for these drugs (15). Patients treated with IFX and ADL can be stratified

based on the characteristics of their response to treatment or lack thereof. Primary non-responders are patients whose disease does not respond to the drug at all, and a certain subset of these may be mediated via early formation of ADA (15, 16). Secondary non-responders are patients who initially respond to the drug but later fail treatment, often due to development of ADA (for IFX, this was reported to develop mostly within 12 months of treatment initiation) (16).

Studies reporting immunogenicity following mAb administration and ADA prevalence have been inconsistent due in part to the various assay formats used to monitor immunogenicity in the clinic (17). Current limitations of each available format might reduce utility in clinical and research settings and complicate data interpretation. Some assays have a poor dynamic range and may generate false negative results because of interfering interaction with another circulating drug, or conversely, false positive results due to the presence of other antibodies such as rheumatoid factor (18). The pros and cons of available ADA detection assays were previously elaborated, and the formation of ADA following treatment with IFX, ADL, and other TNF α antagonists, including newly developed biosimilars, have been extensively studied and reviewed elsewhere (5, 19–21).

Notwithstanding the effort invested in understanding the reasons that mAb immunogenicity and strategies to increase mAb efficacy, little is known about the molecular mechanism that governs the formation of ADA following treatment with an mAb.

In this study, we investigated the molecular landscape of ADA following treatment with TNF α antagonists. First, we developed a simple bio-immunoassay that accurately quantifies ADA levels in patient sera. We further modified the bio-immunoassay to evaluate the neutralization capacity of the ADA. Next, we aimed to profile the immune response following mAb administration. We used flow cytometry to determine the frequency of B cells in the circulation and whether the dynamics of the immune response was akin to vaccine response. Finally, we used next-generation sequencing (NGS) and high-resolution shotgun tandem mass spectrometry (LC-MS/MS) to elucidate the molecular composition of serum ADA. Using our bio-immunoassay we found that ADA levels in sera from 54 patients ranged between 2.7 and 1268.5 $\mu\text{g/ml}$. The modified bio-immunoassay enabled us to differentiate between patients who have high and low neutralization capacity. Interestingly, we found that patients with a high neutralization capacity showed a strong bias in the λ/κ light chain ratio thereby suggesting that *ntADA* exhibits a preference for λ light chains.

To elucidate the nature of the immune response following drug administration we chose to study a patient with IBD who was treated with IFX and who had high ADA levels and neutralization capacity. At 10 days (D10) following IFX infusion, the patient exhibited an ~ 13 -fold increase in the frequency of plasmablasts (PB) and unchanged frequency of activated memory B cells (mBC), compared with the pre-infusion time point (D0). Comparative NGS analysis of the antibody heavy chain variable region (V_H) from isolated PB at D0 and D10, showed a significant temporal decrease in the level of somatic hypermutation (SHM) and an increase in the length of the complementary determining region 3 of the antibody heavy chain (CDRH3).

Moreover, the proteomic analysis of serum ADA supports the observation obtained from the neutralization capacity assays, that a preference for using λ light chains exists. These data suggest a possible mechanism whereby the humoral immune response following the administration TNF α antagonists is governed by a T cell-independent (TI) response. This response may be induced by the formation of immunocomplexes (drug-TNF α -ADA) serving as a strong driver of immunogenicity that in-turn diverts the immune response to TI pathway were B cells are activated by B cell receptor (BCR) cross-linking.

MATERIALS AND METHODS

Over Expression and Purification of rhTNF α

The sequence-encoding residues Val77–Leu233 of human TNF α was cloned and fused to the N-terminal 6xHis tag in pET-28a+ vector (Novagen) and transformed into *Escherichia coli* Rosetta (DE3) cells (Novagen). A single colony was inoculated into 2 ml LB supplemented with Kanamycin at final concentration of 100 μ g/ml and incubated overnight (O.N.) at 37°C, 250 RPM. The culture was next re-inoculated into a 0.5 L Erlenmeyer containing LB supplemented with Kanamycin, and grown at 37°C 250 RPM until O.D.₆₀₀ ~0.6–0.8 was reached. Induction was carried out by supplementing bacterial culture with IPTG (0.1 mM final concentration) and incubating the culture for 3 h at 37°C, 250 RPM. Bacterial cells were harvested by centrifugation at 8,000 RPM, 15 min, at 10°C (SORVALL RC6 Plus, Thermo Fisher Scientific) and cell pellet was stored O.N. at –20°C. Next, pellet was re-suspended in 30 ml of binding buffer (50 mM sodium phosphate buffer pH 8.0, 300 mM NaCl, 10 mM imidazole) and sonicated on ice for 8 cycles of 30 s pulse with 2 min pause (W-385 sonicator, Heat Systems Ultrasonics). Following sonication, cells were centrifuged at 12,000 RPM, 30 min, 4°C (SORVALL RC 6+) and supernatant was applied to a HisTrap affinity column (GE Healthcare) that was pre-equilibrated with binding buffer. All affinity purification steps were carried out by connecting the affinity column to a peristaltic pump with flow rate of 1/ml/min. Column was washed with 5 column volumes (CV) of wash buffer (50 mM Sodium phosphate, pH 8.0, 300 mM NaCl, 10% glycerol, 20 mM imidazole) followed by elution of rhTNF α with 5CV of elution buffer (50 mM Sodium phosphate, pH 6.0, 300 mM NaCl, 10% glycerol, 500 mM imidazole). Elution was collected in 1 ml fractions and were analyzed by 12% SDS–PAGE. Fractions containing clean rhTNF α were merged and dialyzed using Amicon Ultra (Mercury) cutoff 3 K against PBS (pH 7.4). Dialysis products were analyzed by 12% SDS–PAGE for purity and concentration was measured using Take-5 (BioTek Instruments). To test functionality of the produced rhTNF α , 96 well-plate (Nunc MaxiSorp™ flat-bottom, Thermo Fisher Scientific) was coated with 1 μ g/ml (in PBS) of purified rhTNF α and commercial hTNF α (PHC3011, Thermo Fisher Scientific) and incubated at 4°C O.N. ELISA plates were then washed three times with PBST (0.1% v/v Tween 20 in PBS) and blocked with 300 μ l of 2% w/v BSA in PBS for 1 h at 37°C. Next, ELISA plates were washed three time with PBST, and incubated for 1 h, room temperature (RT) in triplicates with anti-TNF α mAb (Infliximab or Adalimumab) in 2% w/v BSA,

PBS at the starting concentration of 50 nM with 3-fold dilution series. Plates were then washed three times with PBST with 30 s incubation time at each washing cycle. For detection, 50 μ l of anti-human H+L HRP conjugated antibody (Jackson) was added to each well (1:5,000 ratio in 2% w/v BSA in PBS) and incubated for 1 h at RT, followed by three washing cycles with PBST. Developing was carried out by adding 50 μ l of 3,3',5,5'-Tetramethylbenzidine (TMB, Southern Biotech) and reaction was quenched by adding 50 μ l 0.1 M sulfuric acid. Plates were read using the Epoch Microplate Spectrophotometer ELISA plate reader (BioTek Instruments).

Over Expression and Purification of IdeS

The coding sequence corresponding to amino acid residues 38–339 of *S. pyogenes* IdeS (numbered from the start of the signal sequence) was sub-cloned into the expression vector pET28b (Novagen). The coding sequencing was sub-cloned at the 3' end of Thioredoxin 6xHis-TEV. The complete construct was sub-cloned as previously described (22) and was kindly donated by Dr. Ulrich von Pawel-Rammingen from the Department of Molecular Biology, Umea University. The transformation of pET-TRX_b plasmid harboring the IdeS encoding gene (pET-IdeS) was carried out as follows: 200 μ l of chemical-competent *E. coli* BL21-DE3 cells were thawed on ice for 20 min. Fifty nanograms of the plasmid pET-IdeS was added to the thawed competent cells and incubated on ice for 20 min with gentle mixing every 5 min. Next, heat shock was applied by incubating the cells at 42°C for 2 min followed by incubation on ice water for 2 min. For phenotypic expression, 800 μ l of LB was added, and cells were incubated at 37°C, 250 RPM for 1 h in a horizontal position. Cells were plated on LB agar supplemented with Kanamycin and incubated at 37°C overnight (O.N). Single colony was inoculated into 2 ml LB supplemented with Kanamycin and incubated O.N. at 37°C, 250 RPM. Next day, 2 ml from the grown cultures were inoculated into two 2 L flasks, each containing each 500 ml LB supplemented with Kanamycin. Over expression and purification of IdeS was carried out as described for rhTNF α with a minor modification as follow: IdeS was eluted with imidazole gradient (50, 150, 500 mM imidazole), total of 20 ml. Twenty fractions of 1 ml were collected from each elution step and evaluated for their purity using 12% SDS–PAGE. All fractions containing clean IdeS were merged and dialyzed O.N. at 4°C against 4 L of PBS (pH 7.4), using SnakeSkin dialysis tubing with 10 kDa cutoff (Thermo Fisher Scientific). Dialysis products were analyzed by 12% SDS–PAGE.

Production of mAb-F(ab')₂

Intact clinical grade IFX or ADL (designated here as mAb) were digested using in-house produced IdeS. Ten milligrams of mAb was incubated with 300 μ g of IdeS in the final volume of 500 μ l PBS for 2.5 h at 37°C, followed by a spike-in of additional 300 μ g of IdeS to achieve full digestion of the Fc fragments. IdeS inactivation was carried out by adding 0.1 M of citric acid pH 3 and incubation for 1 min at RT followed by the addition of PBS (pH 7.4) to neutralized acidic pH. Next, reaction mixture was applied to a 1 mL HiTrap KappaSelect affinity column (GE Healthcare Life Sciences). All affinity purification

steps were carried out by connecting the affinity column to a peristaltic pump with flow rate of 1 ml/min. The reaction mixture was recycled three times through the KappaSelect column to maximize the capture of intact mAb and mAb-F(ab')₂. KappaSelect column was subsequently washed with 5 CV of PBS and eluted with 10 CV of 100 mM glycine-HCl (pH 2.7). Collected 1 ml elution fractions were immediately neutralized with 100 µl of 1.5 M Tris-HCl (pH 8.8). Next, the recovered intact mAb and mAb-F(ab')₂ fragments were applied to a custom packed 1 ml Protein-G agarose column (GenScript). The reaction mixture was recycled three times through the column, which was subsequently washed with 5 CV of PBS and eluted with 10 CV of 100 mM glycine-HCl (pH 2.7). The 10 ml elution fraction was immediately neutralized with 1 ml of 1.5 M Tris-HCl (pH 8.8). The recovered 10 ml mAb-F(ab')₂ fragments were dialyzed overnight at 4°C against 4 L of PBS (pH 7.4) using SnakeSkin dialysis tubing with 10 kDa cutoff (Thermo Fisher Scientific). Recovered mAb-F(ab')₂ sample were evaluated for purity by SDS-PAGE and their concentration measured by Take5 (BioTek instruments).

To test the functionality of the produced mAb-F(ab')₂, 96 ELISA plates (Nunc MaxiSorp™ flat-bottom, Thermo Fisher Scientific) were coated with 1 µg/ml of rhTNFα in PBS and incubated at 4°C O.N. ELISA plates were then washed three times with PBST and blocked with 300 µl of 2% w/v BSA in PBS for 1 h at 37°C. Next, 50 nM of intact mAb and mAb-F(ab')₂ (IFX or ADL) in blocking solution was added to each well in triplicates in a 3-fold dilution series, and plates were incubated at RT for 1 h. Next, plates were washed three times with PBST with 30 s incubation time at each washing cycle. For detection, HRP conjugated anti-human kappa light chain (Jackson) was added to each well (50 µl, 1:5,000 ratio in 2% w/v BSA in PBS) and incubated for 1 h at RT, followed by three washing cycles with PBST. Developing was carried out by adding 50 µl of TMB and reaction was quenched by adding 0.1 M sulfuric acid. Plates were read using the Epoch Microplate Spectrophotometer ELISA plate reader. To evaluate the purity of the mAb-Fa(b')₂ samples (i.e., to make sure there are no traces of intact antibody or Fc fragment in the sample), 96 ELISA plate (Nunc MaxiSorp™ flat-bottom, Thermo Fisher Scientific) were coated with 5 µg/ml of intact mAb and mAb-F(ab')₂ in PBS and incubated at 4°C O.N. Next, plates were washed three times with PBST and blocked with 300 µl 2% w/v BSA in PBS for 1 h at 37°C. Next, plates were washed three times with 300 µl PBST, followed by the incubation with HRP conjugated anti-human IgG Fc antibody (Jackson) diluted 1:5,000 in PBST. Developing was carried out by adding 50 µl of TMB and reaction was quenched by adding 0.1 M sulfuric acid. Plates were read using the Epoch Microplate Spectrophotometer ELISA plate reader (BioTek Instruments).

Generation of ADA Standard

A pool of 17 ADA to IFX positive sera were collected at Sheba Medical Center, and passed through a 2 ml custom packed protein G agarose column (GenScript). The pooled sera was recycled three times over the column, which was subsequently washed with 5 CV of PBS and eluted with 10 CV of 100 mM glycine-HCl (pH 2.7). The 10 ml elution fraction was immediately

neutralized with 1 ml of 1.5 M Tris-HCl (pH 8.8). The purified mAbs were immediately passed over a custom made rhTNFα affinity column (NHS-activated agarose beads, Thermo Fisher Scientific) in gravity mode. The purified mAbs were recycled three times over the column, which was subsequently washed with 5 CV of PBS and eluted with 10 CV of 100 mM glycine-HCl (pH 2.7). The 10 ml elution fraction was immediately neutralized with 1 ml of 1.5 M Tris-HCl (pH 8.8). The purified mAbs were dialyzed overnight at 4°C against 4 L of PBS (pH 7.4) using SnakeSkin dialysis tubing with 10 kDa cutoff (Thermo Fisher Scientific). Purified mAbs were analyzed for purity using 12% SDS-PAGE and concentration was determined by Take3 (BioTek instruments).

To test functionality, 96 ELISA plate were coated with 5 µg/ml of mAb-F(ab')₂ in PBS (pH 7.4) and incubated at 4°C O.N. ELISA plates were then washed three times with PBST and blocked with 300 µl of 2% w/v BSA in PBS for 1 h at 37°C. Next, 50 nM of the purified ADA in blocking solution were added to each well in triplicates with 3-fold dilution series and plates were incubated at RT for 1 h. Next, plates were washed three times with PBST with 30 s incubation time at each washing cycle. Next, anti-human Fc HRP conjugate (Jackson) was added to each well at the detection phase (50 µl, 1:5,000 ratio in 2% w/v BSA in PBS) and incubated for 1 h at RT, followed by three washing cycles with PBST. Developing was carried out by adding 50 µl of TMB and reaction was quenched by adding 0.1 M sulfuric acid. Plates were read using the Epoch Microplate Spectrophotometer ELISA plate reader.

Quantitative Measurement of ADA in Serum

The schematic configuration of the bio-immunoassay for the quantitative measurement of ADA in serum is described in **Figure 3B** and was carried out as follows: ELISA plates that were coated overnight at 4°C with 5 µg/ml produced IFX-F(ab')₂ in PBS (pH 7.4). ELISA plates were then washed three times with PBST and blocked with 300 µl of 2% w/v BSA in PBS for 1 h at 37°C. Next, triplicates of 1:400 diluted serum samples were added at triplicates and serially diluted 2-fold in 2% w/v BSA in PBS, 10% horse serum (Biological Industries) and 1% Tween 20 in PBS (1:400–1:51,200 serum dilution factor). Plates were incubated for 1 h at RT. On the same plate, serial dilutions of 10 nM ADA standard were incubated in triplicate and serially diluted 2-fold in 2% w/v BSA in PBS, 10% horse serum (Biological Industries) and 1% Tween 20 in PBS, to allow the conversion of the tested serum to units per milliliter. ELISA plates were washed three times with PBST and 50 µl of HRP conjugated anti-human IgG Fc was added to each well (50 µl, 1:5,000 ratio in 2% w/v BSA in PBS) and incubated for 1 h at RT. ELISA plate was then washed three times with PBST and developed by adding 50 µl of TMB followed by quenching with 50 µl 0.1 M sulfuric acid. Plates were read using the Epoch Microplate Spectrophotometer ELISA plate reader.

Neutralization Index of ADA

Neutralization capacity was determined using ELISA plates that were coated overnight at 4°C with 5 µg/ml IFX-F(ab')₂ in PBS (pH 7.4). Next, coating solution was discarded and ELISA plates

were blocked with 300 μ l of 2% w/v BSA in PBS for 1 h at 37°C. Blocking solution was discarded and 50 μ l of 200 nM rhTNF α in 2% w/v BSA were added to the positive rhTNF α wells, and 2% w/v BSA in PBS was added to the negative rhTNF α wells for 1 h at RT. Next, triplicates of 1:400 diluted serum samples with/without 200 nM rhTNF α were added to the positive/negative rhTNF α wells, respectively, and serially diluted 2-fold in 2% w/v BSA, 10% horse serum (Biological Industries) and 1% Tween 20 in PBS (1:400–1:51,200 serum dilution factor). Plates were incubated for 1 h at RT. ELISA plates were washed three times with PBST and 50 μ l of HRP conjugated anti-human IgG Fc antibody or anti HRP conjugated His-tag antibody were added at the detection phase (50 μ l, 1:5,000 ratio in 2% w/v BSA in PBS) and incubated for 1 h at RT, followed by three washing cycles with PBST. Developing was carried out by adding 50 μ l of TMB and reaction was quenched by adding 0.1 M sulfuric acid. Plates were read using the Epoch Microplate Spectrophotometer ELISA plate reader.

Neutralization index was calculated as following: an ELISA equation curve was calculated separately for wells with and without rhTNF α , using the GraphPad Prism software. The average triplicate signal which are 3 $\times$ standard deviation above the background signal was substituted in the ELISA equation curve to extract the serial dilution value. The logarithmic difference of the value with/without rhTNF α represents the neutralization index.

Blood Processing

IFX treated patients with IBD cared for in the Department of Gastroenterology at the Sheba medical center were included in the study. All subjects signed an informed consent, and the study was approved by the Ethics Committee of the medical center. All patients received IFX on a scheduled regimen and blood samples were drawn immediately before their scheduled IFX infusion. Blood was collected into a single Vacutainer Lithium Heparin collection tube (BD Bioscience).

For NGS analysis, blood was collected from a male donor treated with IFX, before IFX administration and 10 days after administration. Thirty milliliters of peripheral blood were collected into 3 single Vacutainer K-EDTA collection tubes (BD Biosciences). Collection of peripheral blood mono-nuclear cells (PBMCs) was performed by density gradient centrifugation, using Uni-SepMAXI+ lymphocyte separation tubes (Novamed) according to the manufacturer's protocol.

Fluorescence-Activated Cell Sorting Analysis and Sorting of B Cell Populations

PBMCs were stained for 15 min in cell staining buffer (BioLegend) at RT in the dark using the following antibodies: anti-CD3–PerCP (clone OKT3; BioLegend), anti-CD19–Brilliant Violet 510 (clone HIB19; BioLegend), anti-CD27–APC (clone O323; BioLegend), anti-CD38–APC–Cy7 (clone HB-7; BioLegend), and anti-CD20–FITC (clone 2H7; BioLegend).

The following B cell population was sorted using a FACSaria cell sorter (BD Bioscience): CD3–CD19+CD20–CD27+CD38^{high}.

B cell subpopulations were sorted and collected into TRI Reagent solution (Sigma Aldrich) and frozen at –80°C.

Amplification of V_H and V_L Repertoires From B Cells

Total RNA was isolated using RNeasy micro Kit (Qiagen), according to manufacturer's protocol. First-strand cDNA generation was performed with 100 ng of isolated total RNA using a SuperScript RT II kit (Invitrogen) and oligo-dT primer, according to manufacturer's protocol. After cDNA synthesis, PCR amplification was performed to amplify the V_H and V_L genes using a primer set described previously (23) with overhang nucleotides to facilitate Illumina adaptor addition during the second PCR (Table S1). PCR reactions were carried out using FastStartTM High Fidelity PCR System (Roche) with the following cycling conditions: 95°C denaturation for 3 min; 95°C for 30 s, 50°C for 30 s, and 68°C for 1 min for four cycles; 95°C for 30 s, 55°C for 30 s, and 68°C for 1 min for four cycles; 95°C for 30 s, 63°C for 30 s, and 68°C for 1 min for 20 cycles; and a final extension at 68°C for 7 min. PCR products were purified using AMPure XP beads (Beckman Coulter), according to manufacturer's protocol (ratio $\times$ 1.8 in favor of the beads). Recovered DNA products from the first PCR was applied to a second PCR amplification to attach Illumina adaptors to the amplified V_H and V_L genes using the primer extension method as described previously (24). PCR reactions were carried out using FastStartTM High Fidelity PCR System (Roche) with the following cycling conditions: 95°C denaturation for 3 min; 95°C for 30 s, 40°C for 30 s, and 68°C for 1 min for two cycles; 95°C for 30 s, 65°C for 30 s, and 68°C for 1 min for 7 cycles; and a final extension at 68°C for 7 min. PCR products were applied to 1% agarose DNA gel electrophoresis and gel-purified with ZymocleanTM Gel DNA Recovery Kit (Zymo Research) according to the manufacturer's instructions. V_H and V_L libraries concentration were measured using Qubit system (Thermo Fisher Scientific) and library quality was assessed using the Bioanalyzer 2100 system (Agilent) or the 4200 TapeStation system (Agilent). All V_H libraries were produced in duplicates starting with RNA as the common source template. The V_L were produced with one replicate.

V_H and V_L libraries from sorted B cell were subjected to NGS on the MiSeq platform with the reagent kit V3 2 $\times$ 300 bp paired-end (Illumina), using an input concentration of 16 pM with 5% PhiX.

Raw fastq files were processed using our recently reported ASAP webserver (25). ASAP analysis resulted in a unique, full-length V_H and V_L gene sequences database for each time point. The resultant database was used as a reference database to search the LC-MS/MS spectra.

Proteomic Analysis of the Serum ADA to IFX

Total IgG from each time point (D0, D10) were purified from 7 to 10 ml of serum by protein G enrichment. Serum was diluted 2-fold and passed through a 5 ml Protein G agarose column (GeneScript). The diluted serum was recycled three times over

the column, which was subsequently washed with 10 CV of PBS and eluted with 7 CV of 100 mM glycine-HCl (pH 2.7). A total of 35 fractions of 1 ml were collected and immediately neutralized with 100 μ l of 1.5 M Tris-HCl (pH 8.8). All elution fractions were evaluated for their purity using 12% SDS-PAGE and 11 purified 1 ml IgG fractions were combined and dialyzed overnight at 4°C against 4 L of PBS (pH 7.4) using SnakeSkin dialysis tubing with 1 kDa cutoff (Thermo Fisher Scientific).

Next, 9 mg of total IgG were digested with 100 μ g of IdeS in the final volume of 2 ml PBS for 5 h at 37°C. IdeS inactivation was carried out by adding 0.1 M of citric acid pH 3 and incubation for 1 min at RT followed by the addition of PBS (pH 7.4) to neutralize the low pH. Total serum F(ab')₂ was then applied to a one ml custom made affinity column comprised of IFX-F(ab')₂ coupled to NHS-activated agarose beads (Thermo Fisher Scientific). The purified serum F(ab')₂ were recycled three times over the affinity column, which was subsequently washed with 5 CV of PBS and eluted with 15 CV of 100 mM glycine-HCl (pH 2.7) and collected into Maxymum Recovery Eppendorf (Axygen Scientific). A total of 30 $\times$ 0.5ml elution fractions and 1 $\times$ 50ml flow-through were immediately neutralized with 50 and 100 μ l, respectively, of 1.5 M Tris-HCl (pH 8.8). The purified antigen-specific F(ab')₂ were dialyzed overnight at 4°C against 4 L of PBS (pH 7.4) using SnakeSkin dialysis tubing with 10 kDa cutoff (Thermo Fisher Scientific). Elution and flow-through fractions were trypsin-digested, and resulting peptides were fractionated and sequenced by nanoflow LC-electrospray MS/MS on an Orbitrap Velos Pro hybrid mass spectrometer (Thermo Scientific), in the UT Austin mass spectrometry core facility as described previously (26). MS/MS raw files were analyzed by MaxQuant software version 1.6.0.16 (27) using the MaxLFQ algorithm (28) and peptide lists were searched against the common contaminants database by the Andromeda search engine (29) and a custom protein sequence database consisting of the donor-specific V_H and V_L sequences derived from NGS of individual donor B cells. All searches were carried out with cysteine carbamidomethylation as a fixed modification and methionine oxidations as variable modifications. The false discovery rate was set to 0.01 for peptides with a minimum length of seven amino acids and was determined by searching a reverse decoy database. Enzyme specificity was set as C-terminal to arginine and lysine as expected using trypsin as protease, and a maximum of two missed cleavages were allowed in the database search. Peptide identification was performed with an allowed initial precursor mass deviation up to 7 ppm and an allowed fragment mass deviation of 20 ppm. For LFQ quantification the minimal ratio count was set to 2, and match between runs was performed with three mass-spec injections originating from the same sample. MaxQuant output analysis file, "peptides.txt," was used for further processing. Total peptides that were identified in the elution samples were filtered using the following criteria: (a) were not identified as contaminants; (b) did not match to the reversed decoy database; (c) were identified as peptides derived from the region comprising the CDRH3, J region, FR4 and the ASTK motif (derived from the N-terminal of the C_H1 region). The CDRH3 derived peptides were further characterized as informative CDRH3 peptides (iCDRH3 peptides) only if they map exclusively to a single

antibody clonotype. A clonotype was defined as all sequences that comprise CDRH3 with the same length and identity tolerating one amino acid mismatch, and same V, J family. The intensities of high confidence iCDRH3 peptides were averaged between replicates while including only peptides that were observed in at least two out of the three replicates. Clonotype frequencies within each sample were calculated using only iCDRH3 peptides and were determined to be antigen-specific if their frequency in the elution fraction was at least 5-fold greater than their frequency in the flow-through fraction. The CDRH3 sequences identified by the mapping of high confidence MS/MS peptides were used to generate a complete list of full length VH sequences. These VH sequences were used to analyze the repertoire measures of the antibodies that were identified in the donors' serum.

Same filtering criteria was applied to peptides derived from the constant region of both κ and λ light chains. By quantifying the accumulative intensities of these peptides, we calculated the ratio of κ : λ light chain from antibodies that were derived from the affinity column elution fraction which represent both *nt*ADA and *b*ADA, and in the affinity column flow through fraction which represent the depleted ADA fraction.

Study Population

IFX and ADL treated patients with IBD cared for in the Departments of Gastroenterology at Sheba medical center were included in the study. All subjects signed an informed consent, and the study was approved by the Ethics Committee of Sheba medical center. IFX and ADL and ADA serum levels were routinely measured at trough immediately before infusion. All patients received IFX and ADL on a scheduled regimen. All patients that were included in this study exhibited low through levels of IFX and ADL.

Statistical Analysis

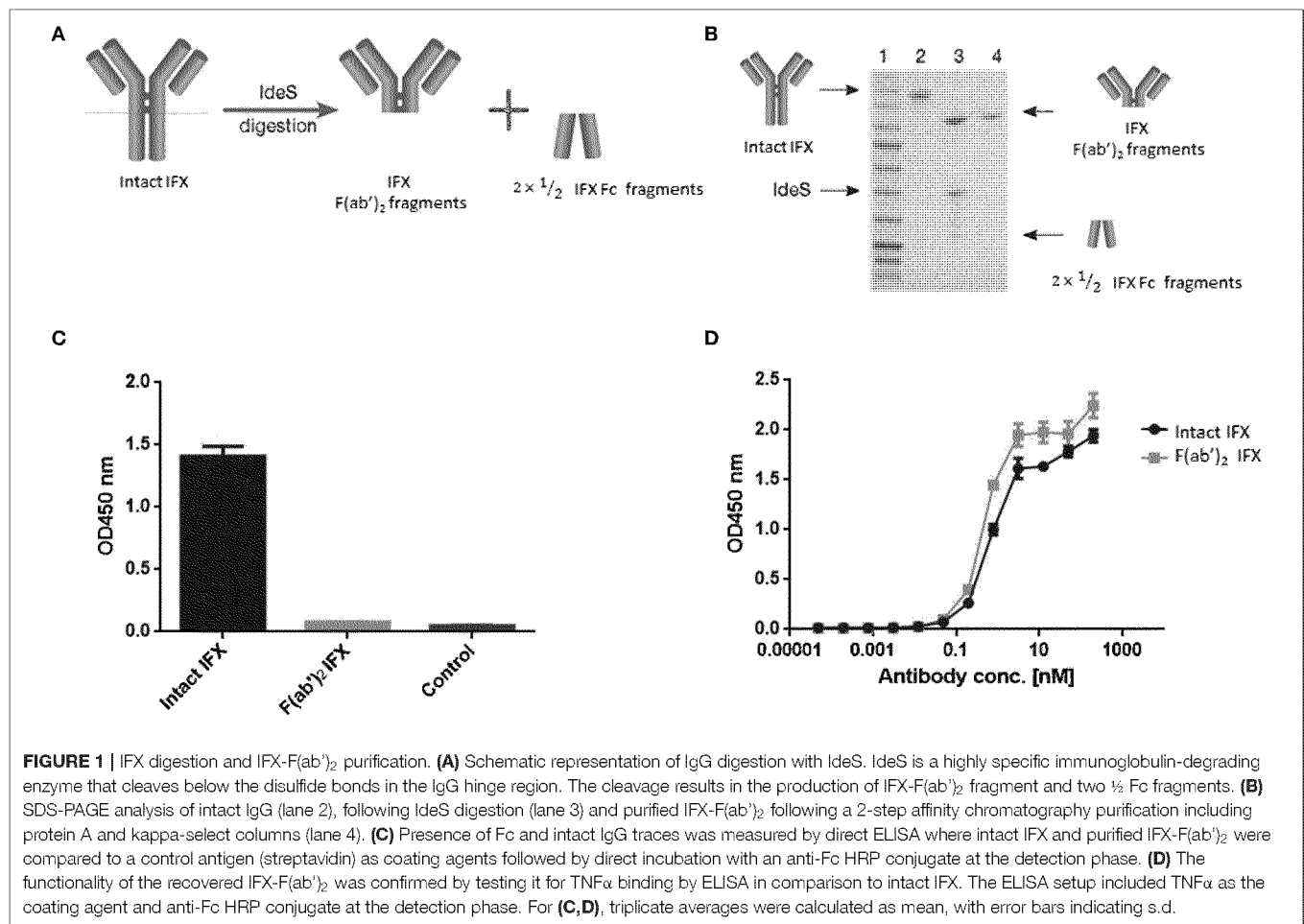
All curves were fitted on a sigmoidal dose-response curve and EC50 of each was calculated. Mann-Whitney test was used to compare continuous variables. All reported *P*-values were two-tailed, and a *P* < 0.05 were considered statistically significant. All statistics were performed with GraphPad Prism software (version 7, San Diego, California).

RESULTS

Production of mAb-F(ab')₂ to Be Used in the Bio-Immunoassay

To investigate the molecular landscape of ADA following mAb administration we first aimed to develop an accurate, sensitive, robust bio-immunoassay to determine ADA levels in sera. The working hypothesis was that anti-idiotypic antibodies dominate the ADA compartment (21) thus, the developed bio-immunoassay was based on the drugs' F(ab')₂ portion to be used as the antigen (i.e., coating agent).

To achieve this, we used the immunoglobulin G (IgG)-cleaving enzyme (IdeS), a cysteine proteinase enzyme that proteolytically cleaves immunoglobulins below the hinge region [(30); Figure 1A]. IFX was digested using IdeS by incubating 10 mg of clinical grade mAb with IdeS to reach near complete



digestion. Next, IFX-F(ab')₂ was purified from Fc regions and undigested full IFX by consecutive affinity chromatography steps comprising protein A and kappaSelect columns.

Recovered IFX-F(ab')₂ purity was evaluated by SDS-PAGE (Figure 1B) and ELISA (Figure 1C) to ensure that the IFX-F(ab')₂ exhibits no traces of IFX-Fc/undigested IFX that will contribute to the background level when using anti-Fc HRP conjugate at the detection phase. Recovered IFX-F(ab')₂ samples were found to be highly pure with basal anti-Fc signal levels similar to the signal observed in the control samples. The produced IFX-F(ab')₂ was tested for functionality by measuring its TNFα binding capacity, using ELISA with TNFα as the coating agent, and was found to show similar functionality as that of the intact IFX (Figure 1D). ADL was subjected to the same preparative pipeline and demonstrated similar results (Figure S1).

ADA Standard Curve

Quantification of total ADA in serum requires a standard reference. Thus, we generated a standard ADA pool that facilitates the quantification of ADA levels in sera of patient treated with IFX. ADA were pooled from several serum samples collected from patients treated with IFX and purified by

consecutive affinity chromatography steps comprising protein G and a custom-made IFX-F(ab')₂ affinity columns. We confirmed the affinity enrichment of ADA by applying the affinity chromatography elution fraction to ELISA with IFX-F(ab')₂ as the coated antigen (Figure 2A). The purity and concentration the recovered ADA were determined by SDS-PAGE (Figure 2B) and nanodrop.

Maximal serum concentration used in a bio-immunoassay (e.g., serum diluted 1:100 or 1:200) is a major factor that may contribute to high background signal levels due to non-specific binding. Screening several maximal serum dilutions showed that 1:400 initial serum dilution demonstrates the lowest background signal (data not shown). To evaluate if serum will affect the signal obtained from purified ADA, we spiked-in purified ADA into negative control serum that was diluted 1:400 in PBS. Serial dilution of spiked-in ADA and purified ADA showed similar signal in ELISA (Figure 2C) indicating that serum does not bias the ADA detection in our developed bio-immunoassay.

Quantitative Measurement of ADA in Serum

ADA detection is technically challenging as both the analyte and antigen are antibodies which may result in the inability

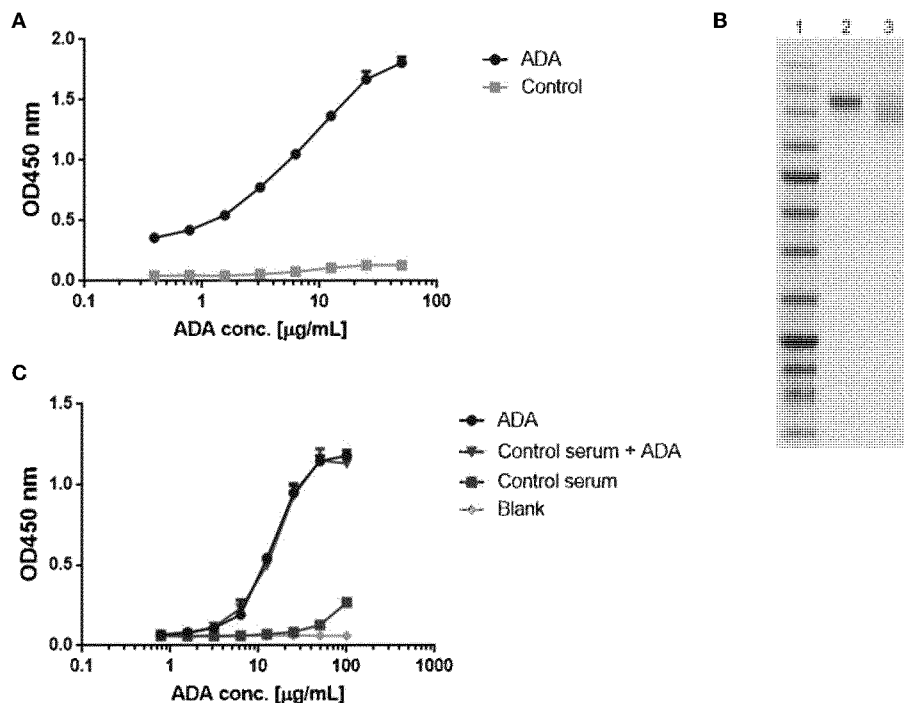


FIGURE 2 | Standard curve for ADA quantification in patients treated with IFX. ADA were purified from sera of 17 patients treated with IFX, utilizing consecutive affinity chromatography steps including protein G and custom made IFX-F(ab')₂ columns. **(A)** Purified ADA were tested in ELISA for functionality. TNF α was used as the coating agent followed by incubation with purified ADA and anti-Fc HRP conjugate at the detection phase. Control included serum obtained from a healthy donor. **(B)** SDS-PAGE analysis of intact IFX (lane 2) and purified ADA (lane 3). **(C)** The effect of serum on ADA standard was tested in ELISA by spiking-in differential concentrations of ADA into ADA negative serum.

to differentiate between the mAb and ADA. To overcome this challenge, many assays were previously developed (5). One of these immunoassays is the anti-human λ chain (AHLC) immunoassay that is used in clinical setups for monitoring the formation of ADA (31). The principle of this assay is to detect ADA comprising λ light chain, thus avoiding cross reactivity with the drug that comprises the κ light chain (Figure 3A).

While AHLC is suitable for monitoring the development of ADA in clinical setups, when one aims to study the molecular composition of ADA there is a need to provide quantitative measures of total ADA in serum. Thus, we developed a new bio-immunoassay based on the F(ab')₂ portion of the mAb. The bio-immunoassay setup is described in Figure 3B and is based on mAb-F(ab')₂ as the coating antigen and anti-Fc HRP conjugate used as the detection antibody. Each of the experimental setups to test ADA in serum included serum from a healthy donor as a control and ADA standard for the quantitation of total ADA.

First, we applied the newly developed bio-immunoassay on two serum sample groups: one negative and one positive for ADA as determined by the AHLC assay [AHLC⁽⁻⁾ and AHLC⁽⁺⁾, respectively]. We also included serum from a healthy subject to serve as a control for the assay specificity (i.e., serum from a subject that was not exposed to IFX). As shown in Figures 3C,D, the ELISA signals obtained when utilizing the new bio-immunoassay were higher compared to the signal

obtained with the AHLC assay. Moreover, applying the new bio-immunoassay on the AHLC⁽⁻⁾ serum (no detected ADA with the AHLC assay) detected relatively high levels of ADA. These results indicate that not all ADA were detected with the AHLC assay as this assay is based on the detection of ADA that comprise the λ light chain only.

Next, to extend and generalize the above results, sera from 54 patients treated with IFX were collected at the Chaim Sheba Medical Center and tested for drug levels and ADA using the AHLC assay. The established cohort showed very low drug trough levels and based on the AHLC results, sera were stratified into two groups: 25 serum samples were identified as AHLC⁽⁻⁾ and 29 as AHLC⁽⁺⁾. Using our newly developed quantitative bio-immunoassay, we found that ADA levels in tested sera ranged between 1.82 and 1268.5 $\mu\text{g/mL}$. Serum ADA levels using AHLC compared to the new bio-immunoassay are summarized in Table 1. More importantly, the new bio-immunoassay demonstrated improved sensitivity compared to AHLC assay manifested in the detection of higher concentrations of ADA in 46 out of the 54 serum samples, of which 17 out of the 54 samples, belong to the AHLC⁽⁻⁾ group. Overall, the average fold increase in ADA detection using the new bio-immunoassay compared to the AHLC assay was 14.13 and 53.26 for the AHLC⁽⁺⁾ and AHLC⁽⁻⁾ groups, respectively.

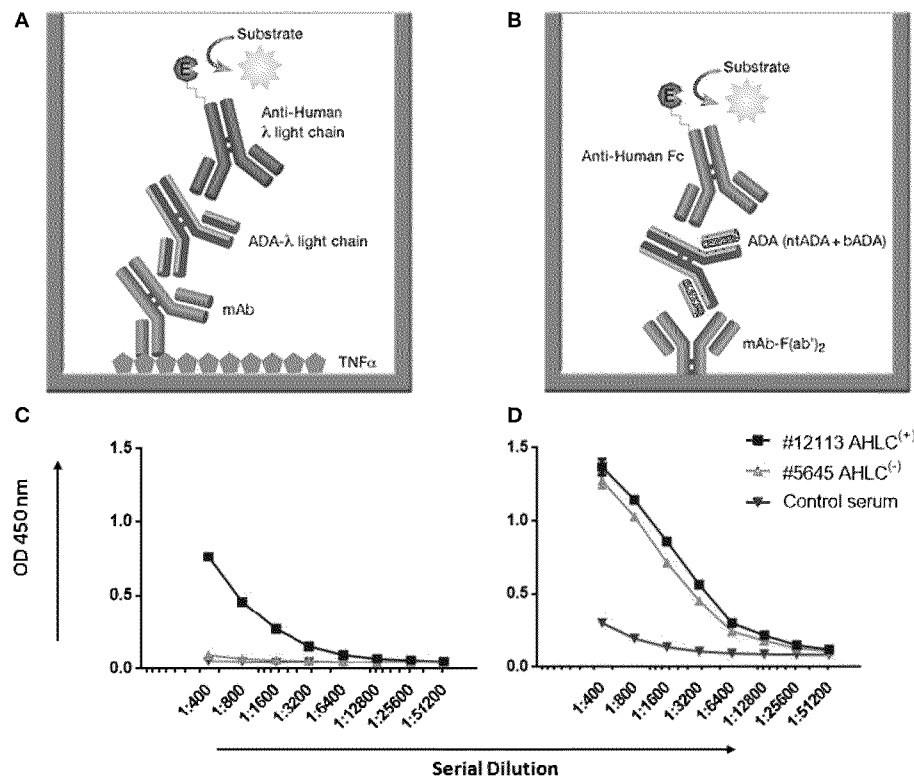


FIGURE 3 | AHLC and the newly developed mAb-F(ab')₂ based bio-immunoassay configuration and their application on serum samples from patients treated with IFX. **(A)** AHLC assay is based on an ELISA where $\text{TNF}\alpha$ is used as the coating agent, following the incubation with the mAb drug followed by serial dilutions of the tested sera. anti- λ HRP conjugate is used at the detection phase. **(B)** The newly developed mAb-F(ab')₂ based bio-immunoassay configuration. The assay is based on an ELISA where mAb-F(ab')₂ is used as the coating agent followed by serial dilutions of the tested sera. Anti-Fc HRP conjugate is used at the detection phase. **(C)** ELISA obtained by utilizing the AHLC assay on two serum samples. Using this assay, one of the tested sera showed detectable levels of ADA [AHLC⁽⁺⁾] and one had no detectable levels of ADA [AHLC⁽⁻⁾]. **(D)** Both serum samples were tested by the newly developed mAb-F(ab')₂ based bio-immunoassay. This assay was able to detect ADA in both sera. For **(C,D)**, averages were calculated as mean from triplicates, with error bars indicating s.d.

Neutralization Index of ADA

Due to high clinical relevance and different mechanism of action of bADA and ntADA, identifying their relative abundances in serum can provide valuable insights regarding the nature of the immune response following mAb administration. We therefore modified our newly developed mAb-F(ab')₂ based bio-immunoassay by blocking the coated IFX-F(ab')₂ binding site with $\text{TNF}\alpha$ in order to obtain a differential signal compared to the unblocked assay (Figure 4A). In order to block the binding site of IFX-F(ab')₂ toward $\text{TNF}\alpha$ and prevent the binding of anti-idiotypic ADA (i.e., ntADA) to the drug, recombinant human $\text{TNF}\alpha$ (rh $\text{TNF}\alpha$) fused to a His-tag was cloned and expressed (see Materials and Methods). In-house production of rh $\text{TNF}\alpha$ was essential, as the N terminal His-tag was used for monitoring the presence of the rh $\text{TNF}\alpha$ throughout the bio-immunoassay.

First, we evaluated the ability of rh $\text{TNF}\alpha$ to inhibit the binding of ADA to the coated IFX-F(ab')₂ by setting up a competitive ELISA where a series of ADA standard concentrations were incubated with a series of fixed rh $\text{TNF}\alpha$ concentrations (data not shown). We observed a competitive effect while rh $\text{TNF}\alpha$ was fixed at the concentration of 5 nM (Figure 4B). This step was

important as it enabled us to determine the ADA equimolar concentration of rh $\text{TNF}\alpha$ to be used that will fully occupy the IFX-F(ab')₂ binding site and will prevent the binding of ntADA to the coated (and blocked) IFX-F(ab')₂. We monitored the presence of rh $\text{TNF}\alpha$ using an HRP-conjugated anti-His tag antibody and observed that if we aim to completely block ADA it is required to use equimolar concentration of rh $\text{TNF}\alpha$ that corresponds to the highest concentration of ADA in the assay (200 nM).

In practice, IFX-F(ab')₂ binding site was blocked with rh $\text{TNF}\alpha$ by prior incubation of serum with the coated IFX-F(ab')₂ hence, the differential signal w/ and w/o the presence of rh $\text{TNF}\alpha$ represent the portion of ADA that could not bind the IFX-F(ab')₂ binding site thus, reflects the neutralization capacity (hereby named neutralization index) of the ADA in the tested serum. Using this assay, we evaluated the neutralization index of the 46 ADA positive sera from patients treated with IFX and 7 ADA positive sera from patients treated with ADL. In sera from patients treated with IFX, we noticed that there are two main neutralization index patterns: those with high differential signal (Figure 5A) and low differential signal (Figure 5B). More interestingly, we found that patients that were

TABLE 1 | ADA concentrations in 54 serum samples from patients treated with IFX.

AHLC ⁽⁻⁾ patients			AHLC ⁽⁺⁾ patients		
Patient #	AHLC $\mu\text{g/ml}$	New bio-immunoassay $\mu\text{g/ml}$	Patient #	AHLC $\mu\text{g/ml}$	New bio-immunoassay $\mu\text{g/ml}$
5645	0	867.33	14655	7.9	121.95
5557	0.9	0	15046	16.6	85.24
5381	1.9	0	15460	21.1	147.89
6497	1.6	26.26	15809	22.8	996.84
6386	1.7	0	15107	6.3	126.5
6259	0.8	41.39	14408	4.8	90.54
6098	1.3	0	4297	8.9	274.6
5993	1.3	0	5048	5.6	49.28
5882	1.7	152.72	5735	6.9	99.67
5822	1.3	0	6393	3.4	289.31
6291	0.7	19.43	6324	6.7	91.14
6616	0.3	84.05	6275	31.8	242.03
7083	1	97.29	6261	27.2	245.52
7041	1	0	6208	5.8	65.35
7004	0.7	4.28	6165	3	2.7
6866	1.3	80.05	6148	7.2	148.88
6788	1.8	46.83	9348	42.2	285.05
6740	1.5	0	8970	59.3	1268.5
14735	0.4	1.86	8816	27.2	396.2
14752	1.2	43.87	7553	46.7	178.83
14879	1.7	1.94	12113	20.9	772.83
14834	0.57	1.96	12104	20.9	441.09
13741	1.3	18	6329	11	87.31
13711	0.3	1.89	8178	7.9	55.11
14278	1.72	1.82	7653	16.1	53.52
			8856	42.4	87.97
			9454	7.8	265.35
			12343	16.5	358.54
			12345	37	329.49

Serum samples were initially stratified into AHLC⁽⁺⁾ and AHLC⁽⁻⁾ based on the AHLC assay used in the clinic. The newly developed bio-immunoassay for the quantification of total ADA was applied on all serum samples and concentration are listed. All ADA concentrations are in $\mu\text{g/ml}$.

stratified as AHLC⁽⁺⁾ have a significantly higher neutralization index compared to those that belong to the AHLC⁽⁻⁾ group (Figure 5C). This suggests that there is a preferential usage of the λ light chain in ntADA as the AHLC⁽⁺⁾ group is *a priori* defined by the presence of ADA comprising the λ light chains. All sera from patients treated with ADL ($n = 7$) were subjected to modified bio-immunoassay and demonstrated high neutralization indexes (Figure S2)

IFX Infusion Induces a Vaccine Like Immune Response

To further investigate the molecular landscape of ADA we explored the dynamics of the B cell response following mAb administration. When investigating well-controlled clinical scenarios such as samples obtained from post-vaccinated individuals, it is convenient to isolate the antigen-specific B cell as they peak at a defined time window (23, 32). However, the characteristics of the humoral response and ADA encoding B

cell dynamics following mAb administration is unknown. Our working hypothesis assumed that the immune response following mAb administration is a vaccine-like response thus; we expected to observe a wave of PB peaking several days after IFX infusion. It was previously demonstrated that boost vaccines induce a strong proliferation of PBs and mBCs that can be detected in the blood circulation several days after the boost (33, 34). To test if IFX administration induces a vaccine like response, we collected blood samples from a patient that was found to be positive to ADA at two time points: prior to IFX infusion (D0) and 10 days after IFX infusion (D10). The second time point (D10) was determined in order to capture an enriched population of antigen-specific PB as well as mBC that enable the establishment of a donor-specific V_H database for the proteomic interpretation of peptides derived from ADA.

Peripheral blood mononuclear cells (PBMCs) were sorted by FACS and the frequency of PB (CD3⁻CD19⁺CD20⁻CD27⁺⁺CD38⁺⁺) and mBC (CD3⁻CD19⁺CD20⁺CD27⁺) subsets were determined.

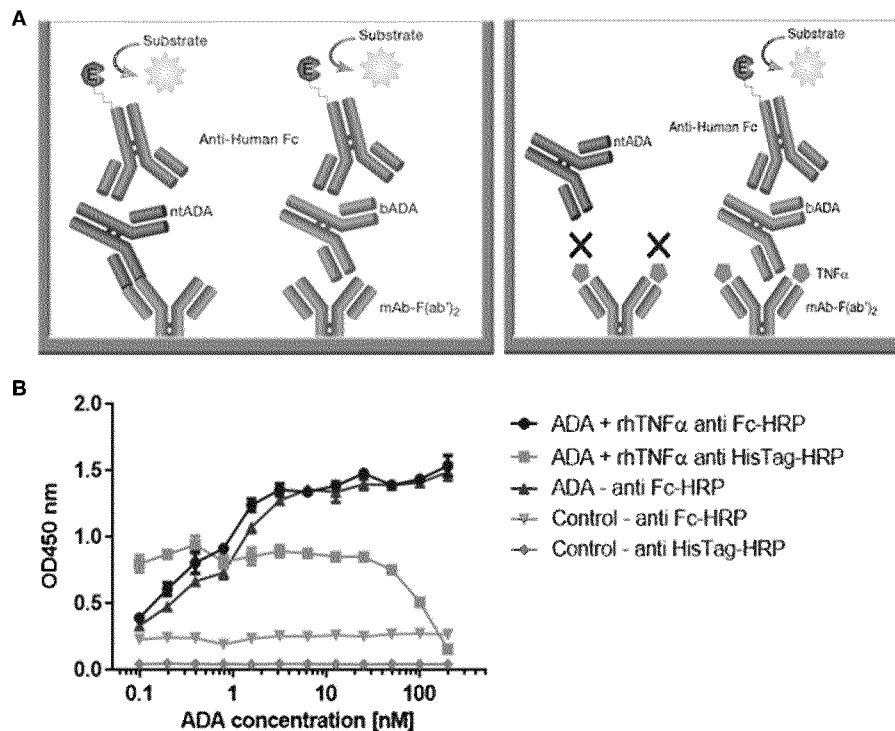


FIGURE 4 | Configuration of the assay for determining the neutralization index of ADA in patient sera and competitive ELISA between ADA and rhTNF α . **(A)** The newly developed mAb-F(ab')₂ based bio-immunoassay configuration (left) and the modified configuration where mAb-F(ab')₂ binding site is blocked by saturating the assay with rhTNF α (right). **(B)** Competitive effect of rhTNF α on ADA binding to IFX-F(ab')₂. ELISA plate was coated with 5 μ g/ml of IFX-F(ab')₂. ADA standard was diluted 3-fold in blocking solution supplemented with 5 nM rhTNF α . ADA diluted 3-fold in blocking solution without the presence of rhTNF α served as a control.

We identified a 13-fold increase in the frequency of PB at D10 and no increase in the mBC compartment. The PB data suggests that the B cell dynamics following IFX infusion exhibits vaccine-like characteristics in accordance with our working hypothesis (Table 2, Figure S3).

Antibody Repertoire of ADA Encoding B-Cells

The waves of PB following challenge is enriched with antigen-specific B cells (23, 32, 35). Based on this, a major fraction of PB at D10 post-mAb infusion is expected to comprise B cell clones responding to the current antigen challenge. Thus, the repertoire of B cells at two time points (pre- and post-infusion) is predicted to represent the overall differences in the ongoing ADA encoding B cell response.

This diversity of antibodies is accomplished by several unique molecular mechanisms, including chromosomal V(D)J rearrangement, somatic hypermutations (SHM) and class switch recombination (25), processes that are mediated by recombination-activating gene (RAG) and activation-induced cytidine deaminase (AID), respectively. The AID enzyme functions mainly in secondary lymph nodes named germinal centers. Next-generation sequencing (NGS) of the antibody variable regions (V-genes) coupled with advanced bioinformatics tools provides the means to elucidate the antigen-specific

antibody repertoire's immense diversity (36). To deep sequences antibodies' V-genes, recovered RNA from sorted PB and mBC was used as the template for first-strand cDNA synthesis, followed by PCR amplification steps to produce barcoded amplicons of the V-genes of the antibody heavy chains (V_H) as described previously (24). While NGS of antibodies is a powerful tool for immune repertoire analysis, relatively high rates of errors accumulate during the experimental procedure. To overcome this challenge, we generated duplicates of the antibody V-gene amplicons and sequenced them using the Illumina MiSeq platform (2 $\times$ 300 bp). The resultant V_H sequences were processed using our recently reported ASAP webserver that was specifically developed to analyze NGS of antibody V-gene sequences derived from replicates (25).

In our analysis, we concentrated on several repertoire measures that collectively provide a molecular level characterization of the ADA: (i) V(D)J family usage; (ii) CDR3 length distribution; (iii) SHM levels, and, (iv) isotype distribution. Our data revealed several interesting antibody repertoire features that may shed light on the molecular mechanism involved in the formation of ADA.

V(D)J Gene Family Usage Is Stable

Examining the V(D)J family usage is important to determine whether the basal gene frequency is similar to the expected

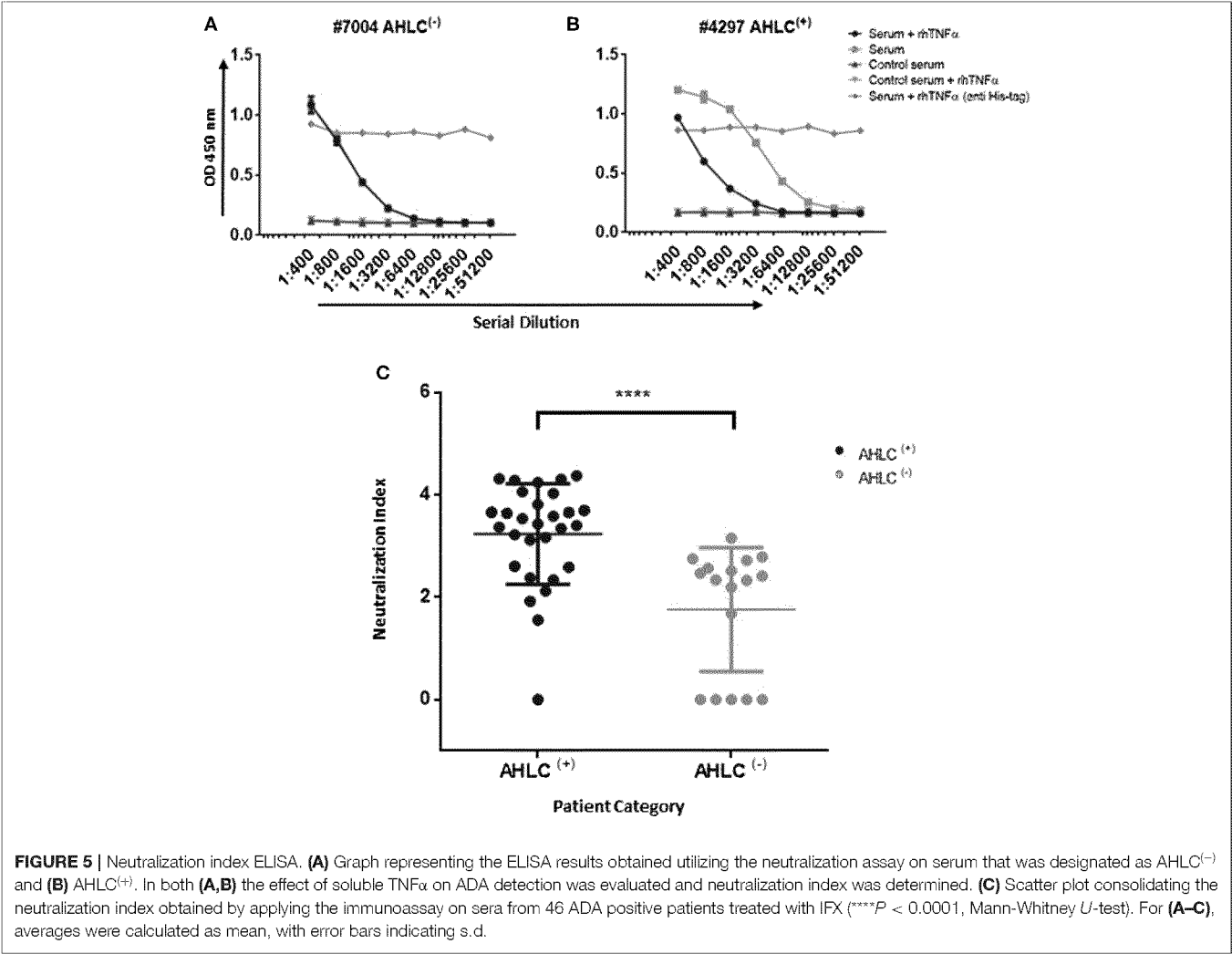


FIGURE 5 | Neutralization index ELISA. **(A)** Graph representing the ELISA results obtained utilizing the neutralization assay on serum that was designated as AHLC⁽⁻⁾ and **(B)** AHLC⁽⁺⁾. In both **(A,B)** the effect of soluble TNF α on ADA detection was evaluated and neutralization index was determined. **(C)** Scatter plot consolidating the neutralization index obtained by applying the immunoassay on sera from 46 ADA positive patients treated with IFX (**** $P < 0.0001$, Mann-Whitney U -test). For **(A–C)**, averages were calculated as mean, with error bars indicating s.d.

TABLE 2 | B cell frequency of a patient treated with IFX.

Time point	B cell subset	% Frequency of sorted cells (out of CD19+ cells) (%)	No. of raw paired-end sequencing reads		No. of filtered paired-end sequencing read			No. of unique IGH clonotypes extracted (Unique CDRH3)
			Replicate A	Replicate B	Replicate A	Replicate B	Joint	
D0	PB	0.9	39,129	63,168	12,863	19,082	2,041	1,294
	mBC	10	714,722	639,984	121,998	111,373	30,725	9,146
D10	PB	11.5	167,859	151,849	49,762	46,192	10,341	5,590
	mBC	9.7	528,765	488,619	143,864	133,879	40,899	19,521

frequency and if the B cell response following IFX infusion drives B cells to exhibit a preferential V(D)J gene usage. Therefore, we examined the frequency of family usage at two time points (D0 and D10), within PB and mBC subsets across isotypes (IgG and IgM). The V(D)J family usage showed no marked difference between the two time points, B cell subsets and isotypes. The frequency of V-gene family usage was also found to have similar frequency profile as previously described (37, 38). For example, the V-gene family frequency showed that the V3, V4, and V1

have the most prevalent representation followed by V2, V5, and V6 that had significantly lower frequencies (Figure 6). The same pattern trends were identified for the D and J family usage.

CDRH3 Length Increases Following IFX Infusion
Composed of the V(D)J join with its inherent junctional diversity, the CDRH3 specifies the antibody V_H clonotype. The V_H clonotype is an important immunological concept because

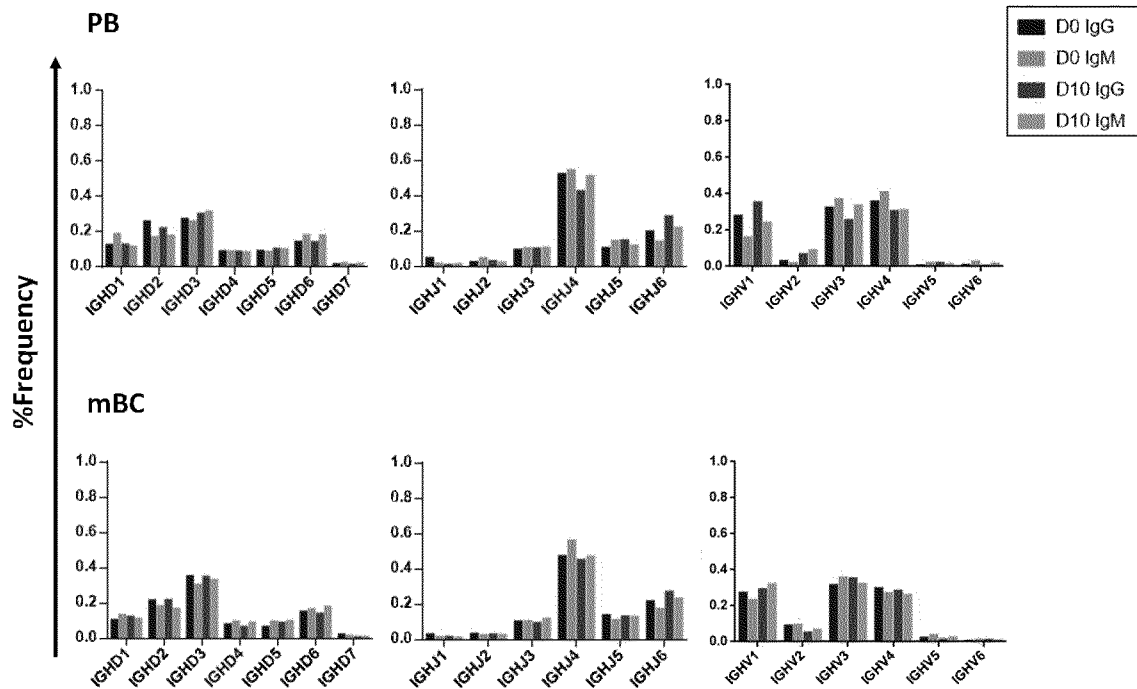


FIGURE 6 | V, D, and J family usage in B cell following IFX infusion. mBC and PB from a patient treated with IFX were collected at two time points (D0, D10) and processed for NGS analysis. The V family usage showed no difference between D0 and D10, different B cell subsets and isotypes. The D and J family usage showed no difference between time points.

it accounts for antibodies that likely originate from a single B-cell lineage and may provide insight on the evolution of the antigen-specific response (39). Here we defined V_H clonotype as the group of V_H sequences that share germ-line V and J segments and have identical CDRH3 sequences. By examining the length distribution of CDRH3 from PB across isotypes and time point we observed a shift toward longer CDRH3 at D10 (Figure 7). Interestingly, this observation is in contrast to previous studies that reported a decrease in the CDRH3 length post-immunization with pneumococcal (40) and hepatitis B vaccines (41) and when comparing antigen experienced B cell to naïve B cells (42).

Somatic Hypermutation Levels Decreases Following IFX Infusion

Examining the level of SHM following vaccination provides insights regarding the extent of the affinity maturation that antigen-stimulated B cell undergo. It was previously reported that boost vaccination induces a substantial increase of the SHM levels when comparing post- to pre-vaccination (41). Despite the vaccine like response following IFX infusion, we observed in the PB compartment a significant decrease in the SHM levels post-infusion, regardless if the mutations were synonymous and non-synonymous (Figure 8).

Proteomic Analysis of ADA

Analysis of serum antibodies provides a comprehensive profile of the humoral immune response and is complementary to

the transcriptomic analysis derived from NGS of the antibody V_H . Applying an approach that integrates NGS and tandem mass spectrometry (LC-MS/MS) has been shown to provide valuable data regarding the composition of antigen-specific serum antibodies and their relationship to B cells and generates new insights regarding the development of the humoral immune response in disease and following vaccination (23, 32). Here we utilized the previously developed omics approach (26, 39) to elucidate the serum ADA composition following IFX infusion. ADA from 10 ml of serum collected at D0 and D10 were subjected to protein G affinity chromatography and total of 9 mg of recovered IgG was digested by IdeS to remove the Fc regions that may mask the MS/MS signal obtained from low abundant peptides. Following 5 h of digestion, the reaction mixture was subjected to custom made affinity column where the IFX- $F(ab')_2$ was coupled to agarose beads and served as the antigen to isolate ADA. Recovered 48.57 μ g polyclonal ADA- $F(ab')_2$ [i.e., IFX- $F(ab')_2$ -specific $F(ab')_2$] in the elution fraction and total $F(ab')_2$ [depleted from ADA- $F(ab')_2$] in the flow through fraction were digested with trypsin and injected to high-resolution tandem mass spectrometer analyzer in triplicates. LC-MS/MS raw data files were analyzed using MaxQuant using label free quantitation mode (LFQ) and searched against the custom antibody V-gene database derived from the NGS data of B cells isolated from the same donor. Identified peptide from the interpretation of the proteomic spectra were stratified into three types of peptides: informative peptides (*i*Peptide) that map uniquely to one antibody clonotype in a region that is upstream

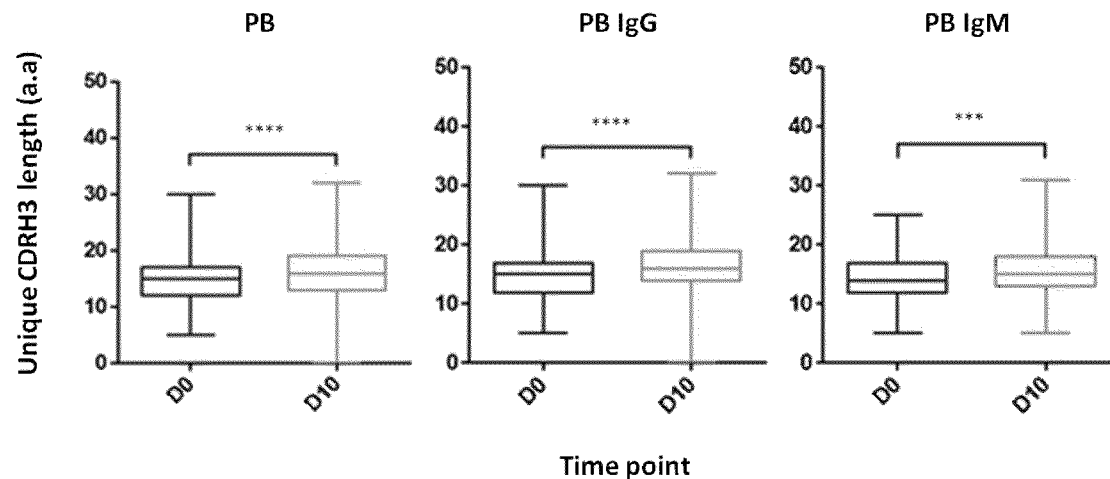


FIGURE 7 | CDRH3 length at two time point and across isotypes. PB from a patient treated with IFX were collected at two time points (D0, D10) and processed for NGS analysis. An increase in antibody CDRH3 length was observed (*** $P < 0.001$; **** $P < 0.0001$, Mann-Whitney U -test).

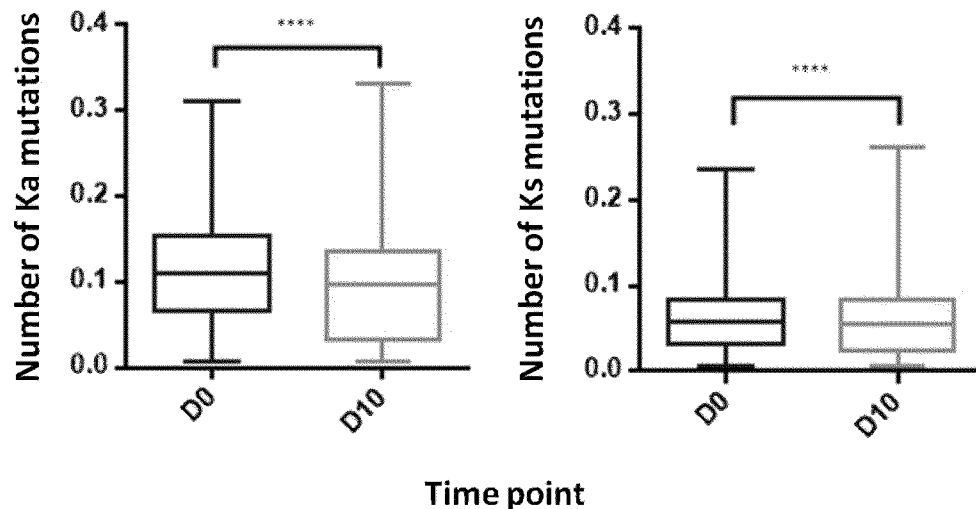


FIGURE 8 | Somatic hyper mutations. PB from a patient treated with IFX were collected at two time points (D0, D10) and processed for NGS analysis. A decrease in the number of Ka mutations (number of non-synonymous mutation per codon) and Ks mutations (number of synonymous mutations per codon) was observed at D10 (**** $P < 0.0001$, Mann-Whitney U -test).

to the CDRH3, non-informative CDRH3 peptides (*niCDRH3*) that map to the CDRH3 region of the antibody but do not map uniquely to a single antibody clonotype and informative CDRH3 peptides (*iCDRH3*) that map uniquely to a single antibody clonotype. Summary of identified peptides in LC-MS/MS are shown in **Table 3**. Beyond the designation as *iCDRH3* peptides, additional filtration steps were applied including peptides that were present in more than 2 replicates, peptides in elution that show 5 × fold frequency than in the flow through. The *iCDRH3* peptides enabled the identification of 62 unique ADA CDRH3 clonotypes with 205 associated full-length V-gene sequences. The resulting V-gene sequences were analyzed to determine their V(D)J family usage and the B cell subset they are mapped to, based on our NGS data (**Figure 9**).

The V(D)J family usage of the antibody variable region sequences that were identified by LC-MS/MS (**Figure 9A**) showed a similar distribution as observed in the NGS data (**Figure 6**). V family frequency analysis showed that the V1, V3, and V4 are the most dominant V families followed by V2 and V5 that had significantly lower frequencies. D family frequency analysis showed that the D6, D3, D2, and D1 have the most prevalent representation, and J family frequency showed that the J4, J5, and J6 have the most prevalent representation.

Next, we examined the distribution of the proteomically identified V-gene sequences to B cell subsets (**Figure 9B**) and found that the V-genes predominantly map to mBC from D0 (46.83%), followed by mBC from D10 (27.8%). Moreover, we found that 23.9% of V-genes map to D10 PB. Based on

the dynamics of antibodies in serum (32), the majority of antibodies produced following a boost challenge are the product of pre-existing mBC cells that were re-activated following drug infusion, much like a response to a vaccine boost (23).

As mentioned above, flow cytometry of B cells following IFX administration allowed us to identify a substantial increase in the frequency of PB at D10, which suggests that the B cell dynamics following IFX infusion exhibits vaccine-like characteristics. Therefore, we expected to find a majority of V-gene sequences mapping to IgG⁺ B cells that underwent class switch recombination in the germinal center. Surprisingly,

TABLE 3 | Summary of identified peptides and the corresponding clonotype and antibody somatic variances in the LC-MS/MS spectra.

	Day 0	Day 10
Total peptides	908	3,177
Total antibody peptides	761	2,805
Total CDRH3	42	224
Present in ≥2 technical replicates	30	166
Frequency ratio E/FT >5	11	81
No of clones	5	62
No of somatic variances	35	205

E, elution; FT, flow-through.

the majority of proteomically identified serum antibodies were mapped to IgM⁺ B cells (**Figure 9C**).

Next, we aimed to provide support to the observation that *nt*ADA preferably use the λ light chain. By quantifying the accumulative intensities of peptides derived from the constant region of both κ and λ light chains, we calculated the ratio of κ:λ light chain in the elution fraction which comprise both the *nt*ADA and *b*ADA (ADA-IgG), and in the flow through fraction that represent ADA-depleted IgG (dep-IgG). The expected κ/λ ratio of IgG in human serum is 2 (66% κ and 33% λ). Indeed, proteomic analysis of the dep-IgG (D0 and D10), resulted in an average κ/λ ratio of 2.1. The same analysis of the ADA-IgG showed a significant shift of the κ/λ ratio to 1.19. The proteomic analysis was carried out on samples from patient that exhibited a high neutralization index (have high levels of *nt*ADA) and designated ADA⁺ using AHLC (detects only ADA with λ light chain). Brought together, this further suggests that *nt*ADA contribute to shift in the κ:λ light chain ratio.

DISCUSSION

The use of therapeutic mAbs in treating a wide range of diseases and disorders is growing exponentially. Nonetheless, a major shortcoming of their use is the development of ADA in patients receiving the mAb. Advances in mAb engineering have enabled the development of fully human mAbs with

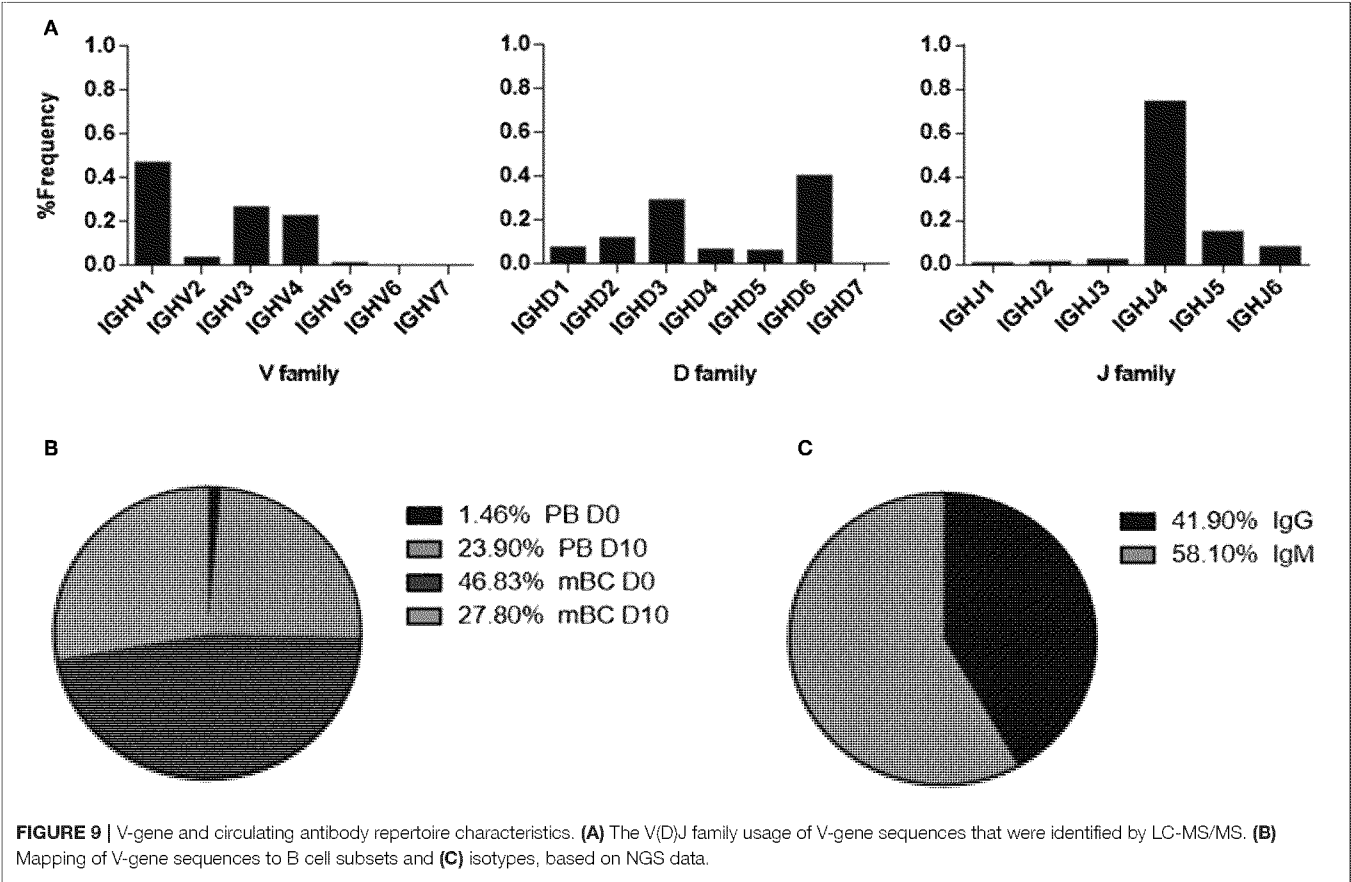


FIGURE 9 | V-gene and circulating antibody repertoire characteristics. **(A)** The V(D)J family usage of V-gene sequences that were identified by LC-MS/MS. **(B)** Mapping of V-gene sequences to B cell subsets and **(C)** isotypes, based on NGS data.

reduced immunogenicity without abolishing it completely. Thus, a mAb administered to a patient can still induce an immune sensitization as reflected by the production of ADA, which is associated with low trough drug levels and can mediate loss of clinical response to the drug (20).

The precise mechanism underlying ADA production is unknown, and many questions related to its development remain unaddressed, including determining precise concentrations of ADA in serum, which portion of the ADA exhibits neutralizing capacity, the immune pathway governing the production of ADA, and ultimately, the molecular composition of ADA at the sequence level. To address these questions, we chose the chimeric TNF α antagonist IFX as the model system. First, we aimed to quantify the ADA level in patient sera. Many methods were previously reported to evaluate serum ADA levels. These assays include radio-immunoassays (43), Biotin-drug Extraction with Acid Dissociation (BEAD) (44), Precipitation and acid dissociation (PANDA) (45), affinity capture elution ELISA (ACE) (46), and Homogenous Mobility Shift Assay (HMSA) (47). While these assays are not limited to the λ light chain detection like the AHLC assay, they provide mostly qualitative measures to assist physicians in deciding the most appropriate intervention when treating patients, and many (if not all) studies underestimated actual ADA levels (19). These assays also lack a standardization methodology that can enable the comparison of ADA levels across health centers.

To provide quantitative measures describing the molecular landscape of ADA, we first developed a bio-immunoassay that would allow quantify ADA levels based on the F(ab')₂ region of the mAb because previous reports indicated that the ADA generated from mAb administration are mostly anti-idiotypic (21). Indeed, the bio-immunoassay demonstrated higher sensitivity compared with the AHLC assay used initially to detect ADA and was able to detect ADA when the AHLC assay could not. Leveraging its improved sensitivity compared to the AHLC assay, we applied our proprietary assay on sera from 54 patients treated with IFX and found that patients designated as AHLC⁽⁺⁾ showed significantly higher levels of ADA (mean: 264 μ g/ml) compared to the AHLC⁽⁻⁾ group (mean: 59.64 μ g/ml). These results support the clinical use of AHLC assay because overall, patients were correctly stratified leading to clinical decision-making that was based on a valid indicative assay. Notwithstanding, the applicability of the AHLC assay, the newly developed F(ab')₂-based bio-immunoassay demonstrated that ADA levels can reach extreme concentrations that were not detected using the AHLC assay.

Some patients who develop ADA in response to IFX present a prolonged remission with maintenance therapy despite repeated indications of high ADA and low IFX trough levels (20). The mechanism of action of these ADA has significant influence on drug efficacy. For example, *b*ADA are most likely to enhance the clearance of a drug whereas *nt*ADA will prevent a drug from binding to its target. Hence, it is important to differentiate between *b*ADA and *nt*ADA, or in other words, a need exists to identify sera with high levels of *nt*ADA that may predict the likelihood of a patient losing a favorable response to an administrated mAb. To achieve this, we further revised our

bio-immunoassay to qualitatively measure the neutralization index of ADA in the serum of patients treated with IFX. Of note, as the neutralization index is a qualitative and not a quantitative index, some patients may exhibit relatively low ADA levels and high neutralization index. Using this assay on sera from the 46 ADA positive patients, revealed that patients who tested positive utilizing the AHLC assay, exhibit a significantly higher neutralization index than patients tested negatively for it [i.e., AHLC⁽⁻⁾]. Noteworthy, the AHLC assay is based on the anti- λ light chain antibody at the detection stage, suggesting that sera with high neutralization index comprise ADA that preferably use the λ light chain (either *b*ADA or *nt*ADA). This phenomenon received additional support from our proteomic analysis in which we compared the changes in the ratio between peptides derived from κ and λ constant light chains from ADA-IgG pool and peptide derived from depleted ADA IgG polyclonal pool (dep-IgG). This analysis demonstrated that the κ/λ ratio in the total IgG compartment is as expected and is decreased in the mAb-specific compartment (κ/λ ratio 2.1 and 1.19 for dep-IgG and ADA-IgG, respectively). The preferential use of the λ light chain in neutralizing antibodies has been previously reported (21, 48), however, the authors of those studies did not provide an explanation beyond the structural adaptability of the light chain toward the target. The relevance of the reported cases showing λ chain bias is not clear. Similar phenomena was reported in B-1 sub-population, unlike follicular B cells, B-1 cells exhibit an increased frequency of lambda light chains (49). The recurrence of BCRs with the enrichment of λ light chain has been considered to result from strong antigen-dependent selection of the B-1 cell repertoire (50).

Repetitive administration of mAbs may induce a strong humoral response manifested in the production of ADA. We hypothesized that mAb administration is similar to the response that occurs following a boost vaccine. Others and we have demonstrated that boost vaccines induce a strong proliferation of PB that can be detected in blood circulation several days after the boost. The “wave” of B cells after the boost vaccine are dominated by antigen-specific B cell (34) thus, repertoire analysis of these cells can provide invaluable data about the antigen-specific antibody repertoires. Utilizing flow cytometry showed an order of magnitude increase in PB compartment 10 days after IFX infusion, suggesting that the immune response following IFX administration is indeed similar to a vaccine response. To the best of our knowledge, this is the first report to identify a vaccine like response following therapeutic mAb administration.

Next, we aimed to provide a comprehensive repertoire profile of the B cells induced after mAb administration. To achieve this, we applied an “omics” approach as previously described (23, 26, 39) that is based on the integration of NGS of the V-genes and proteomic analysis of serum ADA. NGS of V-genes revealed no bias in the V(D)J usage across isotypes, cell types, and time point. These data suggest that the original repertoire that existed before mAb administration and antigen-specific repertoire induced by IFX administration is formed by random recombination processes without preferential use of any particular V(D)J

segment. Comparative repertoire analysis of the V-genes between time points (before and after IFX administration) revealed that post-IFX administration, PB exhibit longer CDRH3 and lower SHM rates. Although the B cell dynamics after mAb administration are similar to those that occur after a boost vaccine, the repertoire measures show a different profile. It was previously reported that the antibodies generated after a boost vaccine exhibit shorter CDRH3, high SHM (40–42).

To explain these data we revisited two reports: the first describes how the immune response in TNF α -deficient mice was “diverted” to the marginal zone instead of to the germinal center (51) and the characteristics of the immune response in the marginal zone is directly affected by low levels of the AID that in turn is reflected in lower SHM rate. The second reported a skewed λ chain usage in B-1 cells (49). Based on these reports we propose a mechanistic model according to which administration of TNF α antagonist blocks the TNF α on one hand and induces a vaccine-like response on the other. Due to the TNF α blockade, immune response of B cells occurs extra follicular where AID is downregulated, thus the encoded ADA exhibit lower SHM rates. Moreover, the data suggests that the immune response following mAb administration may be a T cell independent (TI) response which is governed by the B-1 cell lineage with the characteristics mentioned of an increased usage of λ light chains and little to non-evidence for SHM (49, 52).

Another possible mechanism that should be further explored is the strong TI immune response in the marginal zone that is also induced by a drug/ADA immune-complex (IC). It was previously suggested that many of the immune-mediated adverse effects attributed to ADA require the formation of an IC intermediate that can have a variety of downstream effects (6, 53). In the context of the system we investigated, administration of a TNF α antagonist will divert the immune response extra follicular either by TNF α blockade or by the formation of an IC carrying multiple mAbs that can induce the cross-linking of cognate BCR. The BCR of ADA-encoding B cells will undergo co-clustering leading to their activation in the TI pathway.

Of note, insights from this study are restricted to the immune response following treatment with TNF α antagonists, as it is specifically affected by the drug’s mechanism of action. First, TNF α is a trimer that has the propensity to form immunocomplexes with the drug. Second, blocking TNF α “simulates” a scenario that was observed in TNF α knockout mice. Combined, these attributes contribute to the specific nature of the immune response which is suggested to be diverted to the extra follicular, TI immune response. Moreover, the deep analysis data was obtained from one patient. However, this patient exhibited a set of attributes including high ADA level, high neutralization index, low trough level, and lack of immunosuppressant treatments. These attributes enabled to generate insights that are directly relevant to the drug administration.

In our study we examined molecular aspects related to the formation of ADA. To the best of our knowledge, this is the

first report describing ADA repertoire that resulted in insights about a possible mechanism of ADA formation. Further work will be needed to elucidate additional phenotypic markers of the B cells induced by mAb administration and the role of IC in the activation of the B cell. Moreover, the mechanism described here covers the response to a TNF α -antagonist, and by using the same omics approaches, it will be highly informative to study the B cell response following treatment with other mAbs that induce ADA formation. We envision that high throughput data obtained from such studies can facilitate our understanding on why and how, mAb administration generates ADA and eventually may contribute guidelines for engineering therapeutic mAbs with reduced immunogenicity.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

All subjects signed an informed consent, and the study was approved by the Ethics Committee of Sheba medical center.

AUTHOR CONTRIBUTIONS

SB-H, IB, and YW conceived the research. AV-M, SR, and YW designed the experiments. AV-M, SR, MY, EF, and YD performed the experiments. AV-M, SR, SB-H, MY, EF, BU, UK, and OP collected and processed clinical samples. AV-M, AK, and YW carried out data analysis. AV-M and YW wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2019.02921/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SampPick: Selection of a Cohort of Subjects Matching a Population HLA Distribution

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Immune responses to therapeutic proteins and peptides can adversely affect their safety and efficacy; consequently, immunogenicity risk-assessments are part of the development, licensure and clinical use of these products. In most cases the development of anti-drug antibodies is mediated by T cells which requires antigen presentation by Major Histocompatibility Complex Class II (MHCII) molecules (also called Human Leucocyte Antigen, HLA in humans). Immune responses to many protein therapeutics are thus HLA-restricted and it is important that the distribution of HLA variants used in the immunogenicity assessments provides adequate coverage of the target population. Due to biases inherent to the collection of samples in a blood bank or donor pool, simple random sampling will not achieve a truly representative sample of the population of interest. To help select a donor cohort we introduce SampPick, an implementation of simulated annealing which optimizes cohort selection to closely match the frequency distribution of a target population or subpopulation. With inputs of a target background frequency distribution for a population and a set of available, HLA-typed donors, the algorithm will iteratively create a cohort of donors of a user selected size that will closely match the target population rather than a random sample. In addition to optimizing the HLA types of donor cohorts, the software presented can be used to optimize donor cohorts for any other biallelic or monoallelic trait.

Keywords: immunogenicity, HLA-typing, optimization, donor-selection, simulated-annealing, algorithm

INTRODUCTION

Protein and peptide therapeutics include seven of the 10 top-selling drugs (1) and provide medical interventions for diseases that are otherwise untreatable. Immunogenicity, the undesired immune response to a protein or peptide therapeutic, is a key concern during drug-development and licensure. While the development of some drugs has been discontinued due to immunogenicity-risk (2–5); immunogenicity issues continue to cast a shadow even on marketed drugs. For example, within 5 years, 30–70% of patients receiving TNF- α inhibitors experience “secondary failure” due to immunogenicity (6). Similarly, about a quarter of hemophilia A patients develop so-called inhibitors, i.e., neutralizing antibodies (NABs) to Factor VIII, leading to severely diminished quality

of life and medical costs that can exceed USD 1 million per year. Thus, the most egregious consequence of immunogenicity is not that a drug will fail to be marketed but that medications with a market value of almost 100 billion dollars that treat millions of individuals are sub-optimal.

The immunogenicity risk of a drug-candidate can be determined at two principal steps during drug-development. In early stage drug-development, non-clinical *in silico*, *in vitro*, and *ex vivo* tools can be used to assess the potential for an immune response (7). Although there have been substantive improvements in these technologies in the last decade, it is still not possible to rely entirely on the surrogate markers measured by these methods for estimating the risk of clinical immunogenicity of biologics (8). Consequently, the identification of anti-drug antibodies (ADAs) and NABs is almost always a part of Phase 3 clinical studies (9).

The HLA-type of a patient is one of several risk factors for immunogenicity. The HLA proteins act at the interface between the antigen and the immune system. These receptors bind peptides derived from protein antigens and transport them to the membrane surface where the complex is recognized by T cells which can then initiate the cascade of complex immune responses. Numerous studies indicate that immune responses to therapeutic proteins require T-cell activation (10). Hence antigen presentation via the HLA is a necessary, albeit not a sufficient, condition for therapeutic protein product immunogenicity (8).

From the point of view of assessing the immunogenicity risk of a protein-drug; a population that has a diverse HLA repertoire presents a challenge. Genes for the major histocompatibility complex (MHC), also called the HLA in humans, are the most polymorphic in the vertebrate genome (11). If, and this is often the case, immune responses to the therapeutic-protein are HLA restricted, ensuring that a representative distribution of HLA variants is included in the clinical and non-clinical studies is very difficult.

A testing cohort can be generated from any “available population” such as HLA typed individuals donating at a blood bank, a bio-repository, commercial catalogs of HLA typed cells etc. The immense diversity of the HLA repertoire raises many technical questions in the design of a study. How many HLA variants should be studied? How does one generate a suitable cohort that considers the relative frequencies of HLA variants in different human populations? For an *ex vivo* assay how many samples should be used? What HLA types should the donors of the cells have? The answers to many of these questions will depend on the drug, the disease and the specific question(s) the study is being designed to answer. However, once a decision has been made as to the composition of the “representative cohort” (e.g., a distribution of HLA alleles reflecting the US population, a disease etc.) statistical approaches can be used to select the most appropriate cohort for the study.

Usual methods for donor cohort selection involve either hand selection of donors to ensure that alleles with high frequencies in the population are included in the study, or random selections of donors under the assumption that this random selection will be a representative sample of the population from which it is drawn. While hand selecting donors to cover important alleles

will ensure that these alleles are included in the study, it does not consider the frequencies of the alleles. Additionally, it does not try to model the distribution of the less frequent alleles on the population of interest.

Random selections of donors would address these issues of attaining the proper distribution of alleles assuming the pool of donors is representative of the population from which it is drawn. It is known that some biases will exist in donors in a bio-repository or blood bank (12). In order to confront the biases inherent to the group of samples to choose we propose avoiding random sampling from a biased population and introduce a method that uses simulated annealing to generate a cohort of subjects in which HLA alleles occur at the similar frequencies as they occur in the sub-population of interest. We use a simulated annealing algorithm to select a cohort of subjects that better resembles a background population (*vis-à-vis* relative frequencies of HLA alleles) as measured by the Jensen-Shannon distance (JSD). While there are several distance measures that can be used, this distance measure is often used in the comparison of probability distributions in machine learning applications due to its symmetry and finite bounds.

Simulated annealing is a global optimization algorithm which draws its inspiration from the metallurgical process of annealing. This annealing process involves a scheduled heating and cooling process that serves to strengthen metal and reduce flaws. The basis of Simulated Annealing is the Metropolis-Hastings Algorithm in which a change is introduced into the system. In our case this change is the substitution of one potential member of the selected cohort for another. This new state is accepted with some probability and the algorithm will continue anew. The introduction of the concept of cooling in annealing sets up a schedule for the acceptance of new states. At the start of the algorithm, the system is hotter and has a great deal more free energy (temperature of 1). This temperature will exponentially decrease over the iterations of the algorithm until the system has cooled to a temperature nearing 0. For each iteration, the JSD score is assessed after the substitution is made. If the substitution is an improvement with respect to JSD, then the change is accepted. In addition, if a random number from a uniform distribution between 0 and 1 is below the current temperature, the change is also accepted regardless of whether the score is an improvement. These non-optimal changes are considerably more likely when there is a lot of free energy in the system (higher temperature). As the system “cools” to a temperature of 0, the chance of a substitution being accepted without also being an improvement in score exponentially decreases. The benefit of this algorithm is in its flexibility in dealing with local vs. global minima. If the algorithm has no chance to accept non-optimal solutions, there is a chance that it will miss a lower minimum and just be stuck in the minimum for the region in which it started. By allowing for the algorithm to try out other areas in the solution space, there is a greater chance that the global minimum is found. Importantly, the reduction in temperature means that toward the later parts of the algorithms run, the algorithm would have hopefully found the area around the global minimum and will fine-tune its selection in that region as the system further cools toward 0.

TABLE 1 | The samples used for the creation of the background distribution of HLA-DRB1 alleles for the US population.

Race/ethnic description	DRB1-typed sample counts (1)	% US population race distribution (US Census Bureau estimate) (2)	% US population race distribution (used in this work)
African American	505,250	13.4	13.5
Asian or Pacific Islander	568,597	6	6.0
Caucasian	3,912,440	60.7	61.0
Hispanic	712,764	18.1	18.2
Native American	46,148	1.3	1.3
Total	5,745,199	99.5	100

The allelic distributions for each sub-population are weighted by the demographic distribution of those sub-populations (scaled to 100%).

We demonstrate that a cohort selected using this tool is closer in HLA type distribution to a known background distribution (as measured by the Jensen-Shannon distance) than random selections from biased sets of donors. Finally, we provide illustrative applications of our tool.

METHODS

Obtaining the Background Distribution of HLA DRB1 Alleles

We obtained the population frequencies of 514 HLA-DRB1 alleles from the United States (US) from the Be the Match® bone marrow donor registry (13). This dataset reports the allele frequencies for broad race categories in the US (Table 1) from 5,745,199 haplotyped samples. To calculate the allele frequencies representing the general American population we weighted the racial allele frequencies with the demographic distribution of those races in the population. To obtain the racial distributions, we first retrieved the US Census Bureau's July 1, 2018 estimates on the population demographics and then amended the percentages such that they add up to a 100, by distributing the missing (100%-99.5%) based on the original percentages. In the allele dataset, there were four ambiguous (04:07G, 11:01G, 12:01G, 14:01G) and four not-expressed (07:10N, 12:24N, 15:17N, 15:50N) DRB1 allele types. For standardization purposes we mapped those alleles to their base allele type (e.g., 04:07G reverts to 04:07). The resulting scaled and weighted table of allele frequencies (Supplementary Table 1) was used as a background distribution for the North American Population.

The Source of HLA Typed Donors

As a test case for an available population of samples, all available samples from the CTL ePBM® Searchable Database v1.2.6 were used (Table 2). Samples that had no information for HLA-DRB1 were not considered and sample labeled as ambiguous with a "P" group suffix were excluded.

The Jensen-Shannon Distance

The Jensen-Shannon Distance is used as a metric for scoring the difference between the two probability distributions. This metric was used since it is symmetrical version of the Kullback-Leibler Divergence. As we are using the natural log in the Kullback-Leibler Divergence definition, the Jensen-Shannon Distance is

bounded by 0 and $\sqrt{\ln(2)} \approx 0.83$. It is defined by the following equation:

$$JSD(P||Q) = \sqrt{\lambda * D_{KL}(P||M) + \lambda * D_{KL}(Q||M)}$$

where:

$D(P||Q)$ is the Kullback-Leibler divergence of probability distributions a and b.

λ is a scaling factor for the two distribution (1/2 is generally used).

P is the probability distribution of the Sample of interest.

Q is the probability distribution of the Target Population (Background Distribution).

M is the mean of both distributions defined as:

$$M = \frac{1}{2}(P + Q)$$

The Kullback-Leibler Divergence is defined as:

$$D_{KL}(P||Q) = \sum_{a \in A} P(a) * \log\left(\frac{P(a)}{Q(a)}\right)$$

where:

A is the union of all alleles in the target and sample populations.

Simulated Annealing

A Simulated Annealing algorithm (Figure 1) was used to optimize selection of a sample cohort through iterative resampling of the population. The algorithm allows for the user to select a sample size (N), as well as refine the parameters: number of iterations (I), temperature decrease (α), number of changes (C).

The purpose of this algorithm is to accept changes to the selected cohort which will decrease the JSD between the cohort chosen and target distribution. The reason it will accept non-optimal changes in step VII (ii) is to allow the algorithm to avoid getting stuck on local minima and have a chance to fully explore the search space. It should be noted that the probability of accepting non-optimal changes decreases exponentially over time.

TABLE 2 | All available samples from the CTL ePBMCR Searchable Database v1.2.6 were used as an example of available donors to use in the selection process.

Subject	DRB1 Allele 1	DRB1 Allele 2
LP_7	DRB1*07:01	DRB1*14:02
LP_88	DRB1*04:04	DRB1*08:01
LP_89	DRB1*13:02	DRB1*14:01
LP_91	DRB1*04:07	DRB1*07:01
LP_92	DRB1*15:01	DRB1*15:01
LP_96	DRB1*03:01	DRB1*04:04
LP_98	DRB1*03:01	DRB1*03:02
LP_99	DRB1*03:01	DRB1*08:02
LP_105	DRB1*03:01	DRB1*15:01
LP_118	DRB1*14:02	DRB1*15:01
LP_120	DRB1*04:07	DRB1*08:02
LP_121	DRB1*01:01	DRB1*16:02
LP_123	DRB1*07:01	DRB1*13:01
LP_141	DRB1*01:02	DRB1*13:03
LP_151	DRB1*10:01	DRB1*14:02
LP_161	DRB1*03:01	DRB1*14:06
LP_169	DRB1*11:04	DRB1*15:01
LP_178	DRB1*04:02	DRB1*14:06
LP_180	DRB1*01:02	DRB1*03:01
LP_188	DRB1*04:02	DRB1*13:03
LP_194	DRB1*11:04	DRB1*14:06
LP_198	DRB1*04:07	DRB1*16:02
LP_203	DRB1*01:03	DRB1*16:01
LP_205	DRB1*03:01	DRB1*07:01
LP_208	DRB1*03:01	DRB1*11:02
LP_209	DRB1*08:01	DRB1*15:02
LP_210	DRB1*04:10	DRB1*15:01
LP_211	DRB1*01:02	DRB1*04:04
LP_212	DRB1*08:01	DRB1*15:02
LP_214	DRB1*11:04	DRB1*14:06
LP_231	DRB1*11:01	DRB1*11:01
LP_235	DRB1*01:02	DRB1*04:04
LP_238	DRB1*07:01	DRB1*11:04
LP_239	DRB1*07:01	DRB1*08:02
LP_242	DRB1*08:04	DRB1*14:06
LP_253	DRB1*04:04	DRB1*13:03
LP_258	DRB1*07:01	DRB1*14:02
LP_260	DRB1*14:01	DRB1*15:01
LP_263	DRB1*01:01	DRB1*03:01
LP_264	DRB1*04:03	DRB1*08:02
LP_265	DRB1*01:02	DRB1*13:01
LP_266	DRB1*04:03	DRB1*15:01
LP_267	DRB1*04:07	DRB1*16:01
LP_268	DRB1*04:01	DRB1*08:02
LP_270	DRB1*11:01	DRB1*16:01
LP_272	DRB1*08:04	DRB1*15:03
LP_273	DRB1*01:02	DRB1*04:07
LP_275	DRB1*03:01	DRB1*09:01
LP_277	DRB1*01:03	DRB1*01:03
LP_279	DRB1*13:02	DRB1*14:06
LP_280	DRB1*01:01	DRB1*01:01

(Continued)

TABLE 2 | Continued

Subject	DRB1 Allele 1	DRB1 Allele 2
LP_282	DRB1*04:04	DRB1*12:01
LP_283	DRB1*13:04	DRB1*13:04
LP_284	DRB1*04:07	DRB1*07:01
LP_285	DRB1*03:02	DRB1*07:01
LP_289	DRB1*13:02	DRB1*13:04
LP_290	DRB1*04:01	DRB1*12:01
LP_292	DRB1*14:01	DRB1*15:01
LP_296	DRB1*11:01	DRB1*15:02
LP_297	DRB1*04:04	DRB1*04:07
LP_298	DRB1*04:11	DRB1*16:02
LP_299	DRB1*04:07	DRB1*08:03
LP_301	DRB1*09:01	DRB1*14:02
LP_302	DRB1*14:01	DRB1*15:01
LP_304	DRB1*01:02	DRB1*13:01
LP_305	DRB1*07:01	DRB1*14:01
LP_306	DRB1*11:04	DRB1*13:02
LP_307	DRB1*04:04	DRB1*08:02
LP_311	DRB1*08:02	DRB1*11:01
LP_312	DRB1*14:06	DRB1*15:01
LP_313	DRB1*07:01	DRB1*07:01
LP_314	DRB1*04:02	DRB1*04:02
LP_315	DRB1*13:02	DRB1*13:04
LP_317	DRB1*04:11	DRB1*11:01
LP_318	DRB1*04:04	DRB1*04:04
LP_320	DRB1*01:01	DRB1*16:02
LP_321	DRB1*04:02	DRB1*04:02
LP_323	DRB1*03:01	DRB1*13:02
LP_325	DRB1*12:01	DRB1*13:01
LP_326	DRB1*08:06	DRB1*11:01
LP_327	DRB1*14:02	DRB1*15:03
LP_328	DRB1*01:01	DRB1*11:04
LP_331	DRB1*04:07	DRB1*08:02
LP_332	DRB1*03:01	DRB1*04:03
LP_333	DRB1*04:01	DRB1*07:01
LP_334	DRB1*11:01	DRB1*15:01
LP_335	DRB1*01:02	DRB1*11:02
LP_336	DRB1*01:01	DRB1*04:01
LP_338	DRB1*03:01	DRB1*04:07
LP_340	DRB1*01:01	DRB1*15:01
LP_341	DRB1*01:02	DRB1*13:01
LP_342	DRB1*13:02	DRB1*15:01
LP_343	DRB1*04:01	DRB1*15:03
LP_344	DRB1*04:01	DRB1*07:01
LP_345	DRB1*01:02	DRB1*13:01
LP_346	DRB1*08:01	DRB1*11:01
LP_347	DRB1*13:01	DRB1*13:03
LP_349	DRB1*01:01	DRB1*13:02
LP_350	DRB1*11:04	DRB1*11:04
LP_351	DRB1*11:01	DRB1*13:02
LP_352	DRB1*04:01	DRB1*07:01
LP_353	DRB1*07:01	DRB1*11:01

(Continued)

TABLE 2 | Continued

Subject	DRB1 Allele 1	DRB1 Allele 2
LP_354	DRB1*07:01	DRB1*07:01
LP_355	DRB1*01:01	DRB1*13:02
LP_356	DRB1*04:07	DRB1*16:01
LP_359	DRB1*04:01	DRB1*07:01
LP_360	DRB1*01:03	DRB1*03:01
LP_361	DRB1*04:04	DRB1*13:01
LP_362	DRB1*08:04	DRB1*11:01
LP_363	DRB1*01:01	DRB1*11:04
LP_364	DRB1*09:01	DRB1*15:01
LP_365	DRB1*04:05	DRB1*08:02
LP_366	DRB1*03:01	DRB1*13:01
LP_367	DRB1*11:02	DRB1*13:02
LP_369	DRB1*01:01	DRB1*11:04
LP_372	DRB1*13:01	DRB1*13:02
LP_373	DRB1*03:01	DRB1*08:02
LP_375	DRB1*03:01	DRB1*04:02
LP_377	DRB1*01:01	DRB1*11:04
LP_378	DRB1*13:03	DRB1*16:01
LP_379	DRB1*04:04	DRB1*07:01
LP_381	DRB1*04:05	DRB1*12:02
LP_383	DRB1*01:02	DRB1*13:01
LP_384	DRB1*09:01	DRB1*13:02
LP_385	DRB1*10:01	DRB1*14:06
LP_386	DRB1*03:01	DRB1*04:11
LP_388	DRB1*03:01	DRB1*03:01
LP_389	DRB1*07:01	DRB1*07:01
LP_390	DRB1*01:01	DRB1*13:02
LP_391	DRB1*04:11	DRB1*13:02
LP_392	DRB1*01:01	DRB1*04:01
LP_393	DRB1*03:01	DRB1*09:01
LP_394	DRB1*03:01	DRB1*14:02
LP_395	DRB1*01:02	DRB1*15:03
LP_396	DRB1*04:05	DRB1*15:01
LP_397	DRB1*04:06	DRB1*15:01
LP_398	DRB1*01:02	DRB1*03:02
LP_399	DRB1*04:01	DRB1*15:01
LP_400	DRB1*01:02	DRB1*12:01
LP_401	DRB1*01:02	DRB1*11:02
LP_402	DRB1*09:01	DRB1*15:01
LP_403	DRB1*04:04	DRB1*07:01
LP_404	DRB1*07:01	DRB1*11:04
LP_405	DRB1*11:01	DRB1*13:04
LP_406	DRB1*07:01	DRB1*11:01
LP_407	DRB1*11:02	DRB1*13:02
LP_408	DRB1*03:01	DRB1*16:01
LP_409	DRB1*13:02	DRB1*16:02
LP_411	DRB1*07:01	DRB1*15:01
LP_412	DRB1*08:02	DRB1*16:02
LP_413	DRB1*04:02	DRB1*07:01
LP_414	DRB1*01:03	DRB1*03:01
LP_415	DRB1*11:01	DRB1*15:02
LP_416	DRB1*03:02	DRB1*08:02
LP_417	DRB1*08:03	DRB1*12:02

(Continued)

TABLE 2 | Continued

Subject	DRB1 Allele 1	DRB1 Allele 2
LP_418	DRB1*09:01	DRB1*14:02
LP_419	DRB1*10:01	DRB1*11:01
LP_420	DRB1*13:01	DRB1*14:01
LP_421	DRB1*04:07	DRB1*15:01

Each sample is identified by a unique donor ID and has two HLA-DRB1 alleles identified.

- I. The starting temperature is set to 1.
- II. A random sample of size N is first selected by the algorithm.
- III. This sample is scored using the Jensen-Shannon Distance (JSD) described above.
- IV. C members of the sample are replaced with new subjects from the available samples.
- V. The subjects removed from the sample are put back into the available samples.
- VI. This sample is scored using the JSD.
- VII. This new sample will be accepted if either of two criteria are true:
 - i The new sample has a lower JSD score.
 - ii A number drawn from a uniform distribution, U (0,1), is less than the current temperature.
- VIII. The current temperature is multiplied by (1- α).
- IX. Return to step 4 while the number of iterations is below the target number of iterations.
- X. Output an optimized sample to the user.

The algorithm is written in Python (14). All visualizations were created in R Core Team (15) using the ggplot2 package (16) or in Python using matplotlib (17).

Parameter Testing

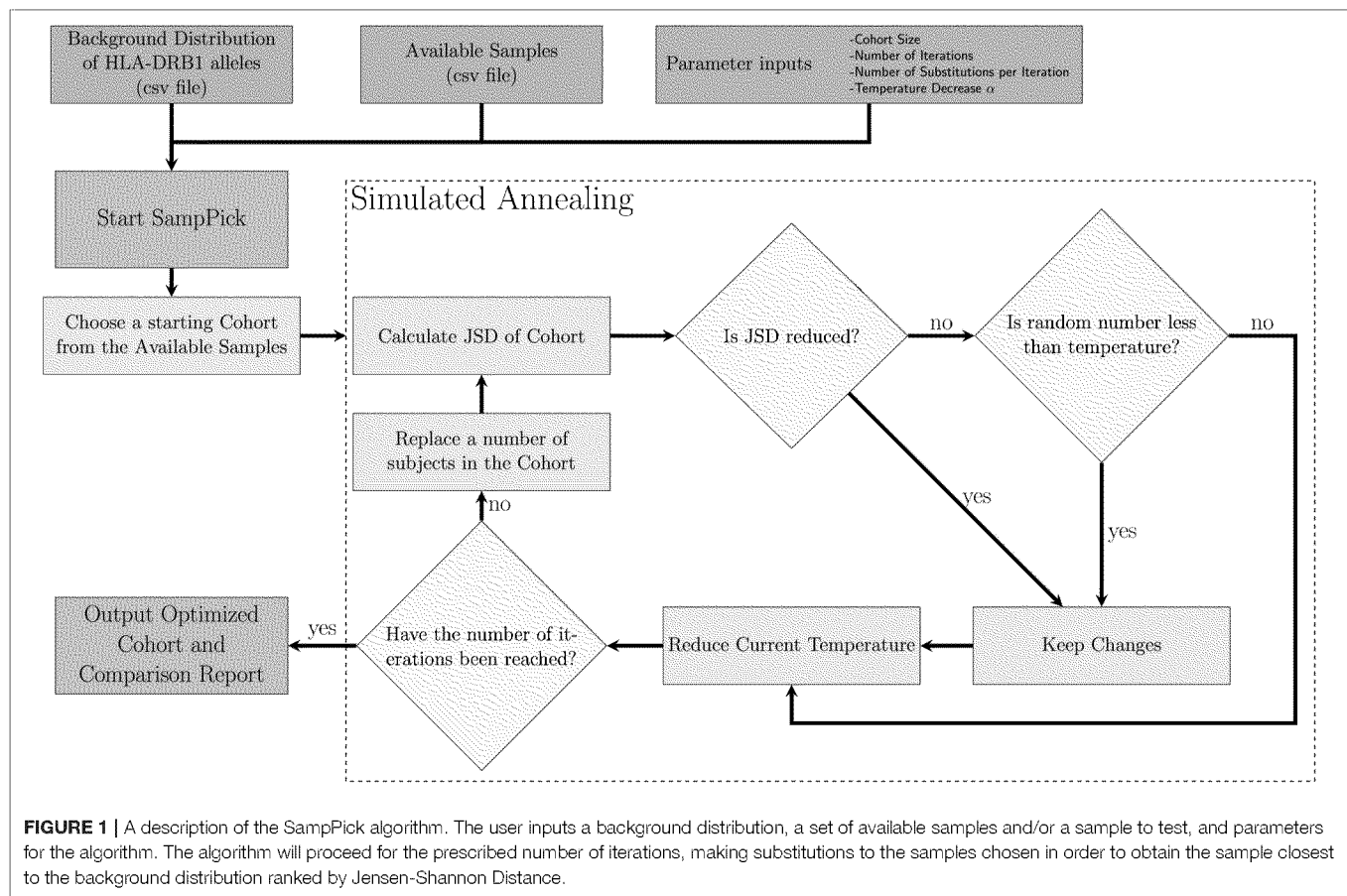
The algorithm was run using the background population of interest and available sample mentioned above. Sample sizes of 10, 20, 30, 40, 50, 75, and 100 were chosen from the available sample of 159 subjects. The number of substitutions during each iteration was all discrete values from 1 to 10. The number of iterations test were: 10^2 , 10^3 , 10^4 , 10^5 , and 10^6 with corresponding values for α of: 0.2, 0.02, 0.002, 0.0001, and 0.00001. These values of α were chosen to allow for the exponential decay of the temperature over the respective number of iterations to approach 0 with sufficient speed.

It should be noted that parameters are highly sensitive to the inputs given and it is often best to try a number of combinations of parameters (as seen in section Optimizing Number of Iterations, Size of Replacement Sub-set, and Cohort Size for Simulated Annealing) to get the best results.

RESULTS

Workflow and Description of Computation Tool

We have developed a computational tool that uses a bio-repository of HLA typed cells, and a background distribution of



allelic frequencies in the global population or a sub-population of choice. In this study we used an on-line catalog of HLA typed frozen cells, ePBMC® to evaluate the algorithm. The number of unique donors in the catalog fluctuates; at the time of our analysis there were cells from 159 unique donors listed who had non-ambiguous HLA-DRB1 typing. The workflow of the computational tool is illustrated in **Figure 1** and described in the section Methods. In our testing of the software, we first developed trials to evaluate the parameters being used in the software. We then present instructive examples that show a variety of uses for this software.

Optimizing Number of Iterations, Size of Replacement Sub-set, and Cohort Size for Simulated Annealing

As illustrated in **Figure 1**, our computational tool selects a cohort from the available pool of donors. The input parameters for simulated annealing are an important factor in finding the most compact, yet representative group of donors to use for an experiment. Our algorithm was tested using different numbers of iterations (100, 1000, 10000, 100000), various numbers of substitutions made per iteration (from 1 through 10) and different cohort sizes from 2 to 157.

The first parameter we evaluated is the number of iterations the algorithm runs for. The JSD scores decrease as one allows the algorithm to run longer. These extra iterations allow for more fine-tuning of the cohort selection. However, there is a limit

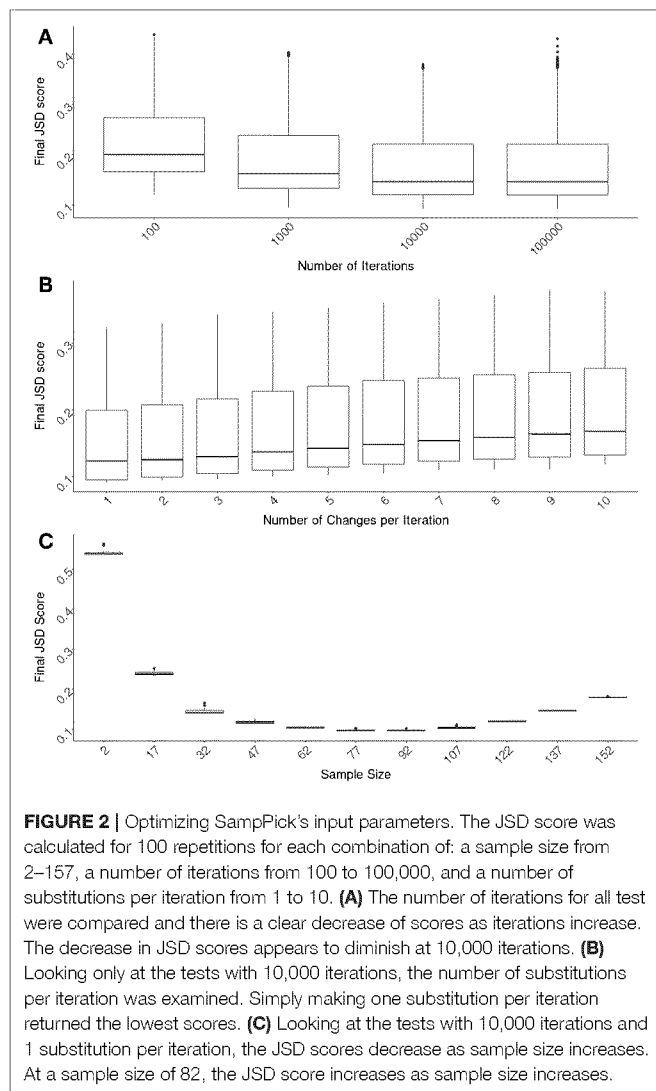
to the usefulness of this fine-tuning and unnecessary iterations increase in computational time (and costs). We found that 10,000 iterations are optimal (**Figure 2A**) and used this number for evaluating the other parameters. It is important to note that the α parameter is directly related to the number of iterations chosen. An α needs to be chosen for each number of iterations such that $(1 - \alpha)^i$ approaches 0 as i approaches the number of iterations. The values we used, as shown in section 2.5 could be used as a guideline for how quickly $(1 - \alpha)^i$ approaches 0.

Using test runs that all had 10,000 iterations, we evaluated the number of changes allowed per iteration. In these tests we found that the lowest JSD scores were obtained when there was only one change made per iteration (**Figure 2B**).

The size of the cohort chosen is related to considerations outside of the scope of this algorithm including cost, samples availability, etc. We have assessed several different sample sizes using these original parameters of 10,000 iterations with an α of 0.002 and one change per iteration (**Figure 2C**). The variation in JSD scores in relation to sample size will be helpful for the user in deciding on an appropriate sample size.

Performance of the Computational Method and the Range of JSD Scores

Based on the results shown in **Figure 2**, the following parameters were selected for evaluating the performance of our algorithm: Cohort size (N) = 50; Replacement number per iteration (C) = 1, number of iterations (I) = 10,000. Using our pool of 159



subjects we randomly selected 10-million cohorts of 50 subjects each. The JSD scores for these (compared to the frequencies of HLA variants in the US population) are depicted in the histogram in **Figure 3**. We also used the same pool of subjects to run our algorithm to select 100 cohorts (of 50 subjects each). The mean of the JSD scores for the cohorts selected by our algorithm (0.11) was 7 standard deviations below the mean JSD scores for the random samples. In addition, 1,000 random sets of 100 alleles, drawn with replacement from the 30 most common in the North American population, were generated and scored according to JSD. These allele sets had the same number of alleles as a cohort of 50 donors, but the alleles were uniformly distributed with respect to HLA type. These sub-sets were used as a negative control to examine the scores of cohorts that were not drawn from the given population distribution at all. These cohorts of random alleles were found to have a higher JSD score than the random donor cohorts, 0.365. This progression of shows that while the random cohorts drawn from actual donors are more representative of the

population of interest, the optimized samples that result from our algorithm are far better.

The frequencies of HLA alleles in the pool of 159 subjects compared to the frequencies of the same alleles in the US population (**Figure 3A**) illustrate considerable discrepancies. A much smaller cohort of 50 subjects drawn from this pool using our algorithm however shows a far closer match to the frequencies of HLA alleles in the population (**Figure 3B**).

The Diminishing Returns of Increasing Sample Size

One important utility of the SampPick algorithm is that not only does it choose samples that more closely match the population of interest, but it will often choose fewer samples than available in the pool. **Figure 2C** shows the JSD scores from 25 runs of the algorithm with sample sizes ranging from 2 to 157 (out of 159 available samples in the pool). It is not surprising that the JSD scores decrease as sample size increases from 2 to ~80. What is crucial for the setup of experiments using a limited donor pool such as this is that at a certain point the JSD score will start to increase, tending toward the sub-optimal score of the whole donor pool. Similar to the constraints imposed on a cohort of extremely small sizes, where it is not possible to generate a representative cohort with 2 to 7 subjects, larger cohorts with 152 or 157 samples are also constrained. In these almost complete sub-sets of the whole donor pool, the algorithm is only able to exclude small numbers of donors to match the population of interest. Our approach therefore not only results in cohorts more representative of the population but also (by limiting the size of the cohort) saves cost, labor, and time.

Is There a Need for Our Computational Method?

Assessing the Distribution of a Donor Cohort for an *ex vivo* Study of Inhibitor Development to Factor VIIa (FVIIa) Analogs Created to Reduce Immunogenicity

A cohort of 50 HLA-typed donors were used in T-cell proliferation and ELISA assays to assess the T-cell responses of three variants engineered to reduce the potential immunogenicity of FVIIa (18). In this experiment, donors were manually chosen with the intention of being representative of the North American allelic distribution. As shown in **Figure 4**, the optimized sample shows a closer match to the North American allelic distribution than the samples selected by hand. In addition to avoiding overrepresenting common alleles such as HLA-DRB1*15:01, HLA-DRB1*13:02 and HLA-DRB1*13:02, the algorithm selects alleles such as HLA-DRB1*04:02 and HLA-DRB1*04:03. The use of our algorithm caused a reduction of JSD from 0.198244 in the hand-picked sample to 0.111660 in the optimized sample.

Assessing a Cohort Used in a Clinical Study for Association of HLAs With Neutralizing Antibodies to Factor VIII (FVIII)

A cohort of 57 HLA typed subjects with Hemophilia A was used in a study of neutralizing antidrug antibodies to FVIII (19). As

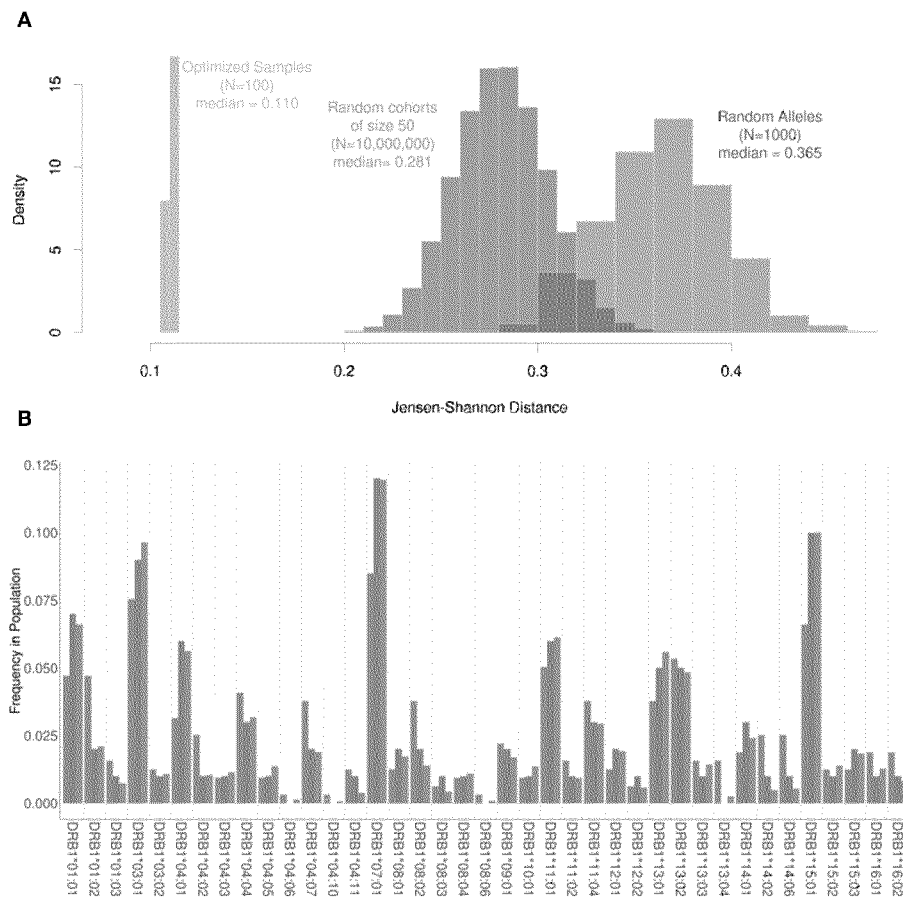


FIGURE 3 | The range of Jensen-Shannon distances. **(A)** 10,000,000 random samples of 50 subjects were drawn from our pool of donors and their similarity to the background distribution of North Americans was calculated using JSD (Orange) with a median value of 0.281. Additionally, 100 samples were created using our optimization algorithm (Green). These 100 samples all had a sample size of 50 and were run using one substitution per iteration and 10,000 iterations. The scores obtained using the algorithm were roughly 7 standard deviations below the mean of the scores for the random samples with a median score of 0.110. In addition, 1000 sets of completely random alleles were selected (Blue). These random sets, which were meant as a negative control, have a median score of 0.365. **(B)** The frequency of alleles in the background distribution of alleles in the North American Population (blue) was compared to both the entire set of 159 subjects available (red) and a smaller subset of 50 subjects selected by the algorithm (green). Although the optimized sample is much smaller than the available samples, it is much closer to the desired distribution of alleles.

the study was conducted in the US, we estimated the JSD score (0.29) between this cohort and the North American population with respect to HLA-DRB1 allele frequency (**Figure 5A**). We also generated 1,000,000 random samples of 57 subjects each from the ePBM samples to obtain a distribution of JSD scores from this population. Since the population in the study and the source of the ePBM samples is similar (North American), it is not surprising that the study population fit within this distribution. We then ran our algorithm 100 times to create optimized samples from the same pool of ePBMs. These optimized samples have a much lower JSD score when compared to the allele frequencies in the North American population. In addition to showing a global decrease in the JSD score (**Table 3**), we also demonstrate that compared to the study cohort our optimized cohort better matches the frequencies of individual HLA variants in the North American population (**Figure 5B**). Our analysis illustrates that the distribution of HLA alleles in a typical clinical study is sub-optimal if

the aim is to have a cohort that is representative of the general population.

Comparing the Distribution of HLA Variants in a Biased Cohort, Randomly Selected Individuals, and a Cohort Optimized Using SampPick

A study carried out in Iraq (20) recruited self-selecting individuals from a hospital and not a randomly selected cohort. This cohort has a much higher JSD score than the distribution of JSD scores of 1,000,000 randomly selected 48 individual samples from the ePBM[®] databank as compared to a background distribution of Middle Eastern and North African donors due to the inclusion of some very rare alleles (i.e., DRB1*14:02) (**Figure 6A**). An optimized cohort of 48 subjects had a lower JSD score when compared to either the randomly selected subjects or the subjects included in the study (**Figure 6A**). This analysis provides another example where study subjects are sub-optimal with respect to distribution of HLA variants (**Figure 6B**).

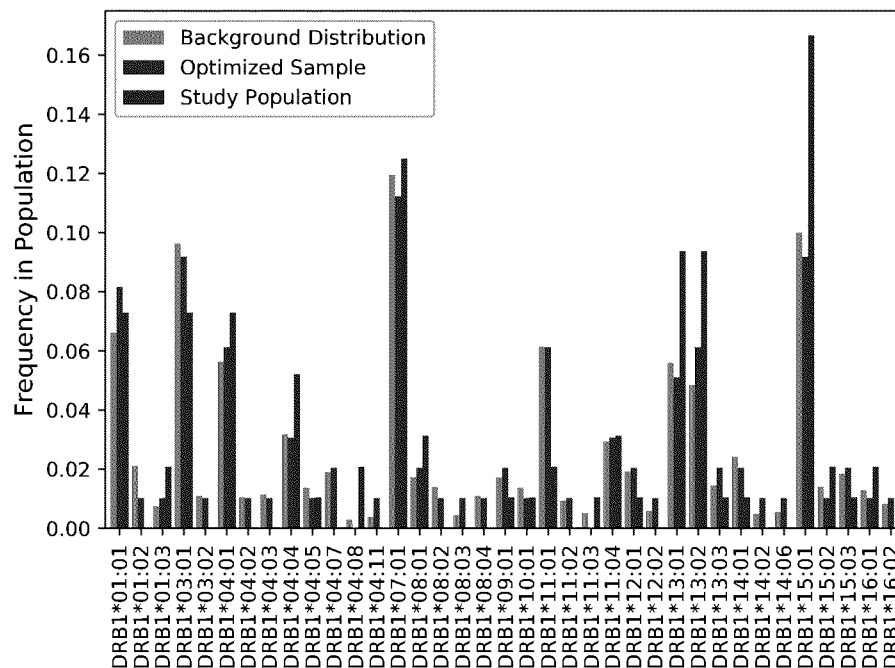


FIGURE 4 | Optimizing selection of a hand-picked donor cohort. A cohort of 50 HLA-typed donors were used in a T-cell proliferation and ELISA assay to assess the T-cell responses of three variants engineered to reduce the immunogenicity of a FVIIa. The frequency of each allele found in the assay is shown in black. The frequency of each allele in the optimized sample is shown in blue. The frequency of each allele in the background distribution of alleles in the North American population is shown in green. The use of our algorithm resulted in a reduction of JSD from 0.198244 in the hand-picked sample to 0.111660 in the optimized sample when each was compared to the background distribution of HLA variants in the US population.

Evaluating the Distribution of HLA Variants in Cohort of Subjects With Rheumatoid Arthritis (RA) Using SampPick

A study by Lee et al. (21) evaluated 1364 HLA typed Korean subjects, 744 patients with RA and 620 healthy controls. Consistent with previous findings, this study demonstrated an association between some HLA haplotypes and RA. We computed the JSD scores for the HLA alleles in the patient population and control subjects as well as a distribution of theoretical samples drawn from the pool of alleles in the population, weighted by allele frequencies found on the Allele Frequencies Net Database (22, 23) (Figure 7A). While the large sample sizes of these theoretical groups coupled with the fact that they were drawn using the theoretical frequencies of the background distribution should render a nearly perfect match, the results show that they still have some distance from the background distribution, with median scores of 0.062 and 0.067. The distribution of HLA alleles in both populations deviated from the distribution of alleles in the Korean population (JSD scores of 0.1289 and 0.2398 for the control and RA populations, respectively) (Table 3). This finding is consistent with our findings in Figures 4–6 and shows that the relative frequencies of HLA variants in randomly picked individuals, deviates from that found in the population. This bias could be due to some bias in either the sample selected or in the background distribution. However, the difference between the JSD scores for the control and patient populations suggests that specific

alleles are represented in the patient population. Figures 7B,C show that the differences in allelic distribution is due to large discrepancies in certain HLA-DRB1 alleles in the RA cases as compared to the Korean population that are not exhibited in the controls.

DISCUSSION

In the last decade novel and critical medical interventions have revolutionized the treatment of many devastating diseases. Their immunogenic potential poses a critical safety and efficacy threat. Therefore, immunogenicity needs to be considered at every step of drug-development and licensure (8). For most biologics, immunogenicity risk is evaluated through clinical trials, but the size of the trial (and donors for *ex vivo* non-clinical immunogenicity assessments) can range from thousands of patients to <100, depending on the disease. A critical unresolved concern when setting up clinical trials is that adequate and representative HLA distribution is achieved so that the results can be extrapolated to the affected population. Multiple studies have shown that HLA alleles play a central role in the immunological cascade and some immune responses are HLA-restricted (24). The HLA repertoire is the most diverse in the human genome and different HLA alleles occur at different frequencies in different human sub-populations (23). This is illustrated by the difference in relative frequencies of HLA alleles between human ethnicities (25, 26) disease conditions (27, 28), etc. Currently, given the

difficulties of putting together a cohort that is representative of the HLA repertoire, most studies do not even collect information on the HLAs represented in the cohort. As shown above, based

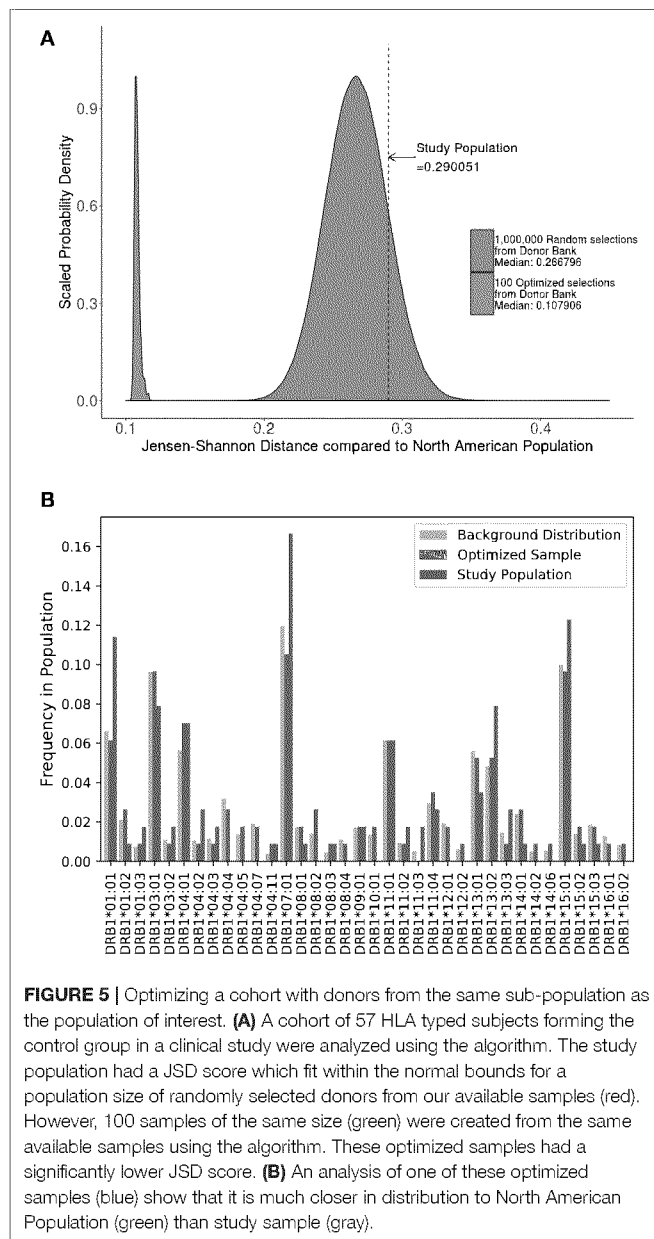


TABLE 3 | A summary of studies that were analyzed in the testing of SampPick.

Study	Size of background distribution	Sample size in study	Jensen Shannon distance score of study population	Jensen Shannon distance score of optimized cohort selection
FVII (18)	5,745,199 (13)	50	0.198	0.112
FVIII (19)	5,745,199 (13)	57	0.290	0.108
Psoriasis (20)	70890 (13)	90	0.603	0.168
RA Cases (21)	4280 (22)	744	0.240	N/A*
RA Controls (21)	4280 (22)	620	0.129	N/A*

Included are the size of the population used to create the sub-populations background distribution of alleles, the cohort size analyzed, and the JSD score of both the cohort analyzed and the optimized cohort.

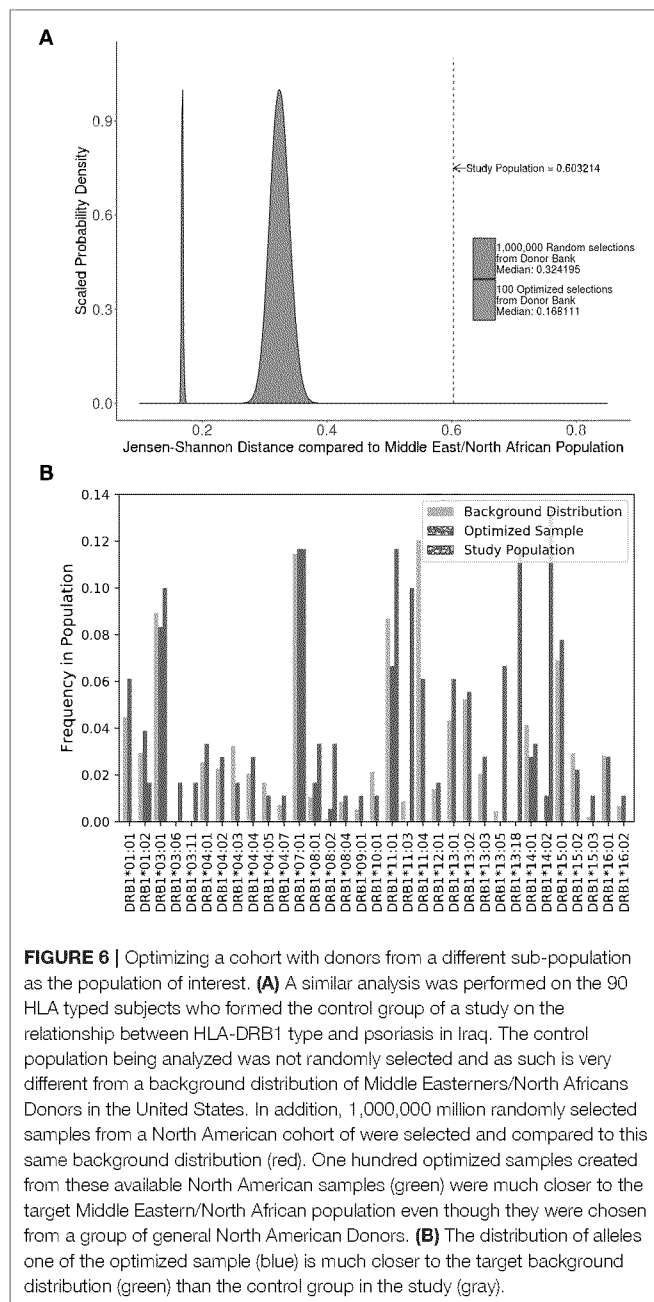
*These examples do not include optimized samples. They are examples of post-hoc analysis of two study samples.

on a JSD-score (see methods and results) the HLA distribution of study subjects in some studies where HLA typing was performed (Figures 4–7) was not representative of the population from which the population was drawn. This is expected as we find that the HLA distributions of as many as 700 randomly selected individuals deviates from the distribution of HLA variants in the same population to some degree (Figure 7A), but larger trials are expensive, and can delay the access of patients to drugs.

One proposed solution to the seemingly intractable problem of generating a reasonably-sized cohort that includes HLA variants at frequencies comparable to any desired population is to use simulated annealing (29). As previously discussed, self-selection bias and the “healthy donor effect” (12, 30) will cause for some bias in the available samples in a blood bank. If a cohort of donors is selected from these biased samples, the HLA allele distribution will closely match unknown, biased population of blood donors rather than the target population. To counteract this, samples can be compared to a known distribution. We assume that the actual distribution of HLA alleles in a population is known or can be estimated using larger samples such as the Be the Match® bone marrow donor registry (13). By optimizing cohort selection to a known distribution, rather than randomly sampling a biased sub-population, the selected cohort of subjects will be a better match of HLA type distribution.

For instance, we demonstrate an improvement in the JSD-score for cohorts of 50 subjects selected using SampPick that is 7 standard deviations below the mean score of 10-million randomly selected cohorts of 50 each (Figure 3A). Importantly, the cohorts of 50 generated using SampPick were constructed from a pool of only 159 HLA typed donors. This indicates that this tool can improve the HLA distribution efficiency of subject selection even in the absence of large numbers of available subjects.

A second advantage of the SampPick algorithm is the ability to generate cohorts based on background distributions of any type. As is seen in the case of RA patients in Korea, the distribution of allele is different than the distribution in healthy donors (Figures 7B,C). By using the frequencies in this background distribution, one could use the algorithm to select a donor cohort to more closely match Korean RA patients. In this case, a chosen population would more closely match the population of people who are receiving a certain treatment.

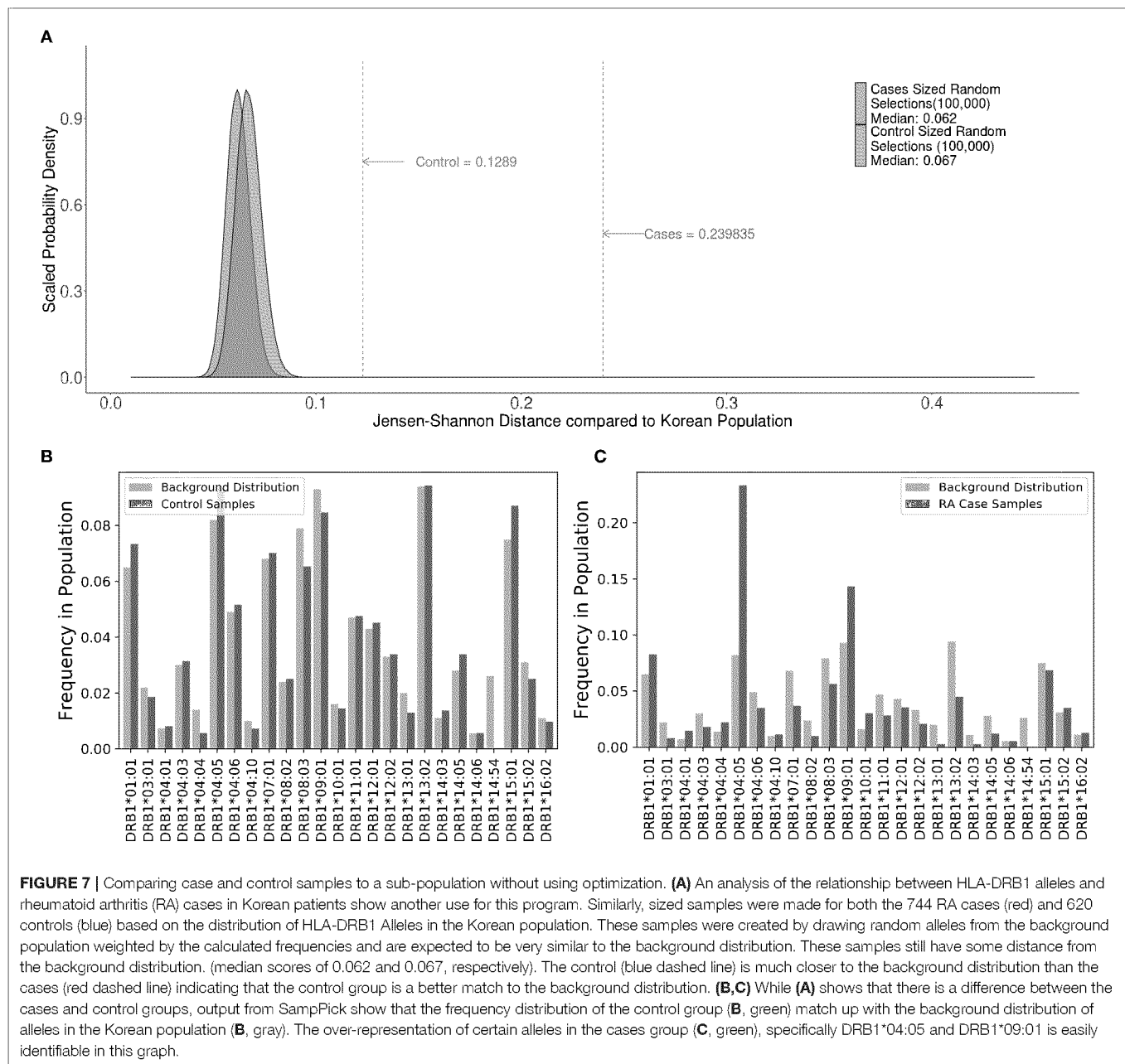


A third advantage of the SampPick algorithm is the ability to make donor cohorts the match a given subpopulation while drawing from any donor pool. Two insights are gained from the analysis of the cohort of patients from in Iraq (20) (section Is There a Need for Our Computational Method?). First, the scores of randomly chosen samples from a catalog of North American donors is higher when compared to a background distribution of alleles in a population of Middle Eastern/North African donors, medians of 0.32 vs. 0.27 (Table 3); second, optimized samples selected from North American donors, compared to a Middle Eastern/North African background distribution, have a higher score than optimized samples selected from North

American donors compared to the North American background distribution. These results should be obvious as drawing a sample from one distribution that would match a completely different distribution by chance while possible; is extremely unlikely. What is notable is that the samples from the catalog of North American donors, optimized to match the Middle Eastern/North African allelic frequencies outperformed random selections of North American donors compared to the North American allelic frequencies. This exhibits the flexibility in the allelic distribution of donor pools while selecting a cohort matching a given subpopulation's allelic distribution.

In clinical studies it may often be impractical to use HLA typing as an acceptance or rejection criterion. In addition, specific HLAs may be more frequent in certain disease populations. Further, the risk of developing NABs varies for different therapeutics. This makes it difficult to justify selection of subjects based on their HLA. Nonetheless, as HLA typing becomes more accessible, there is value in evaluating the HLA distribution in the trial cohorts and any deviation between the study population from the population that will be treated. This information can contribute to the totality of the evidence used to evaluate the immunogenicity risk of a therapeutic or a class of products, which includes experience with similar proteins, presence of a comparable endogenous protein, results from non-clinical *ex vivo* studies, etc. Post-marketing, patients with HLA alleles not included in the clinical study but adjudged high-risk based on secondary non-clinical evidence could be monitored more carefully, bringing us closer to personalized medicine.

Another potential application of SampPick is during the development of generic peptides. Peptides smaller than 40 amino acids are considered small molecule drugs and not biologics. In the US, once regulatory protection expires, it may be possible to develop generic copies of synthetic peptides, however; concern about their immunogenicity potential can preclude their licensing in the absence of clinical trials. In some instances, when the immunogenicity of the product is well-understood, bio-analytical studies can provide sufficient information to establish that the generic versions do not pose an increased immunogenicity risk in the absence of clinical trials (5). Such approaches require multiple bioanalytical, *in silico*, and *in vitro* immunological assessments and commonly include assays (e.g., ELISpot assays, T-cell proliferation assays and DC-T assays) that use human PBMC (10). To ensure that these methods provide a meaningful assessment of clinical risk it would be advantageous to use cells from a cohort of donors that is representative of the population that will receive the drug. Such a cohort can be aided by using SampPick to select the donors. Catalogs of banked HLA typed blood and PBMC samples are readily available and we have demonstrated that it is possible to use one such catalog with a listing of 159 donors to generate a cohort of 50 with HLA frequencies comparable to those observed in the US population (Figure 4). Finally, it is increasingly recognized that some HLAs are associated with specific diseases (28) and can have an impact on the responses to medications (31, 32). In studies designed to identify or validate such associations SampPick could prove to be a useful tool.



One potential drawback to SampPick is that it is used on a single feature in our test cases, HLA-DRB1. This is not a problem when working under the assumption that there is a genotype that is the main driver of a systemic response; however, this could be an issue when using this method in other situations. For instance, it has been demonstrated (33) that for peptides derived from the Dengue Virus the overall magnitude of CD4⁺ T cell responses is higher in HLA-DRB1 compared to other HLA class II alleles. However, when antigens (proteins) encoded by the Dengue polyprotein were tested, for some of the proteins the magnitude of CD4⁺ T cell response was higher for HLA-DQ or HLA-DP. Thus, in designing experiments for cases where alleles other than HLA-DRB1 are

of interest our software allows addition of a functionality for the creation of individual features describing combinations of multiple alleles. This extension of the software is described in the documentation and enables the users to optimize populations for other biallelic traits, haplotypes or phenotypic traits which may find applications in pharmacogenomic studies. It is worth noting that the increasingly smaller joint frequencies of combinations of alleles or traits will most likely require larger sample sizes to ensure adequate coverage of genotypic combinations.

Additionally, the efficacy of this algorithm is dependent on the sample from which to draw from. While we have shown that cohorts with HLA distributions representing racially different populations can be created using the same pool of donors, it is

preferable to use a pool of donors that is similar to the desired population. This is shown in the comparison of the two examples of sample optimization (**Figures 6A, 7A**). A sample of North American donors was used to generate two cohorts: (i) Matching the HLA distribution of the North American population and (ii) matching the HLA distribution of the Middle Eastern/North African population. We obtained lower JSD scores for the former compared to the latter (0.108 vs. 0.168).

In summary, we have described the development of a computational tool, SampPick, that can be used to assess the distribution of HLA frequencies in cohort of subjects as well as to generate a cohort that is closely matched vis-à-vis HLA frequencies to a target population. We have also provided several examples showing that SampPick could prove useful during the selection of patients or blood donors to improve the development of protein therapeutics, therapies that target the immune system and in clinical studies that evaluate these products. The use of this tool can facilitate the translation of results from *ex-vivo* studies and clinical trials to the patient population.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

JM and OY conceptualized and designed the study under the supervision of HY and ZS. Data acquisition was performed by JM and OY. Software and data visualizations were created by JM.

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SUPPLEMENTARY MATERIAL

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Software is available for download at: <https://www.github.com/fda/SampPick>.

Supplementary Table 1 | The weighted and scaled frequencies of HLA-DRB1 variants in the North American population.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Neutralizing Anti-Rituximab Antibodies and Relapse in Membranous Nephropathy Treated With Rituximab

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Membranous Nephropathy (MN) is an autoimmune disease associated with antibodies against podocyte proteins: M-type phospholipase A2 receptor (PLA2R1) or thrombospondin type-1 domain-containing 7A (THSD7A) in 70 and 3% of patients, respectively. Antibody titer is correlated with disease activity: rising during active disease and decreasing before remission. Therefore, decreasing PLA2R1-Antibodies titer has become an important goal of therapy. Rituximab a chimeric monoclonal antibody induces remission in 60–80% of primary MN patients. All monoclonal antibodies such as rituximab can elicit antidrug antibodies, which may interfere with therapeutic response. We aim to analyze the relevance of anti-rituximab antibodies on the outcome of MN after a first course of rituximab. Forty-four MN patients were included and treated with two 1 g infusions of rituximab at 2-weeks interval. Anti-rituximab antibodies, CD19 count, and clinical response were analyzed. Then, we (i) analyzed the association of anti-rituximab antibodies at month-6 with response to treatment: remission, relapse and the need for another rituximab course; (ii) confirmed if anti-rituximab antibodies could neutralize rituximab B-cells depletion; and (iii) tested whether anti-rituximab antibodies could cross-inhibit new humanized anti-CD20 therapies. Anti-rituximab antibodies were detected in 10 patients (23%). Seventeen patients received a second rituximab course after a median time of 12 months (7–12), following nine cases of resistance and eight relapses. Anti-rituximab antibodies were significantly associated with faster B-cell reconstitution at month-6 (75 [57–89] vs. 2 [0–41] cells/ μ l, $p = 0.006$), higher proteinuria 12 months after rituximab infusion (1.7 [0.7; 5.8] vs. 0.6 [0.2; 3.4], $p = 0.03$) and before treatment modification (3.5 [1.6; 7.1] vs. 1.7 [0.2; 1.7] $p = 0.0004$). Remission rate 6 months after rituximab was not different according to anti-rituximab status ($p > 0.99$) but the rate of relapse was significantly higher for patients with anti-rituximab antibodies ($p < 0.001$). These patients required more frequently a second course of rituximab infusions (7/10 vs. 10/34, $p = 0.03$). Anti-rituximab antibodies neutralized rituximab activity in 8/10 patients and cross-reacted with other humanized monoclonal antibodies

in only two patients. Three patients with anti-rituximab antibodies were successfully treated with ofatumumab. Anti-rituximab antibodies could neutralize rituximab B cells cytotoxicity and impact clinical outcome of MN patients. Humanized anti-CD20 seems to be a satisfying therapeutic alternative for patients with anti-rituximab antibodies and resistant or relapsing MN.

Keywords: membranous nephropathy, rituximab, anti-CD20 monoclonal antibody, immunogenicity, immuno-monitoring

INTRODUCTION

Membranous Nephropathy (MN) is a renal autoimmune disease defined by sub-epithelial immune complex deposits inducing a dysfunction of the glomerular basement membrane. Most cases are associated with antibodies against podocyte proteins: M-type phospholipase A2 receptor (PLA2R1) or thrombospondin type-1 domain-containing 7A (THSD7A) in 70% and 3% of patients, respectively (1, 2). The pathogenic role of anti-PLA2R1 antibodies (PLA2R1-Ab) is not yet demonstrated, but antibodies titers correlate with disease activity i.e., rising during active phases and decreasing before clinical remission (3). One third of patients enter into spontaneous remission while another third progresses to end stage kidney disease (4). High titers of PLA2R1-Ab at diagnosis are associated with poor clinical outcome (5). Reducing anti-PLA2R1 antibody levels is a major goal of treatment.

Rituximab is a chimeric monoclonal antibody including human IgG1 constant regions and a murine anti-human CD20 variable region which can lyse lymphocytes B (CD20+ cells) (6). Rituximab was first used in non-Hodgkin lymphoma treatment (7), but is now used in many auto-immune diseases (8–13) including MN (3, 14–17), with an excellent efficacy and tolerability in comparison to more conventional treatment regimens (18, 19). In fact, rituximab induced clinical remission in 60–80% of patients with primary MN in several non-randomized studies (3, 14–16) and its efficacy was established in a recent controlled study after an extended follow-up (17). Rituximab efficacy increases with regimens using high doses (1 g D0 and D15 i.e., 2 g) with 67% of remission at month-6 vs. low doses (375 mg/m² D0 and D7 i.e., 1.4 g) with 33% of remission at month-6 (20).

While rituximab efficacy seems to be well-established in MN, many factors could modify rituximab response. Rituximab pharmacokinetic is largely variable among patients, related to genetic factors or disease, which could impact on B-cell lysis and clinical response (21–23). However, rituximab may be lost in the urine of nephrotic patients and a close monitoring of rituximab residual level could help to retreat patients underexposed to rituximab after a first line (23, 24). Some cases of resistance after rituximab have been described in lymphoma by a decreasing of CD20 expression after repeated rituximab therapies (25). Moreover, monoclonal antibodies such as rituximab can elicit antidrug antibodies, which may interfere with therapeutic response.

First generation fully murine monoclonal antibodies led to very high levels of antidrug antibodies and the murine

constant region of these antibodies interacted poorly with human FcRn on endothelial cells with little recycling and rapid clearance from the body. In second-generation chimeric monoclonal antibodies, such as rituximab, human constant regions replaced the corresponding murine regions, resulting in decreased immunogenicity and increased serum residual levels. Humanized or fully human monoclonal antibodies were later developed to further decrease immunogenicity. For example, IgG antibodies to infliximab developed in about 60% of patients with Crohn's disease blunting treatment response (26). In six multinational trials evaluating bococizumab, a monoclonal antibody targeting PCSK9, antidrug antibodies significantly attenuated the decreasing of LDL cholesterol levels (27). A recent study demonstrated that immunization to rituximab is more frequent in systemic autoimmune diseases (31.1%) than rheumatoid arthritis (8.6%) (28). In pemphigus vulgaris and ANCA-associated vasculitis, patients with anti-rituximab antibodies presented disease relapses (11, 12). In systemic lupus erythematosus (SLE), antibodies to rituximab correlated with poor B-cell depletion and negative outcomes (22), impaired normalization of dsDNA titers and predict infusion-related reactions (29). In previous MN studies, Fervenza et al. detected anti-chimeric rituximab antibodies in 6 of 15 patients but these antibodies were not associated with remissions (16).

The improved patient-outcomes and cost-effectiveness have led to the development of other anti-B cells agents (30). New monoclonal antibodies targeting CD20 are currently studied in non-Hodgkin lymphomas and autoimmune diseases (7, 31), including two humanized IgG1: obinutuzumab and ocrelizumab (Roche®); and a fully-human IgG1: ofatumumab (GSK®).

We aim to monitor development of anti-rituximab antibodies in a cohort of patients treated for primary MN and to assess whether resistance or relapse of MN after rituximab treatment could be associated with the development of anti-rituximab antibodies. We then tested whether new humanized and fully human anti-CD20 monoclonal antibodies could be used as therapeutic alternatives.

MATERIALS AND METHODS

Drug Minimal Cytotoxic Concentration Assessment

We assessed *in vitro* minimal anti-CD20 monoclonal antibody cytotoxic concentration. Anti-CD20 monoclonal antibodies (rituximab, obinutuzumab, ocrelizumab and ofatumumab) at 6.25, 12.5, 25, and 50 ng/ml were incubated with 1.5×10^3

purified B-cells (MACSprepTM HLA B Cell Isolation Kit, Milteny Biotec) for 30 min at room temperature in 60-well Terasaki plates (Dutcher[®] Strasbourg, France) in duplicates of 1 μ l per well. Then, 5 μ l per well of standard rabbit complement (Cerdarlane[®] Ontario, Canada) were added for 45 min at room temperature. Dead cells were then revealed after adding 2.5 μ l per well of Fluoroquench AO/EB staining/quench (Ingen[®] Chilly-Mazarine, France) for 10 min in darkness.

Two blinded independent evaluators read the percentage of dead cells using a fluorescent microscope (Axiovert 100 Carl Zeiss[®] Göttingen, Germany).

Patient Population

Patients were included after signing informed consent (NCT02199145). They were recruited in Nice in the Department of Nephrology-Dialysis-Transplantation at Pasteur University Hospital between July 2014 to January 2018. Inclusion criteria were: (a) biopsy-proven MN; (b), idiopathic MN defined by the absence of anti-nuclear antibodies, negative hepatitis B and C serologies, and negative cancer investigations (whole-body CT-scan, gastro-intestinal endoscopy, PSA for men and mammography for women); (c) persistent nephrotic proteinuria (i.e., urinary protein/creatinine ratio >3.5 g/g) after 6 months of maximal antiproteinuric treatment or early deterioration of kidney function, or complications of the nephrotic syndrome; (d) follow-up of at least 1 year.

Patients received two 1 g infusions of rituximab at 2-weeks interval after 6 months of symptomatic treatment and persistent nephrotic syndrome or earlier in cases of kidney failure or thrombosis. Patients did not receive concomitant immunosuppressive treatments except 100 mg of methylprednisolone dose at each rituximab infusion according to protocol. Serum and urine samples were prospectively collected before the first infusion, at months 3, 6, and 12 to measure rituximab serum levels, and anti-PLA2R1 antibodies, CD19 positive cells, serum creatinine and proteinuria. Anti-rituximab antibodies were measured at month-3 and also month-6 due to their delayed ability to be detected. In fact, the assay only measures free anti-rituximab antibodies and before month-6, rituximab is still detected, and circulating antibodies link to the drug.

Remissions were defined according to the 2012 KDIGO guidelines. Complete remission was defined by a proteinuria <0.3 g/g with normal serum albumin levels and preserved kidney function. Partial remission was defined by proteinuria <3.5 g/g with over 50% reduction of proteinuria, increasing, or normalization of albuminemia levels and preserved kidney function (serum creatinine levels increase from baseline below 30%). Remissions were counted at month 6 and subsequently before any treatment modification. A relapse was defined by an increase of proteinuria over 3.5 g/g after remission and an increase of anti-PLA2R1 antibodies for PLA2R1 related MN.

A second course of rituximab was needed for resistance to a first course of rituximab (i.e., persistent anti-PLA2R1 activity for anti-PLA2R1 related MN and active disease, after 1 year of follow-up) or relapse [increasing proteinuria (active disease) after

complete or partial remission and positive anti-PLA2R1 activity, for anti-PLA2R1 related-MN].

Detection of Anti-PLA2R1 and Anti-THSD7A Antibodies

Total IgG anti-PLA2R1 level was measured by ELISA (EUROIMMUN, Germany) and was considered positive above 14 RU. Total IgG anti-THSD7A was detected by indirect immunofluorescence (EUROIMMUN, Germany) at 1:10.

Measurement of Rituximab by ELISA

Serum rituximab level was measured by ELISA, according to the manufacturer's instructions (LISA- TRACKER, Theradiag[®] Croissy Beaubourg, France). This assay measures only free rituximab. The limit of detection defined by the manufacturer was 2 μ g/ml, with an intrarun variability of 8% and interrune variability of 10%.

Anti-Rituximab Antibodies Detection

Anti-rituximab antibodies were detected by ELISA according to the manufacturer's instructions (LISA- TRACKER, Theradiag[®] Croissy Beaubourg, France). This assay measures only free anti-rituximab antibodies. The limit of detection for anti-rituximab antibodies defined by the manufacturer was 5 ng/ml with an intra-run variability being at 9.1% and inter-run variability at 10.6%.

Neutralization of Anti-CD20 Monoclonal Antibodies by Anti-Rituximab Antibodies *in vitro*

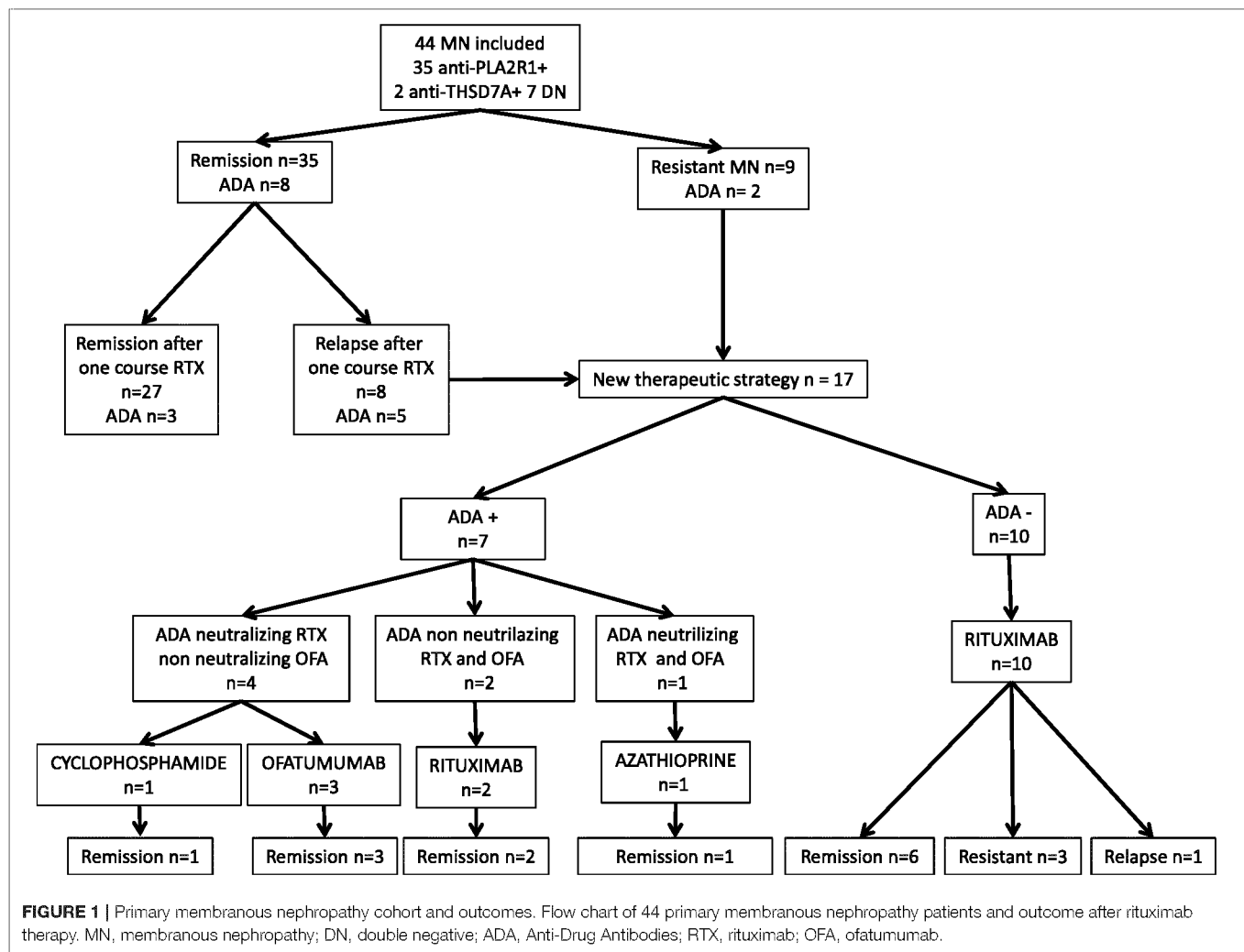
Serum samples incubated in the presence of rituximab were used to test the potential neutralizing effect of anti-rituximab antibodies.

In vitro Complement-Dependent Cytotoxicity Assay

B-cell cytotoxicity was measured in different conditions as described by Terasaki. Ten microliters of serum from patients with anti-rituximab antibodies were incubated with 10 μ l of different anti-CD20 monoclonal antibodies (rituximab, obinutuzumab, ocrelizumab, or ofatumumab) at 50 ng/ml. Sera from healthy donors were used as negative controls. Each sample was pre-incubated for 2 h at room temperature, before adding 1.5×10^3 purified B-cells for a 30-min incubation at room temperature in 60-well Terasaki plates in duplicates of 1 μ l per well. Then, 5 μ l per well of standard rabbit complement were added for 45 min at room temperature. Dead cells were then revealed after adding 2.5 μ l per well of Fluoroquench AO/EB staining/quench for 10 min in darkness. Two blinded independent evaluators estimated the percentage of dead cells using a fluorescent microscope.

Antibody-Dependent Complement-Independent Cell Cytotoxicity Assay

After a Ficoll separation, 1.8×10^6 peripheral blood mononuclear cells (PBMC) from healthy donor were incubated overnight at 4°C with 20 μ l of anti-CD20 monoclonal antibodies (rituximab,



obinutuzumab, ocrelizumab, or ofatumumab) at 50 ng/ml; pre-incubated with 20 μ l of serum from patients with anti-rituximab antibodies or from healthy donor diluted at 1:2. All samples were heated at 54°C for 30 min to inhibit complement activity. Cell viability was assessed using 10 μ l of trypan blue added to 90 μ l of PBMC incubated in each condition. Numbers of dead and alive cells were counted in four different fields. The cells were washed three times in 3 ml of PBS (Cell Wash BD Biosciences® Erembodegem, Belgium) at 4°C and incubated 30 min in darkness with a panel of antibodies specific for T, B, and NK cells: anti-CD3, anti-CD4, anti-CD8, anti-CD45, anti-CD19, anti-CD16 et CD56 (6-color TBNK Reagent BD Biosciences). Then, lysing Solution was added, and samples were incubated 10 min in darkness. The percentages of the T-lymphocytes (CD3+), B-lymphocytes (CD19+), and NK-cells (CD3- CD19-) were determined using Cytometer BD FACS Canto II.

Endpoints

We first compared the association of anti-rituximab antibodies at month-6 with response to treatment: remission, relapse

and the need for a second course of rituximab. We then confirmed if anti-rituximab antibodies could neutralize rituximab B-cells depletion and tested whether anti-rituximab antibodies could cross-inhibit new humanized anti-CD20 monoclonal antibodies.

Statistical Analyses

For descriptive statistics, data are presented as median (ranges) (for variables with non-Gaussian distribution) or mean $\pm$ standard deviation (for variables with Gaussian distribution). We used the Shapiro-Wilk test to test if a variable has a normal distribution. Comparison of qualitative criteria was performed using Chi-square test or Fisher's exact test (according to the terms of use). Comparison of quantitative variables was performed using the Student *t*-test or Wilcoxon-Mann-Whitney test (according to normal distribution). A *p* < 0.05 indicated statistical significance. Survival curves were calculated using Kaplan-Meier estimates for survival distribution. Statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA).

RESULTS

Population Characteristics

A total of 44 patients with idiopathic MN treated with two perfusions of 1 g-rituximab at 2-weeks interval were included and followed for a median time of 30 months [24–60]: 35 (80%) had anti-PLA2R1 antibodies, two (4%) had anti-THSD7A antibodies, and seven patients (16%) were double negative (**Figure 1**). **Table 1** shows the characteristics of the study cohort. All patients were on Renin-Angiotensin System inhibitor. Remission (partial or complete) was obtained in 35 of 44 patients (79%) in a median time of 3 months [3; 9] and nine patients were resistant to a first rituximab course. Residual serum rituximab level at month 3 was inversely correlated with proteinuria at month 6 ($r = -0.70$; $p < 0.0001$) (**Supplementary Figure 1**). After remission, eight patients relapsed in a median time of 15 months [10.5; 24]. At least, 17 patients were resistant or relapsed and benefited from a new therapeutic strategy in a median time of 12 months (7–12) (**Table 2**). Patients who required a second rituximab course exhibited an increase in proteinuria and anti-PLA2R1 antibodies titer before retreatment (**Supplementary Figures 2A,B**): we observed similar outcomes for relapsing and resistant patients (**Supplementary Figures 3, 4**). Twenty-seven patients were still in remission after one course of rituximab at last observation.

Detection and Assessment of Anti-Rituximab Antibodies

Anti-rituximab antibodies could be detected at month-6 in 10 patients (23%) (undetectable at month-3) and persisted in nine patients (20%). These patients had similar characteristics at diagnosis but during follow-up patients with anti-rituximab antibodies had higher level of CD19 counts at month 6, since patients with anti-rituximab antibodies showed faster B-cells reconstitution (75 [57–89] vs. 2 [0–41], $p = 0.006$) (**Figure 2A**), higher proteinuria at month-12 (1.7 [0.7–5.8] vs. 0.6 [0.2–3.4] $p = 0.03$), and before treatment modification (3.5 [1.6; 7.1] vs. 1.7 [0.2; 1.7] $p = 0.0004$) (**Figure 2B** and **Table 3**). Remission rate was not different according to anti-rituximab status [8/10 (80%) vs. 27/34 (79%) $p > 0.99$] but relapses were associated with anti-rituximab antibodies [5/10 (50%) vs. 3/34 (9%) $p = 0.009$]. Patients who developed anti-rituximab antibodies required a higher number of rituximab infusions [7/10 (70%) required a second course of rituximab vs. 10/34 (29%) $p = 0.03$] (**Table 3**). **Figure 2** shows evolution of proteinuria, anti-PLA2R1 antibodies titer and CD19+ B-cells rate according to the anti-rituximab antibodies status. Anti-PLA2R1 antibodies levels stop decreasing or increased after month-3 post-rituximab in anti-rituximab antibodies immunized patients and tended to be different before treatment modification (8 [0; 34] vs. 0.5 [0; 15] $p = 0.09$) (**Table 3** and **Figure 2C**).

Only one patient in each group presented a drug infusion reaction that did not require treatment discontinuation.

Relapses and Anti-Rituximab Antibodies

Eight patients relapsed after achieving remission ($n = 35$), with a median time to relapse of 15 months [10.5; 24]. Five of these patients had anti-rituximab antibodies (**Figure 1**). In

TABLE 1 | Cohort characteristics and outcome data.

Characteristics	Value
Age (years)	67 ± 15
Gender (female/male)	14/30
BASELINE CHARACTERISTICS	
Proteinuria (g/g)	5.9 [4.7–7.8]
Serum creatinine (μmol/l)	107 [85–147]
Serum albumin (g/dl)	2.0 ± 0.7
Anti-PLA2R1 titer (RU/ml)	152 [60–271]
CD19 at MO (cell/μl)	200 [114–299]
CHARACTERISTICS AT MONTH 3	
Proteinuria (g/g)	2.9 [1.2–5.9]
Serum creatinine (μmol/l)	103 [87–143]
Anti-PLA2R1 titer (RU/ml)	3 [0–18]
CD19 (cell/μl)	0 [0–2]
Patients with anti-rituximab antibodies	0 (0%)
Serum rituximab level (μg/ml)	2.27 [0.19–7.5]
CHARACTERISTICS AT MONTH 6	
Proteinuria (g/g)	1.7 [0.9–4.5]
Serum creatinine (μmol/l)	104 [84–136]
Anti-PLA2R1 titer (RU/ml)	0 [0–1]
CD19 (cell/μl)	9 [2–70]
Patients with anti-rituximab antibodies	10 (23%)
Serum rituximab level (μg/ml)	0 [0–0]
CHARACTERISTICS AT MONTH 12	
Proteinuria (g/g)	0.9 [0.3–2.9]
Serum creatinine (μmol/l)	101 [87–130]
Serum albumin (g/dl)	3.6 ± 0.6
Anti-PLA2R1 titer (RU/ml)	1 [0–7]

contrast, among the 27 patients who did not relapse, only three patients had anti-rituximab antibodies and two had persistent anti-rituximab antibodies ($p = 0.007$) (**Table 4**).

There was no significant difference between patients with or without relapse for age, proteinuria and PLA2R1-antibodies titers at baseline (**Table 4**). Relapsing patients were more likely to have higher CD19 count at month-3 (0 [0; 9] vs. 0 [0; 0] $p = 0.03$), higher proteinuria at month-3 (5.9 [1.8; 6.9] vs. 2.2 [0.9; 6.2] $p = 0.02$) and month-6 (3.3 [1.6; 7.3] vs. 1.4 [0.5; 1.9] $p = 0.04$), higher anti-PLA2R1 antibodies titer at month-6 (5 [0; 30] vs. 0 [0; 2] $p = 0.04$) and anti-rituximab antibodies at month-6 [5/8 (63%) vs. 3/27 (11%) $p = 0.007$] (**Table 4**).

Because the analysis of the relapse data is too complicated for logistic regression and each relapse occurred at different time points, we performed a time-to-event analysis of renal survival. Renal event was defined by achieving relapse within 2 years after the first course of rituximab. The rate of relapse was significantly higher for patients with anti-rituximab antibodies ($p < 0.001$) (**Figure 2D**).

Neutralizing Effect of Anti-Rituximab Antibodies

The minimal cytotoxic concentration, defined, as the lowest dose required producing $\geq 50\%$ of B-cells cytotoxicity,

TABLE 2 | Treatment history for rituximab resistant or relapsing patients.

	ADA status	First line	Outcome	Second line	Outcome	Third line	Outcome	Fourth line	Outcome
Patient 1	+	RTX	Relapse (12)	RTX	Relapse (12)	RTX	Relapse (12)	RTX AZA	PBC Remission (6)
Patient 3	+	RTX	Relapse (6)	RTX	Remission (3)				
Patient 4	+	RTX	Resistant	RTX	Resistant	OFA	Remission (3)		
Patient 5	+	RTX	Resistant	RTX	Resistant	OFA	Remission (3)		
Patient 7	+	RTX	Relapse (48)	OFA	Remission (30)				
Patient 9	+	RTX	Resistant	RTX	Remission				
Patient 10	+	RTX	Resistant	CYC	Resistant				
Patient 11	-	RTX	Resistant	RTX	Remission (6)				
Patient 12	-	RTX	Resistant	RTX	Remission (6)				
Patient 13	-	RTX	Resistant	RTX	Remission (6)				
Patient 14	-	RTX	Resistant	RTX	Remission (6)				
Patient 15	-	RTX	Resistant	RTX	ESKD				
Patient 16	-	RTX	Resistant	RTX	ESKD				
Patient 17	-	RTX	Relapse	RTX	Resistant	RTX	ESKD		
Patient 18	-	RTX	Relapse	RTX	Relapse	RTX	Relapse	RTX	Remission (6)
Patient 19	-	RTX	Relapse	RTX	Relapse	RTX	Relapse	RTX	Remission (6)
Patient 20	-	RTX	Relapse	RTX	Remission				

ADA, Anti-Drug Antibodies; RTX, Rituximab; AZA, Azathioprine; OFA, Ofatumumab; CYC, Cyclophosphamide. PBC, Primary Biliary Cholangitis.

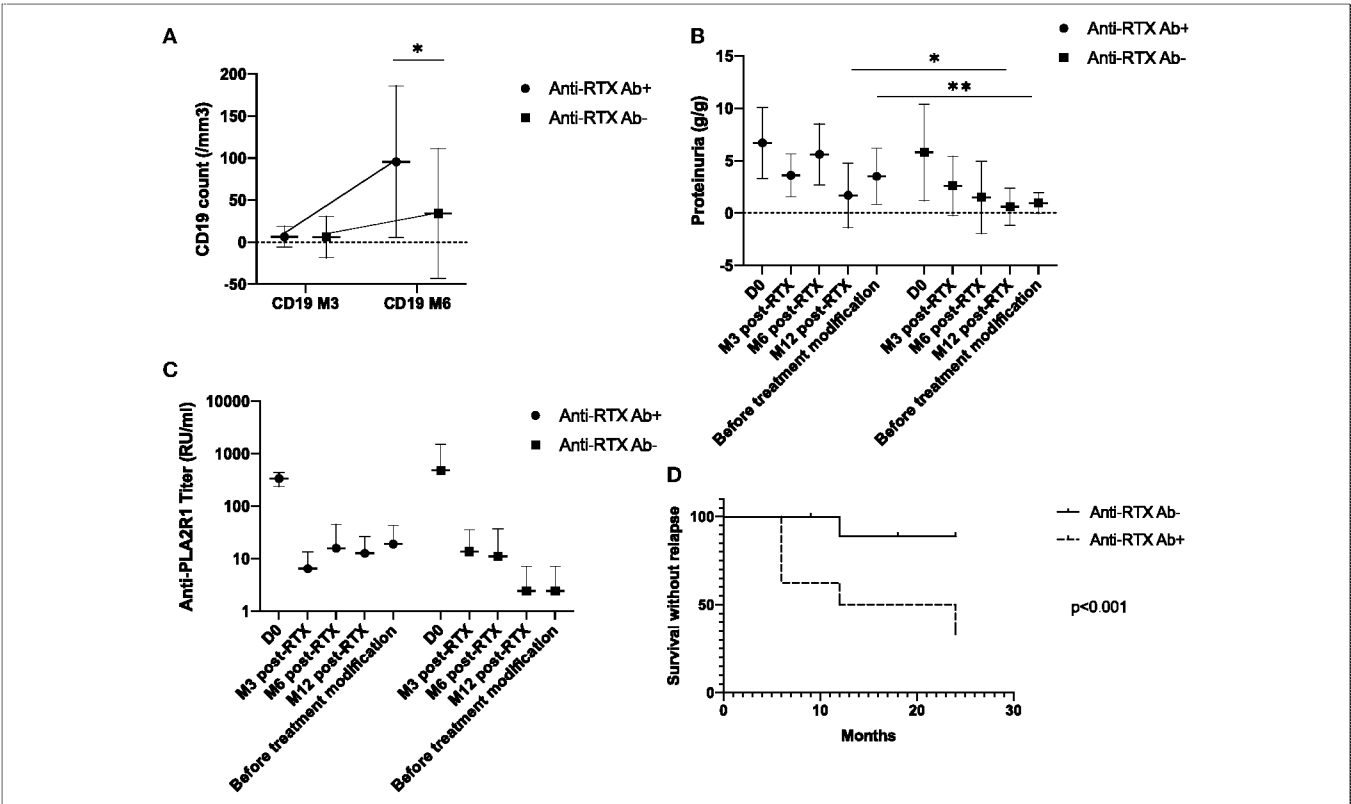


TABLE 3 | Clinical characteristics and outcome in rituximab-immunized or non-immunized patients.

	Anti-RTX antibodies + n = 10	Anti-RTX antibodies- n = 34	p-value
Age (years)	60 ± 13	60 ± 14	0.99
Gender (female/male)	3/7	11/23	0.99
BASILINE CHARACTERISTICS			
Proteinuria (g/g)	6.7 [4.7–10.6]	5.8 [4.6–7.7]	0.42
Serum creatinine (μmol/l)	127 [105–151]	107 [78–149]	0.30
Serum albumin (g/dl)	1.9 ± 0.6	2.1 ± 0.7	0.74
Anti-PLA2R1 titer (RU/ml)	148 [72–243]	159 [45–288]	0.99
CD19 (cell/μl)	236 [190–287]	143 [62–290]	0.08
CHARACTERISTICS AT MONTH 3			
Proteinuria (g/g)	3.6 [2.3–5.9]	2.6 [0.9–5.9]	0.15
Serum creatinine (μmol/l)	109 [89; 140]	100 [86; 143]	0.84
Anti-PLA2R1 titer (RU/ml)	3 [1–15]	2 [0–18]	0.86
CD19 (cell/μl)	0 [0–19]	0 [0–0]	0.46
Serum rituximab level (μg/ml)	3.59 [0.14–7.69]	2.27 [0.59–8.32]	0.74
CHARACTERISTICS AT MONTH 6			
Proteinuria (g/g)	5.6 [2.0–6.4]	1.5 [0.6–4.3]	0.13
Serum creatinine (μmol/l)	104 [86–159]	102 [84–136]	0.29
Anti-PLA2R1 titer (RU/ml)	2 [0–68]	0 [0–10]	0.43
CD19 (cell/μl)	75 [57–89]	2 [0–41]	0.006*
CHARACTERISTICS AT MONTH 12			
Proteinuria (g/g)	1.7 [0.7–5.8]	0.6 [0.2–3.4]	0.03*
Serum creatinine (μmol/l)	94 [84–110]	106 [88–129]	0.39
Serum albumin (g/dl)	3.0 ± 0.8	3.1 ± 0.6	0.88
Anti-PLA2R1 titer (RU/ml)	10 [0–27]	1 [0–3]	0.27
CHARACTERISTICS BEFORE TREATMENT MODIFICATION			
Remission after one course	8/10 (80%)	27/34 (79%)	>0.99
Relapse	5/10 (50%)	3/34 (9%)	0.009*
Serum creatinine (μmol/l)	101 [86; 130]	107 [88; 165]	0.75
Anti-PLA2R1 titer (RU/ml)	8 [0; 34]	0.5 [0; 15]	0.09
Proteinuria (g/g)	3.5 [1.6; 7.1]	1.7 [0.2; 1.7]	0.0004*
Time of follow-up	32 [13.5; 92.0]	30.0 [24.0; 96.0]	0.90
2nd course of rituximab required	7/10 (70%)	10/34 (29%)	0.03*

was 50 ng/ml for all anti-CD20 monoclonal antibodies (Supplementary Figure 5).

In a complement-dependent cytotoxicity assay, rituximab (50 ng/ml) spiked into healthy donor serum in the presence of rabbit complement induced 60% B-cell death while healthy donor serum alone had no effect (Figure 3A). The rituximab effect occurred at a concentration matching *in vivo* therapeutic conditions (7.4 μg/mL). When rituximab was spiked in patients' sera containing anti-rituximab antibodies, its effect was blocked for eight out of 10 patients (Figure 3B). In contrast, anti-rituximab antibodies did not prevent cell death induced by anti-pan B antibodies. We could notice that anti-rituximab antibodies titer did not correlate with neutralizing effect or transitory antibodies.

In a complement-independent B-cell cytotoxicity assay, we tested the effect of rituximab (30 and 50 ng/ml) on the induction of B-cell apoptosis in the absence of complement activation,

TABLE 4 | Baseline characteristics and outcome data in relapsing and non-relapsing patients.

Patients characteristics	Relapse n = 8	No relapse n = 27	p-value
BASILINE CHARACTERISTICS			
Age	63 ± 13	62 ± 14	0.85
Sex Ratio (F/M)	3/5	9/18	0.99
Proteinuria (g/g)	6.3 [5.2–9.4]	5.4 [4.5–7.6]	0.29
Serum albumin (g/dl)	2.1 ± 0.9	2.1 ± 0.7	0.77
Serum creatinine (μmol/l)	126 [94–146]	104 [76–98]	0.40
Anti-PLA2R1 titer (RU/ml)	102 [25–171]	152 [60–253]	0.33
CHARACTERISTICS AT MONTH-3			
Proteinuria (g/g)	5.9 [1.8; 6.9]	2.2 [0.9; 6.2]	0.02*
Serum creatinine (μmol/l)	116 [92; 240]	99 [79; 141]	0.16
Anti-PLA2R1 titer (RU/ml)	15 [0–18]	1 [0–14]	0.35
CD19 count (cell/μl)	0 [0–9]	0 [0–0]	0.03*
Serum rituximab level (μg/ml)	2.70 [0.01–7.41]	2.24 [0.28–9.39]	0.62
CHARACTERISTICS AT MONTH-6			
Proteinuria (g/g)	3.3 [1.6; 7.3]	1.4 [0.5; 1.9]	0.04*
Serum albumin (g/dl)	3.0 ± 1.0	3.2 ± 0.6	0.43
Serum creatinine (μmol/l)	105 [104; 157]	93 [82; 133]	0.08
Anti-PLA2R1 titer (RU/ml)	5 [0–30]	0 [0–2]	0.04*
CD19 count (cell/μl)	5 [2–115]	3 [0–63]	0.60
Anti-rituximab antibodies	5/8 (63%)	3/27 (11%)	0.007*

by flow cytometry. Overnight incubation of rituximab spiked into complement-depleted sera from healthy donors added to a total lymphocyte population led to a decrease of CD19+ cell counts as compared to incubation in the absence of rituximab (Figures 4A,C). In contrast, when rituximab was spiked in a representative complement-depleted patient's serum containing anti-rituximab antibodies, the proportion of CD19+ cells increased from 4.6 to 17.8% (Figure 4B, 30 ng/ml of rituximab) and from 0.4 to 23.9% (Figure 4D, 50 ng/ml of rituximab). Collectively, these studies showed that eight of the 10 patients with anti-rituximab antibodies produced neutralizing antibodies at concentrations sufficient to block the cytotoxic effects of rituximab with and without complement activity.

Cross-Reactivity of Neutralizing Anti-Rituximab Antibodies With New Anti-CD20 Monoclonal Antibodies

We tested whether anti-rituximab antibodies inhibited cytotoxicity of humanized and fully human anti-CD20 monoclonal antibodies. Results are summarized in Figures 1, 5A,B.

Two patients' profiles were observed. Anti-rituximab antibodies from patients 1 and 2 blocked B-cell cytotoxicity for obinutuzumab, ocrelizumab, and ofatumumab (Figure 5A) whereas anti-CD20 monoclonal antibodies efficacy was not impaired for the other eight patients (Figure 5B).

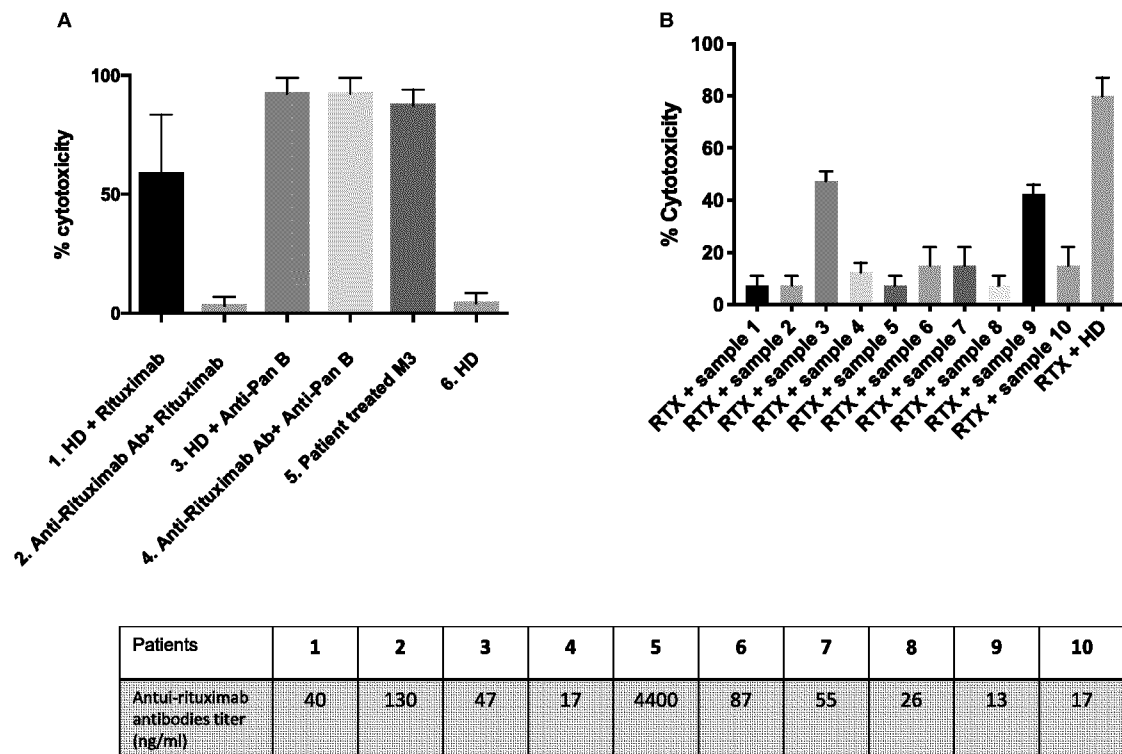


FIGURE 3 | Detection of neutralizing anti-rituximab antibodies. **(A)** Anti-rituximab antibodies neutralized complement dependent cytotoxicity induced by rituximab. Purified B cells were incubated for 30 min with the following samples: (1) Serum from a healthy donor (HD) pre-incubated 2 h at room temperature at a ratio 1:2 with rituximab 50 ng/mL; (2) Serum from a patient with anti-rituximab antibodies (Patient 1) pre-incubated 2 h at room temperature at a ratio 1:2 with rituximab 50 ng/mL; (3) Serum from a healthy donor pre-incubated 2 h at room temperature at a ratio 1:2 with anti-pan B antibody 10 ng/ml (without specific for CD20) (Ingen®); (4) Serum from a patient with anti-rituximab antibodies pre-incubated 2 h at room temperature at a ratio 1:2 with anti-pan B antibody 10 ng/ml (Ingen®); (5) Serum from a patient treated with rituximab 3 months earlier (with residual rituximab concentration of 7.41 µg/ml) and no anti-rituximab antibody; (6) Serum from a healthy donor. The histogram shows the percent of lysed B-cells after adding rabbit complement. About 60–80% of B cells were lysed upon rituximab addition. Note that the percent of lysis decreased to 10% when rituximab was pre-incubated with anti-rituximab antibodies sera and that the inhibitory effect of anti-rituximab antibodies is specific for rituximab mediated lysis (no effect on pan-B mediated lysis). Error bars represent SD of the mean. The lysis is positive when more than 40% of the cells are lysed. **(B)** Screening of patients with anti-rituximab antibodies for inhibition of rituximab induced complement dependent cytotoxicity. The first 10 histograms represent each of 10 patients with anti-rituximab antibodies by ELISA at month-6. Note that only two patients (#3 and #9) presented non-neutralizing anti-rituximab antibodies. The last histogram represents serum from healthy donor pre-incubated 2 h at room temperature at ratio 1:2 with rituximab 50 ng/ml (positive control of lysis). *Mean statistically significant.

Evolution and Personalized Care According to Rituximab Immunization

Patients' evolution is summarized in Figure 1.

We adapted our therapeutic strategy to this profile: using ofatumumab or another immunosuppressive therapy when anti-rituximab antibodies were detected.

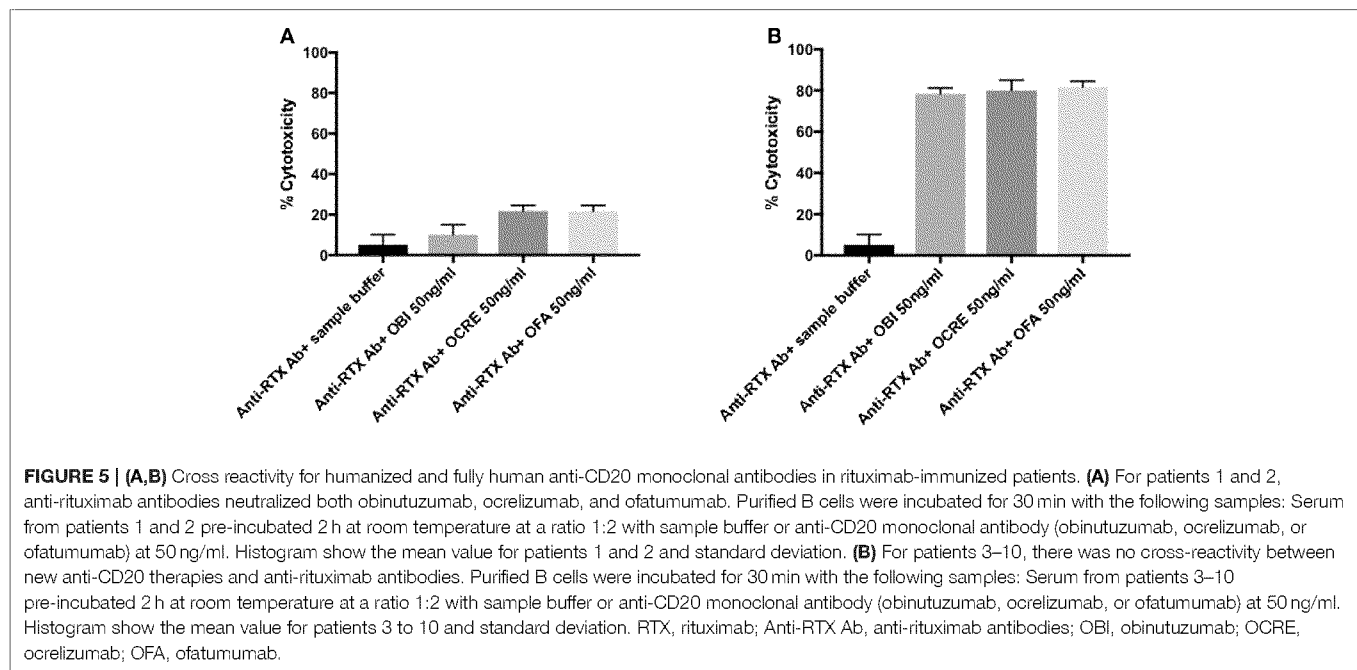
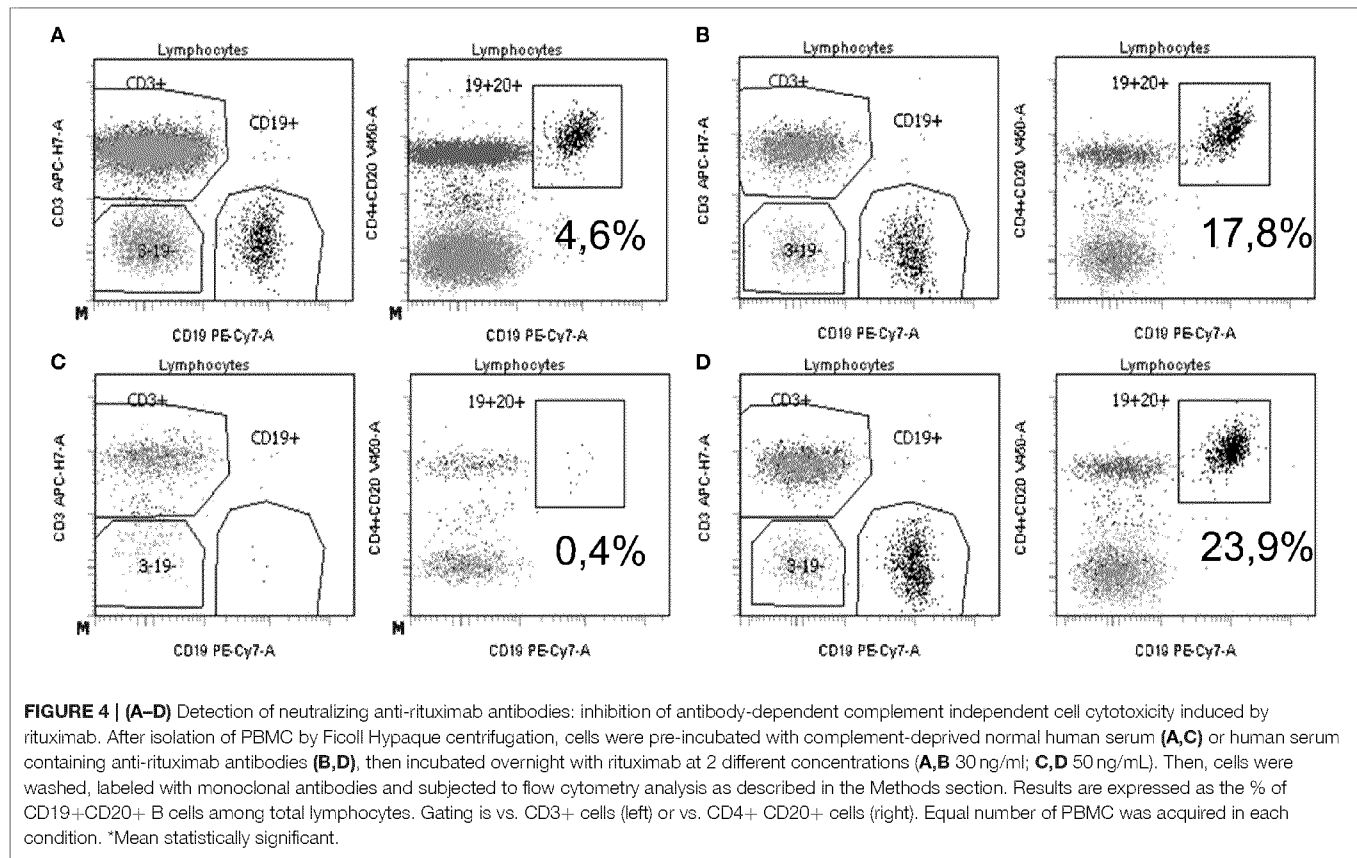
Patients 4, 5, 7, and 10 had anti-rituximab antibodies that did not cross-react with new anti-CD20 monoclonal antibodies and were treated with ofatumumab (300 mg on day 1 and 1,000 mg on day 8 ± day 21 according to clinical and immunological response) and cyclophosphamide when ofatumumab was not available. Patients 4 and 5 were resistant after two courses of rituximab; patient 7 relapsed after one course of rituximab. Those three patients achieved remission at month-3 after ofatumumab therapy. After ofatumumab, anti-rituximab antibodies disappeared at month-3 for patient 4 (Figure 6A) and were persistent at the same high titer for patient 5 (Figure 6B).

Patient 1 was treated with four courses of rituximab for MN relapses. During the last relapse, he developed primary biliary cirrhosis associated with anti-M2 and anti-gp120 antibodies. These antibodies were detected after his first relapse but before the onset of biliary cirrhosis symptoms and were negative at diagnosis. Due to cross-reactivity with new anti-CD20 monoclonal antibodies and the development of primary biliary cirrhosis, he was switched to azathioprine and finally achieved remission for his MN.

Patients 3 and 9 developed non-neutralizing anti-rituximab antibodies and achieved remission on rituximab therapy.

DISCUSSION

Anti-rituximab antibodies were detected in 23% of patients treated with rituximab for idiopathic MN and were associated with the need for at least two courses of rituximab for resistant



MN or relapses. These results are similar to findings of antidrug antibodies following anti-TNF α therapy (31–35) and rituximab in ANCA-vasculitis, SLE (11, 36) and multiple sclerosis (37). As described by Fervenza et al., we found that anti-rituximab antibodies were not associated with lower remission (15) but we described their association with MN relapses. Conversely, we

confirmed that residual serum rituximab levels at month 3 were associated with remission (20, 23). Initial CD19+ B cell depletion was seen in all patients; but in patients who developed anti-rituximab antibodies, or in patients with undetectable residual rituximab serum level at month-3 as we previously described (23), B cells started to recover earlier.

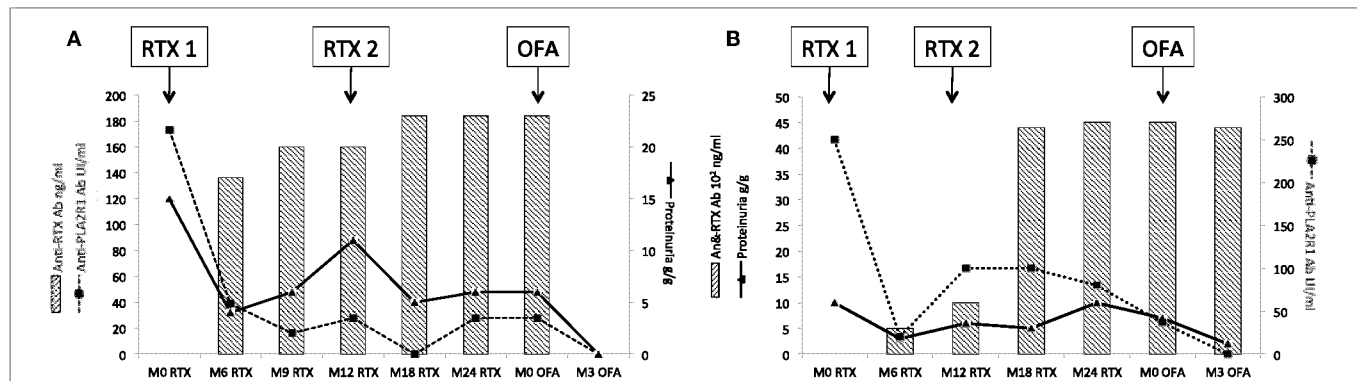


FIGURE 6 | Evolution of rituximab-resistant patients treated with ofatumumab. Patients 4 and 5 were resistant after two courses of rituximab and developed neutralizing anti-rituximab antibodies. Anti-rituximab antibodies did not neutralize *in vitro* B-cells cytotoxicity for ofatumumab. These two patients were treated with ofatumumab (300 mg on day 1 and 1,000 mg on day 8 and day 21). **(A)** Evolution for patient 4: patient 4 developed anti-rituximab antibodies (17 ng/mL at month-6 and reached 23 ng/mL at month-24). After ofatumumab infusions, anti-rituximab antibodies get negative within 3 months. Anti-PLA2R1 antibodies became also negative and proteinuria decreased to 0.4 g/g. **(B)** Evolution for patient 5: patient 5 developed anti-rituximab antibodies (500 ng/mL at month-6 and reached 4,500 ng/mL at month-24). After ofatumumab infusions, anti-rituximab antibodies were still detected and stable at 4,400 ng/mL at month-3. Anti-PLA2R1 antibodies became negative and proteinuria decreased to 2 g/g. RTX, rituximab; OFA, ofatumumab.

Anti-rituximab antibodies probably appear before month-6 but we are able to detect them only at month-6 on their free fraction. B cell depletion is possible until circulating rituximab persists. Between month-3 and month-6, anti-rituximab antibodies could block B cell depletion. In fact, in non-immunized patients, B cell depletion is prolonged until month-6 while in immunized patients, they recover B cells earlier. This might be associated with relapse or incomplete response to treatment while B cells repopulation might be a more intricate mechanism. In clinical practice in case of resistant or relapse MN, anti-rituximab should be considered to help optimized therapeutic strategy, some of them would not respond to a second course of rituximab.

We established with two different methods that anti-rituximab antibodies neutralized rituximab mediated B-cell depletion and could negatively affect clinical response. Unfortunately, we could not use samples from non-immunized patients as controls. Inhibition of rituximab activity could favor the persistence of pathogenic memory B-cells and induce disease relapse, with an earlier reconstitution of B-cell compartment (38). Distinctions have been made between non-neutralizing antibodies that do not inhibit the clinical effect of a drug and neutralizing antidrug antibodies. However, the presence of neutralizing antidrug antibodies is not always associated with a decreased therapeutic effect. Pharmacological efficacy depends on the balance between drug concentrations and antidrug-antibodies levels in some cases drug levels are sufficient to induce the therapeutic drug effect. By contrast, non-neutralizing antidrug antibodies, link to a portion far from the paratope of the drug molecule and does not neutralize its therapeutic activity (e.g., to the allotope). In such cases, the formation of antibodies is triggered by polymorphisms expressed in the constant portion of the immunoglobulin, which vary between individuals. The biologic effect of non-neutralizing antidrug antibodies is less well understood (39–42), but the formation of immune complexes may accelerate drug clearance by the reticuloendothelial system

(42). However, our patients with non-neutralizing anti-rituximab antibodies showed a favorable outcome after two courses of rituximab.

It is reported that patients with antidrug antibodies present more infusion-related reactions (29), in this study we did not confirm this finding as reported in two other studies (11, 40).

If we consider that immunogenicity is an important factor that should be considered in the overall treatment strategy, we should take actions to reduce antidrug antibodies formation: modifying drug administration; increasing dose; decreasing immunogenicity by adding immunosuppressive agents to the regimen or using new drugs which are supposed to be less immunogenic such as humanized or fully human monoclonal antibodies. In this perspective, we evaluated *in vitro* whether anti-rituximab antibodies developed in some of MN patients could inhibit B-cell depletion by three new humanized anti-CD20 therapies. To our knowledge, this is the first study looking at cross-reactivity between antidrug antibodies developed in patients treated with rituximab and new anti-CD20 molecules. For two patients, anti-rituximab antibodies blocked both obinutuzumab, ocrelizumab, and ofatumumab B-cells cytotoxicity, suggesting an anti-idiotypic activity (43). In contrast, non-neutralizing antidrug antibodies might bind to allotopes and human neo-antigens at the hinge of fusion proteins. Three of the MN patients with neutralizing anti-rituximab antibodies that did not interfere with new anti-CD20 monoclonal antibodies activity were successfully treated with ofatumumab. One patient was treated with cyclophosphamide because ofatumumab was not available. But using new anti-CD20 therapy should be considered to avoid side effects (18). After ofatumumab infusion, anti-rituximab antibodies disappeared for two patients, and were persistent at the same titer for the other one. This suggests plasma cells secreting anti-rituximab antibodies are long memory plasma cells or derived from B cells which may have lost CD20 expression as reported in lymphoma where CD20 expression level has been related to acquired rituximab resistance (25). Case reports

and one study reported ofatumumab-effectiveness in children with resistant nephrotic syndrome (44, 45). To our knowledge, study or report on adult nephrotic syndrome and MN treated with ofatumumab are lacking. In these articles, ofatumumab use is not supported by an immunological rationale or based on drug monitoring.

The main limitation of our study is that it is a monocentric retrospective study analyzing a relatively small number of patients. Nevertheless, our study remains original and innovative. Drug monitoring and development of antidrug antibodies have been well-described in anti-TNF α but studies on rituximab are recent and rare in nephrology field (16, 23). Our work is the first to suggest the value of immune-monitoring in adapting the therapeutic strategy in MN, particularly in resistant or relapsing cases. First, neutralizing anti-rituximab antibodies are not rare and their presence at month-6 is associated with subsequent relapses. Anti-rituximab antibodies might be an useful biomarker adding to residual rituximab monitoring (23), anti-PLA2R1 antibodies titer in anti-PLA2R1 antibodies related MN (46), epitope spreading (47, 48) to predict clinical outcomes, and it need to be tested in prospective studies. Secondly, rituximab immuno-monitoring might also be a helping tool for PLA2R1-negative MN patients. Then, anti-rituximab antibodies may or may not interfere with new humanized anti-CD20 monoclonal antibodies, allowing tailored rescue therapies. All last, basing on immunological and clinical arguments, new humanized anti-CD20 seems to be a satisfying therapeutic alternative in adult patients with rituximab-resistant or relapsing MN or rituximab intolerance like serum sickness. Further studies are needed to develop personalized therapeutic strategies in primary MN based on drug monitoring and immunogenicity testing.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by CPP CHU Nice. Written informed consent to participate in this study was provided by all participants.

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AUTHOR CONTRIBUTIONS

SB-S, BS-P, and VE conceived and designed the study and wrote the paper. SB-S and BS-P analyzed and interpreted the data. All authors contributed reagents, materials, analysis tools, or data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2019.03069/full#supplementary-material>

Supplementary Figure 1 | Correlation of serum rituximab level at month 3 and proteinuria at month 6.

Supplementary Figure 2 | Evolution of proteinuria (A) and anti-PLA2R1 titer (B) according to therapeutic strategy required: one course of rituximab needed $n = 27$ and new therapeutic strategy required $n = 17$.

Supplementary Figure 3 | Evolution of proteinuria (A), anti-PLA2R1 titer (B), and CD19 count (C) in resistant ($n = 9$) and non-resistant ($n = 35$). Membranous Nephropathy.

Supplementary Figure 4 | Evolution of proteinuria (A), anti-PLA2R1 titer (B), and CD19 count (C) in relapsing ($n = 8$) and non-relapsing ($n = 27$). Membranous Nephropathy (excluding resistant MN).

Supplementary Figure 5 | Determination of anti-CD20 monoclonal antibody minimal cytotoxic concentration for *in vitro* complement-dependent cytotoxicity assay. For both, the minimal concentration required to produce $\geq 50\%$ of cytotoxicity was 50 ng/ml. (A) Assessment for rituximab. (B) Assessment for obinutuzumab. (C) Assessment for ocrelizumab. (D) Assessment for ofatumumab. RTX, rituximab; OBI, obinutuzumab; OCRE, ocrelizumab; OFA, ofatumumab; Ab, antibodies.

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Factor VIII: Perspectives on Immunogenicity and Tolerogenic Strategies

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Therapeutic treatment of bleeds with FVIII can lead to an antibody response that effectively inhibits its function. Herein, we review the factors that contribute to this immunogenicity and possible ways to overcome it.

Keywords: factor VIII, tolerance, hemophilia, inhibitors, regulatory T (Treg) cells

INTRODUCTION

Self-non-self-discrimination is one of the basic tenets of the immune system. The general failure to respond immunologically to antigens in our bodies is learned during ontogeny, as aptly recognized by Ray Owen in the 1940's in seminal studies with dizygotic cattle twins which shared hematopoietic cells during fetal development (1). Thus, these siblings failed to react to red blood cell antigens or skin grafts of their unrelated twin because their immune systems had learned that they must be "self" during ontogeny. This phenomenon of "actively acquired (immunologic) tolerance" was experimentally verified by the Nobel Prize-winning experiments of Billingham, Brent and Medawar in mice (2). This process is specific because responses to unrelated antigens remains intact.

What happens in the case of a patient who fails to express a given human protein during ontogeny and is then subsequently exposed? The classic case is hemophilia A and B, where patients lack all or part of the factor VIII (FVIII) or factor IX procoagulant proteins, respectively, and therefore have never "acquired" tolerance to that protein as "self." Prophylactic or on-demand treatment of bleeds with recombinant or plasma-derived FVIII can lead to an antibody response to this human (but "foreign") protein that effectively neutralizes or inhibits its function in the coagulation pathway; these antibodies are called "inhibitors."

We discuss here some recent approaches, focusing on several developed in our laboratories, to characterize anti-FVIII immune responses and to promote durable peripheral tolerance to exogenously administered FVIII.

FACTOR VIII IMMUNOGENICITY

Thus, while lack of tolerance (non-self) to FVIII explains its immunogenicity, there are other factors that potentially play a role. These are listed in **Table 1** and are discussed below. Clearly, non-self-proteins tend to be recognized as foreign, as the process of c selection has not occurred. The developing immune system simply hasn't seen the T- and/or B-cell epitopes in the protein. Interestingly, FVIII is usually administered to patients intravenously (i.v.), a normally tolerogenic route to safely administer foreign antigens, yet it is highly immunogenic compared to many other therapeutic proteins, with approximately one in four patients developing a clinically significant inhibitor. Indeed, many foreign proteins that pass through the lymphatic or venous system into lymphoid organs are ignored, unless they provide additional signals or so-called adjuvanticity, often referred to as "danger" signals (3). This is because they lack properties that can stimulate

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TABLE 1 | Factors affecting the immunogenicity of human protein therapeutics.

Product-related	Patient-related
Self or non-self	HLA/genetics of patient
Presence of new (neo) epitopes	Route of administration
Biologic (enzymatic) properties	Underlying infection or pathology
Innate signaling properties	Immunosuppression
Absence of regulatory epitopes	Other medications
Formulation or aggregation	
Glycosylation (extent and type)	SNPs and other immunogenomic variants
Post-translational modifications including oxidation	
PEGylation or other protein engineering	
Organ and cell type (e.g., if delivered via gene therapy)	

the innate immune system, e.g., by interacting with Toll-like receptors (TLR) or through other innate immune processes either directly or indirectly. Efforts to demonstrate this property in FVIII have included culturing of human monocyte-derived dendritic cells (MoDCs) with FVIII, or thrombin-cleaved FVIII, or FVIII complexed with its carrier protein von Willebrand factor (VWF); interestingly, neither the maturation nor the T-cell stimulatory capacity of the MoDCs were affected (4). Skupsky et al. suggested that the biologic activity of FVIII in the clotting cascade, which leads to accelerated thrombin activation, provided an alternative mechanism of stimulating innate immune signaling (5). They found that treatment of mice with the anticoagulants warfarin or hirudin, which inactivate thrombin, reduced the immunogenicity of human FVIII in hemophilia A mice. In contrast, Meeks and co-workers, who engineered human FVIII proteins having several amino acid substitutions that neutralized its procoagulant activity, found that the immunogenicity of these non-active FVIII proteins was highly similar to that of active FVIII, thus leaving the role of its biological activity leading to immunologic “danger” as moot (6). If thrombin activation contributes to immunogenicity, then one might expect factor IX to also be unusually immunogenic when administered to hemophilia B patients. Inhibitor development in hemophilia B is actually rare, but this is likely due to the fact that most hemophilia B patients actually circulate a dysfunctional factor IX protein. They therefore could only respond to far fewer epitopes than patients with null mutations.

Uptake and processing by antigen-presenting cells (APC) is the first step in the immune response to protein antigens. Proteolytic processing leads to presentation of peptides in major histocompatibility complexes on the APC surface, e.g., dendritic cells (DC). As noted above, peptides for which no thymic deletion has occurred may be immunogenic provided that they can be processed and presented on MHC Class I or Class II on mature DC, and that a T-cell receptor (TCR) on a circulating T cell recognizes and engages the resulting MHC-peptide complex. Interestingly, exposure to FVIII does not provoke a CD8⁺ immune response in hemophilia A patients or in murine FVIII^{-/-} mice, whereas CD4⁺ T-cell help (7) is

essential for the development of high-titer anti-FVIII antibodies (8). MHC Class II peptide presentation provides “signal one” to effector CD4 T cells in the peripheral repertoire. In contrast, it has been proposed that many proteins may contain promiscuous peptide sequences that preferentially activate T *regulatory* rather than CD4 effectors; these have been termed “Tregitopes” (9, 10). These peptide sequences are commonly found not only in immunoglobulins but in many infectious agents, which may enable them to modulate and reduce the immune response to those agents. The potential role of Tregitopes in modulating FVIII immunogenicity, however, has not yet been established.

Last, but not least, is the physical properties of the FVIII antigen that may influence immunogenicity, such as post-translational modifications or physical aggregation, especially when the antigen is stored or administered at high concentrations. This may be due to an intrinsic or extrinsic property of the antigen, e.g., caused by charge changes, or by physical perturbations resulting from heating or formulation (11, 12). Differences in glycosylation patterns, e.g., according to the type of cell expression system, and covalent modifications to extend protein half-life (PEGylation, fusions of FVIII with other proteins or domains, etc.), and B-domain removal all could affect the immunogenicity of FVIII. The recent, prospective SIPPET study showed a significantly higher inhibitor incidence in previously untreated patients receiving a recombinant FVIII product, compared to plasma-derived FVIII (13). The biological basis for this difference remains to be identified.

Beyond the above properties, one must consider additional factors that influence immunogenicity which may be manifested in the recipients of FVIII replacement therapy. While there is no clear linkage to the HLA of the patient, HLA does affect which peptides will bind to the MHC on DC. Indeed, HLA Class II-restricted epitopes in FVIII were identified years ago by peptide proliferation assays (14–19). Subsequent isolation of FVIII-specific T-cell clones by classical limiting dilution (20) or by using HLA Class II tetramers loaded with FVIII peptides (7, 21–24) provided unambiguous identification of specific high-avidity epitopes (25). At the level of the repertoire, one must consider the nature of the mutation in the FVIII gene (*F8*) that leads to delayed or absent pro-coagulant activity: patients with a major deletion or other mutation precluding expression of the FVIII protein should lack tolerance to all of the epitopes in FVIII. On the other hand, those with missense mutations, which generally are associated with mild or moderate severity hemophilia A due to a partially disabled but still full-length FVIII protein, have a lower risk of developing an inhibitor response following FVIII infusions. In addition to FVIII mutations, other genetic factors, as well as environmental differences including “danger” resulting from trauma or surgery, influence the risk of hemophilia A patients developing an inhibitor (26, 27). Meunier et al. recently determined the frequency of FVIII-specific CD4⁺ T cells in the periphery of non-hemophilic blood donors and found approximately equal numbers of memory and naïve cells (28). Earlier studies had documented both FVIII-reactive antibodies (29, 30) and FVIII-specific T cells (16) in healthy control subjects. These studies demonstrated that FVIII is an unusually immunogenic self-protein, as also indicated by the rare

autoimmune antibody response to FVIII known as “acquired hemophilia A.”

Several studies have suggested that hemophilia A patients with Black African or Hispanic ancestry experience a higher incidence of inhibitors, compared to white patients (31–33). There are multiple naturally occurring, non-hemophilia causing variants of the *F8* gene in the human population, including non-synonymous single nucleotide polymorphisms (ns-SNPs) that encode amino acid variants (34). Thus, it is conceivable that hemophilia A patients who express a dysfunctional FVIII protein, and are exposed to a therapeutic FVIII having a different amino acid sequence, could mount an immune response to the neo-epitope corresponding to this amino acid sequence (35). Although this is a plausible scenario, statistical analyses of inhibitor incidences in patients whose *F8* sequence at these sites was known (33, 36–38), as well as tetramer-guided epitope mapping to detect CD4⁺ T cells specific for these “mismatched” sequence (36), indicated that immune responses to these potential neo-epitopes occur rarely, if at all, and are therefore unlikely to contribute significantly to the immunogenicity of therapeutic FVIII.

FVIII is usually administered intravenously (i.v.), whereupon it rapidly binds to von Willebrand factor, which may modify its immunogenicity (39–41). The i.v. route is usually tolerogenic when infusing aggregate-free proteins into mice (42). This has been interpreted to suggest that i.v.-administered proteins fail to activate DC and to be processed in an immunogenic manner. However, in contrast to soluble proteins like ovalbumin, which is not immunogenic without adjuvant, FVIII is highly immunogenic when administered i.v. to the majority of FVIII knockout (E16) mice (5, 43, 44). Indeed, administering FVIII mixed with OVA can lead to an anti-OVA response, consistent with the intrinsic adjuvanticity of FVIII (5).

Finally, one has to consider other extrinsic properties of the host aside from HLA or other genetic factors. That is, an underlying infection will create significant inflammation which can tilt the response from tolerance to immunity. This would be a potential concern if a hemophilia A patient has an indwelling cannula which gets infected. On the other hand, a number of medications, especially steroids, are immunosuppressive and can tilt the immune response non-specific toward tolerance (45). Interestingly, both murine model studies and statistical analyses of patient outcomes indicate that immunizations do not affect inhibitor risk (46, 47).

The immunogenicity of FVIII that results in formation of inhibitors is a major impediment for the prevention and treatment of bleeds. While bypassing agents, including the FVIII-mimetic antibody emicizumab (48), or recombinant factor VIIa (49, 50), or FEIBA (Factor Eight Inhibitor Bypassing Agent, which is essentially a plasma-derived pro-coagulant protein cocktail) can facilitate clotting, are critically important lifesaving agents (51), they do not overcome the need to induce tolerance to FVIII. In particular, FVIII remains an essential component of the clinical armamentarium to support surgery, and to restore hemostasis following trauma, whereas the bypassing agents may be less efficient and/or carry a risk of thrombosis if doses are not carefully monitored. The relative risk/benefit ratios of utilizing FVIII vs. recently introduced novel bypass agents to control

bleeding in specific clinical scenarios will become more apparent with further research and clinical “real world” experience.

MODULATION OF FVIII IMMUNOGENICITY

Numerous methods to induce specific tolerance have been described for decades (52, 53). In terms of tolerance therapies to eradicate and prevent reoccurrence of inhibitors in hemophilia A patients, the standard clinical practice is intravenous repeated FVIII administration, which is called Immune Tolerance Induction (ITI). This protocol, first described by Brackmann and Gormsen in 1977 (54), is based on the high dose tolerance described by Mitchison in the 1960's (55) and essentially entails antigen overload, as well as maintaining higher trough levels of FVIII for continuous antigen exposure. This procedure is more often successful with patients having low titered inhibitors but often fails in patients with higher titers. Moreover, it is expensive and challenging for patients and families, due to the need for frequent (often daily) infusions. Alternative methods to induce tolerance have primarily been tested in animal models, and most have not reached standard clinical practice. Below is a summary of several approaches in our labs, but it is not meant to be inclusive.

Following on the work of Weigle and colleagues (42, 56) with ultracentrifuged IgG as a model tolerogen, Borel utilized fusions or haptens and antigens on IgG carriers as tolerogens (57, 58), the latter being dependent on the presence of the IgG Fc fragment (9, 10). This would presumably crosslink the B-cell receptor with inhibitory Fc gamma receptors, an approach we will return to below. Based on the tolerogenicity of IgG fusions, we used retroviral transduction of FVIII domains with an IgG heavy chain in B cells as a tolerogenic protocol. This platform was successful in several autoimmune model systems as well (59–62); ironically, this approach was dependent on MHC class 2 presentation of peptides by B cells that led to the generation of regulatory T cells (Tregs) for both its induction and maintenance (63, 64).

Indeed, recent development of Fc fusions of clotting factors FVIII and FIX, designed for a longer half-life *in vivo* (65), have turned out to be tolerogenic in murine models and to induce Tregs (66, 67). This was initially supported by anecdotal cases reports of hemophilia A patients that suggest that FVIII-Fc is potentially tolerogenic (68–70); more highly powered clinical trials are in progress (NCT02234323, NCT03093480, and NCT03103542). Whether the tolerogenicity of Fc fusions is due to the regulatory epitopes in the constant region (9, 71) that turn on Tregs, and/or inhibitory Fc receptors (72, 73) is not clear.

While polyclonal human regulatory T cells (Tregs) have been proposed to treat autoimmune diseases and transplant rejection, and are already in clinical trials, the frequency of specific Tregs is very low. Moreover, the risk of non-specific immunosuppression and viral reactivation is real (74). Expansion of specific Tregs using peptide/APC and IL-2 has recently been achieved (75). Our labs have approached this issue by expressing specific receptors (or antigen) in expanded polyclonal Tregs or CD8 T cells, based on the seminal work by Eshhar (76, 77) and on clinical success of chimeric antigen receptor (CAR) T cells as reported by June and colleagues (78, 79). Since these studies have recently been

published (80–83) and reviewed (52, 84), we will provide only a brief outline of these approaches to induce immune tolerance.

Starting with Tregs purified from healthy donors, our efforts to engineer specificity into polyclonal Tregs used retroviral transduction of specific T-cell receptors (TCR) (80) or CARs (scFv) (81), or even antigen (as B-cell Antibody Receptor = BAR) (83). In the first application, we cloned TCRs from FVIII reactive T-cell clones obtained from mild hemophilia patients (24). These clones recognized a peptide in the FVIII C2 domain restricted to *HLA DRB1*01:01* (21,22,24). The expanded TCR-transduced human Tregs suppressed proliferation and cytokine production by effector CD4 T cells even when the responders were in excess. Interestingly, the TCR-transduced Tregs also suppressed anti-FVIII B-cell responses *in vitro* and *in vivo* across a xenogeneic barrier (80). Interestingly, although the engineered TCR recognizes a single peptide in the large FVIII protein, the antibody response to other major epitopes of FVIII was also suppressed. Thus, engineered FVIII-specific Tregs exhibit bystander suppression, an effect also seen with a TCR specific for a myelin peptide in a model of multiple sclerosis, an effect which appears to be due to uptake of IL-2 produced by effector T cells (85).

TCR-transduced Tregs are MHC class II restricted, thus limiting their eventual utility only to patients sharing the same HLA allele. Therefore, in the second approach, we collaborated with Anja Schmidt and Christoph Königs in Frankfurt, who provided a single chain Fv (scFv) that recognized the FVIII A2 domain. Like the CARs used in cancer therapy, these recognize conformational determinants and are not MHC restricted. Transduction of one of these scFv, called ANS8, into human Tregs also led to significant suppression of anti-FVIII responses *in vitro* and *in vivo*. Extensive dose response comparisons have not been performed as yet with these two types of engineered Tregs; the advantages (and disadvantages) of these specific Tregs are discussed elsewhere (Scott DW, *Molecular Therapy* submitted 2019).

Lastly, we hypothesized that it might be possible to directly target FVIII-specific B cells by expressing FVIII domains on the surface of Tregs. We refer to these as BAR Tregs, reflecting the fact that surface IgM/IgD on B cells react with these antigens. This was successfully achieved with both human BAR Tregs that expressed FVIII A2 and/or C2 domains and suppressed anti-FVIII responses *in vitro* and *in vivo* (83). The target of these BAR Tregs was proven to be the B cell, based on cell mixing experiments (83). Interestingly, in an allergy model, the target may also include sensitized mast cells, based on results of passive anaphylaxis experiments (86).

An alternative approach utilized transduced cytotoxic CD8 T cells expressing the targeted FVIII domains (82), as was done by Ellebrecht et al. with desmoglein 3 for possible therapy of pemphigus vulgaris, a devastating skin disease (87). [They refer to their antigen-expressing CD8 T cells as chimeric autoantigen receptor T cells (CAAR)]. Whether they are called BAR or CAAR, the cytotoxic T cells are highly specific and do not display bystander effects. There are situations when such specificity and lack of bystander effect might be necessary to carefully target part of a large antigen, but Tregs might be preferred if one doesn't know the targeted domains, and in the case of large multi-domain protein antigens such as FVIII.

Finally, a nanoparticle approach has been developed that can provide an alternative to engineered cellular therapies for tolerance; nanoparticles have also been used for drug delivery and vaccine development (88). Such nanoparticles can contain drugs such as rapamycin and are delivered with the target antigen (either attached or concomitantly) and presumably are taken up by dendritic cells, which act as tolerogenic APC and induce Tregs (89, 90). The use of rapamycin-containing nanoparticles for tolerance was successfully used by our group for FVIII (91), and by others for modulating autoimmunity (89) or the immune response to therapeutic immunotoxins, which are highly immunogenic (92, 93).

Several other approaches, in addition to the above strategies, are being developed to promote tolerance to FVIII. These include hepatic gene therapy, oral tolerance, and trans-placental delivery of FVIII. These are discussed more comprehensively in a recent review (84).

DISCUSSION

In conclusion, while there are multiple factors that influence the immunogenicity of therapeutic proteins, novel approaches such as those described here have the potential to modulate such immunogenicity. Time will tell which of these approaches may become cost-effective clinical therapies in the future.

AUTHOR CONTRIBUTIONS

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Major Histocompatibility Complex Binding, Eluted Ligands, and Immunogenicity: Benchmark Testing and Predictions

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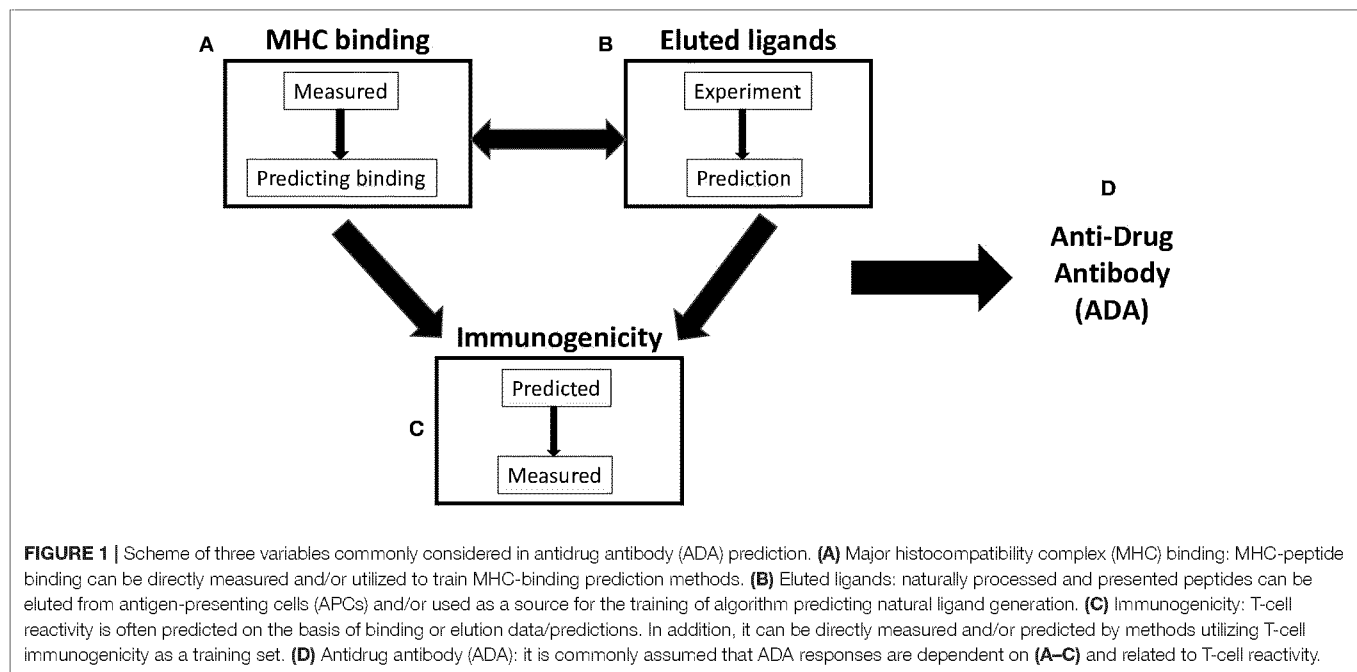
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Antidrug antibody (ADA) responses impact drug safety, potency, and efficacy. It is generally assumed that ADA responses are associated with human leukocyte antigen (HLA) class II-restricted CD4+ T-cell reactivity. Although this review does not address ADA responses *per se*, the analysis presented here is relevant to the topic, because measuring or predicting CD4+ T-cell reactivity is a common strategy to address ADA and immunogenicity concerns. Because human CD4+ T-cell reactivity relies on the recognition of peptides bound to HLA class II, prediction, or measurement of the capacity of different peptides to bind or be natural ligands of HLA class II is used as a predictor of CD4+ T-cell reactivity and ADA development. Thus, three different interconnected variables are commonly utilized in predicting T-cell reactivity: major histocompatibility complex (MHC) binding, capacity to be generated as natural HLA ligands, and T-cell immunogenicity. To provide the scientific community with guidance in the relative merit of different approaches, it is necessary to clearly define what outcomes are being considered. Thus, the accuracy of HLA binding predictions varies as a function of what the outcome predicted is, whether it is binding itself, natural processing, or T-cell immunogenicity. Furthermore, it is necessary that the accuracy of prediction is based on rigorous benchmarking, grounded by fair, objective, transparent, and experimental criteria. In this review, we provide our perspective on how different variables and methodologies predict each of the various outcomes and point out knowledge gaps and areas to be addressed by further experimental work.

Keywords: anti drug antibodies (ADA), CD4 T cell, MHC-prediction, prediction benchmarking, immunogenicity

INTRODUCTION

As discussed in general and in more detail in other contributions to this special issue, protein-drug immunogenicity is of concern, as it can lead to safety issues and can impact drug efficacy and potency. It is further widely assumed that immunogenicity at the level of CD4 T cells recognizing human leukocyte antigen (HLA) class II epitopes is a key and necessary step in the development of antidrug antibodies (ADAs), because CD4 T cells are generally required for antibody affinity maturation and isotype switching, which is of relevance because ADA is in general IgG and other subclasses that require immunoglobulin gene rearrangements.



As a result, a variety of strategies have been developed to assay and/or predict different steps in the process of the development of ADA. This review will focus on efforts and available data benchmarking different methodologies and outcomes relating to HLA class II binding, elution of natural HLA class II ligands, and T-cell immunogenicity *in vitro*. The interconnection between these different methodologies at the level of actual experimental data vs. bioinformatic prediction is graphically illustrated in **Figure 1**. This paper is mostly reflective of our work in the context of Immune Epitope Database and Analysis Resource (IEDB), and we fully acknowledge the seminal contributions of several other groups, as also detailed in other contributions to this special issue. Likewise, this review does not address other variables that are appreciated to impact ADA and T-cell immunogenicity, such as induction of T-cell tolerance, self-similarity, protein-drug dosing and schedule, aggregation state, and general immune responsiveness of the drug recipient. We emphasize that the present study is a review, and as such, we do not present primary data presented elsewhere. In each paragraph, the specific papers and sources of the primary data are referenced, to allow the reader a more in-depth analysis if desired.

HUMAN LEUKOCYTE ANTIGEN BINDING AND ELUTED LIGANDS

HLA class II binding, more generally major histocompatibility complex (MHC) binding, is measured by *in vitro* utilizing preferably synthetic peptides and purified HLA class II molecules. The most accurate and reproducible “gold standard” assay on hand is a classic radiolabeled probe displacement receptor ligand assay, developed by Gray, Sette, and Buus and Unanue,

Babbitt, and Allen in the mid-1980s (1, 2). Other assay platforms that have been previously described suffer from difficulties in controlling peptide degradation (live-cell assays) (3) or a low throughput (plasmon resonance assays) (4). Furthermore, radiolabeled probe displacement receptor ligand assay has been run for many different HLA class II allelic variants with a large number of synthetic peptides (5, 6), and it is thus associated with the most numerous volume of accurate and directly comparable data. Accordingly, these data have been used, as described in more detail in the following sections, to train predictive algorithms, which have increased efficacy and accuracy throughout the past three decades (**Figure 1A**).

Like in the case of all MHC molecules, the vast majority of peptide-binding sites of HLA class II is occupied by natural ligands, derived from antigens processed into small peptides and displayed on the surface of antigen-presenting cells (APCs). These natural ligands can be eluted and characterized (4). In the context of application to the characterization of protein, drug-derived peptides with the acronym MAPPs, which stands for MHC-associated peptide proteomics (MAPPs), are frequently used (7, 8). Recent years have witnessed an explosion of availability of sequences of natural ligands, thanks to the ever-increasing power of mass spectrometry (MS) sequencing techniques (9). As a result, these eluted ligand data can also be used to train predictive algorithms (**Figure 1B**), as also described in the following sections. It is perhaps intuitively expected that the two different training sets might yield largely overlapping results, with binding data being the most effective in predicting binding capacity and eluted ligand being the most effective to predict eluted ligands but not necessarily HLA binding *per se*.

T-CELL IMMUNOGENICITY

In order for an epitope to be recognized by CD4T cells, it needs to be capable of binding HLA class II molecules and of being generated by natural processing. Accordingly, binding and natural ligand assays and predictions are routinely utilized to predict T-cell immunogenicity. However, it should be kept in mind that these measures, by definition, do not necessarily relate to immunogenicity at the T-cell level, as other variables are also involved (e.g., the degree of similarity to self-antigens). Furthermore, it is often not clear which thresholds are associated with the optimal prediction of T-cell epitope, on the basis of either measured or predicted elution or binding data.

Alternative strategies use T-cell immunogenicity data to train agnostic predictors or use *in vitro* immunogenicity assays to predict or rank the immunogenicity of protein drugs *in vivo* in humans (Figure 1C). Here as well, considerable challenges and opportunities for further research exist, as it is unclear how specific and sensitive these assays are and how they do correlate with *in vivo* immunogenicity. Likewise, it is unclear whether T-cell immunogenicity *in vitro* in unexposed naïve individuals can predict T-cell immunogenicity in exposed individuals. Finally, and of the greatest relevance, data that demonstrate that T-cell immunogenicity measured by currently used assays does, in fact, correlate with ADA titers in human patient populations are very limited (Figure 1D). Figure 1D is presented here to point out a knowledge gap, and no data for ADA are reviewed herein. Several studies are starting to generate data relevant to this respect, in the context of protein therapeutics that are either human or humanized and foreign proteins such as asparaginase and glucarpidase. These topics are addressed in other papers presented in this issue and are not within the scope of this review. In the context of this paper, we simply point out that the volume of data is as yet insufficient to perform a systematic and unbiased evaluation.

THE CONCEPT AND NECESSITY OF BENCHMARKING PREDICTIVE ALGORITHMS

To rigorously evaluate the performance of any predictive algorithm, it is generally necessary to define objective measures of performance. Commonly utilized measures are sensitivity [what fraction of true positives (TPs) are predicted vs. false positive (FP)] and specificity [what fraction of the predictions are TPs vs. false negatives (FN)]. The prediction rates are plotted to generate an area under the curve (AUC) and AUC values, which are an overall numeric assessment of performance (with an AUC of 0.5 being associated with random predictions and an AUC of 1.00 corresponding to a perfect prediction).

Once the method to be used for evaluation is defined, it is necessary to define datasets that are going to be used to assess the algorithm's performance. The evaluation dataset should be distinct from the one used to derive the method, to avoid overfitting. This is particularly the case for heuristic and machine learning approaches, where the method will fit the data without a

predefined hypothesis or model. The process by which a different methodology is objectively and rigorously evaluated is generally referred to as “benchmarking.”

In our opinion, to have true scientific value, a benchmarking needs to fit three fundamental characteristics. First, it needs to be objective, following predefined metrics and an accepted methodology. Second, it needs to utilize independent datasets, not used to train the methodology and preferably not available to the method developer while the method was trained. Third, it needs to be transparent, using publicly available code, preferably published in the peer-reviewed literature, and the results must be verifiable and reproducible by anyone in the scientific community.

BENCHMARKING HUMAN LEUKOCYTE ANTIGEN CLASS II BINDING PREDICTIONS

To the best of our knowledge, the first comprehensive rigorous benchmarking of different prediction methodologies was reported for HLA class I by Peters et al. (2). In those studies, predictions for over 48 MHC alleles, 88 datasets, and 48,828 IC₅₀ values were considered, with 50–300 data points per dataset. In general, the performance of different methodologies was similar, and the main factor influencing predictive power was found to be how many data points were available for training predictions for a given allele. Since then, the process of benchmarking was automated and is periodically performed by the IEDB (10).

Following the same thought process and methodologies, we have recently instituted a platform for automated benchmarking of HLA class II predictions (11). On a weekly basis, the absolute and relative predictive performance of all participating tools on data newly entered into the IEDB is assessed before it is made public. This unbiased assessment of available prediction tools is fully automated, and results are posted on a publicly accessible website (http://tools.iedb.org/auto_bench/mhcii/weekly/). The initial benchmarking included six commonly used prediction servers. The results from that process have room for improvement, predictions were reasonably accurate with median AUC values for the various class II molecules of around 0.8 for the best methods (NetMHCIIpan and NNalign). Since the publication of the study, additional gains have been realized with an AUC value of 0.835 for NetMHCIIpan (11). The current benchmarking evaluates MHC binding, and we plan to extend this automated benchmarking to eluted ligand data and eventually T-cell immunogenicity data.

It is important to realize that this benchmarking only assesses the performance on class II binding predictions, in terms of predicting binding itself, and should not be interpreted to assess how well-binding prediction predicts immunogenicity or ADA. Although this would seem self-apparent, we often encounter statements to the extent that the “MHC binding predictions do not work because I have immunogenicity data that” Obviously, although the binding is necessary for immunogenicity, it is not the sole condition. The current efforts to objectively assess the performance of HLA class II

binding (predicted or measured) as a predictor of HLA class II immunogenicity are described in a section further below.

NATURAL LIGAND AND PROCESSING PREDICTIONS

As mentioned above, the recent years witnessed a dramatic increase in the availability of data relating to HLA class II eluted ligands. In this context, a reasonable line of investigation would be to examine if the eluted ligand data could be utilized to learn some “processing motif,” present in natural ligand but not associated with HLA-binding motifs. A recent study by Paul et al. (12) used this approach. MHC II ligand elution data collected from IEDB were further filtered to generate a high-quality dataset. The result was the delineation of a predictive cleavage motif for eluted ligands. A combination of cleavage and binding predictions improved ligand predictions. Strikingly, however, incorporating the processing motif in combination with binding predictions did not improve predictions of which sequences would be actual T-cell epitopes. Similar results were also obtained in a study from Nielsen’s group (13), who detected a footprint of antigen processing, which improved predictions of eluted ligands but did not improve predictions of which sequences would be actual T-cell epitopes. These results are remarkably similar to what was previously observed in the case of class I molecules where it was found that processing predictions were not affording increased efficacy in predicting actual T-cell epitopes, either by themselves or in combination with binding predictions.

Previous data by Jurtz et al. (14) demonstrated that directly using eluted ligand data to train neural networks (NNs) was associated with increased capacity to predict eluted ligands, as compared with NN trained in HLA class I binding data. Garde et al. (15) demonstrated that training in class II eluted data increases the accuracy of predicting eluted ligands, just as previously observed in the case of class I. Thus, training NN algorithms with MS eluted ligands improves the capacity to accurately predict eluted ligands for both HLA class I and II alleles (14–16).

COMPARISON OF BINDING AND ELUTED LIGAND DATA

In terms of comparing these two different data types, a first question to be addressed is how the measured binding and experimental elution data compare with each other. An analysis performed more than 2 years ago (17) demonstrated that T-cell and MHC-binding data were mostly related to non-self, whereas elution ligands are mostly self. This is largely a reflection of the fact that HLA binding and epitope studies have prevalently been focused on infectious diseases and allergy targets, whereas ligands encountered that are naturally occupying the HLA class II binding site are predominantly of self-origin. Therefore, the problem is just that the particular peptide sets that happen to be studied in the two approaches are non-overlapping, complicating direct comparison but not necessarily leading to

different predictions. This is not a reflection of the fact that self and non-self peptides differ in their capacity to bind or to be generated by natural processing. The fact that MHC class II molecules bind indiscriminately the self and non-self peptides were established in the early 1990s (18). The disparity in the number of self vs. non-self peptide data available in the literature and associated with the two techniques is simply a reflection of the investigational bias of MHC-binding and T-cell mapping studies being mostly focused on infectious diseases and allergen targets, whereas in the case of natural MHC ligands, the most abundant species (and therefore more easily sequenced species) are of self-origin. **Tables 1, 2** present numbers of peptides eluted from MHC class II molecules. These are the data available through the IEDB as of Q3 2019, which contain the specific peptide sequences and specific MHC class II molecules.

Table 1A presents an updated analysis (as of Q3 2019) focused on HLA class II. This analysis highlights how comparing measured HLA binding and eluted data is problematic in general and for HLA class II in particular because the two datasets are only minimally overlapping. This knowledge gap is starting to be addressed by several studies in the context of murine class I molecules (19, 20). Croft et al. utilized the vaccinia virus (VACV) as a model system in the context of the murine MHC class I molecules Kb and Db (19). Further benchmarking of the dataset (21) reveals that the majority of eluted peptides are within expected binding ranges, but a large fraction of binders are not identified by the elution of experiments (**Table 1B**). This is not unexpected and is likely reflective of the impact of protein expression/abundance also shaping it; in concert with a binding capacity of the actual peptides, the repertoire of natural ligands bound to MHC. A compensatory relation between binding and expression was indeed noted by Abelin and coworkers, who states. “This revealed a multiplicative relationship between expression and affinity, in which a 10-fold increase in expression could approximately compensate for a 90% decrease in binding potential” (22).

Generating datasets where for a given model antigen we can address which peptides are experimentally found to bind and isolate as natural ligands in the context of HLA class II molecules should be considered a priority for the general field of benchmarking of binding and elution data.

HOW DO ALGORITHMS PERFORM IN PREDICTING THE “OTHER” VARIABLE?

As mentioned above, it is intuitively expected that binding data might be most effective in training to predict binding capacity, but not necessarily eluted ligands. Likewise, training with eluted ligand might be expected to be the most effective to predict eluted ligands but not necessarily HLA binding *per se*. This point was formally addressed by Garde et al. (15). The authors expanded the NNalign approach by adding a second output neuron, and training is performed on both data binding and eluted data simultaneously. The resulting model

TABLE 1 | Composition of epitopes available in IEDB.

Classification based on	Type	MHC class II		
		T-cell epitopes	MHC binders	Eluted ligands
(A) MHC-BINDING DATA				
Host	All	62,380	21,885	66,304
	Human	41,577	18,944	53,810
	Rodents/rabbit	21,351	3,644	13,335
	Non-human primates	269	106	NA
	Other hosts	2,181	60	NA
Antigen source	Self	6,251	3,542	63,766
	Non-self	39,983	12,943	1,495
	Viruses	25,834	9,575	484
	Allergen	1,924	1,342	784
	Bacteria	9,358	1,409	151
	Parasites	2,071	553	74
	Fungus	796	64	2
Predicted binder or not				
		Binder	Non-binder	Total
(B) NATURAL LIGAND ELUTION DATA				
Eluted or not	Eluted	124	42	166
	Non-eluted	10,397	646,239	656,636
	Total	10,521	646,281	656,802

IEDB, Immune Epitope Database and Analysis Resource; MHC, major histocompatibility complex.

is able to predict binding affinity value and the likelihood of peptide being an eluted ligand. This study demonstrated that training in class II eluted data increases the accuracy of predicting eluted ligands, but not to predict binding, and that vice versa training in binding data increases the accuracy of predicting binding data, but not to eluted data. In conclusion, these data reiterate that caution must be exercised when algorithms generated to predict a certain variable are used to predict outcomes linked to a different, albeit related, variable. It further sets the stage for the next level of benchmarking, namely, how do HLA class II binding and eluted data and predictions perform when used to predict HLA class II-restricted T-cell immunogenicity?

MAJOR HISTOCOMPATIBILITY COMPLEX
BINDING AFFINITY DATA AS A
PREDICTOR OF IMMUNOGENICITY

In the case of HLA class I, it was originally reported that ~80% of epitopes bind with $K_d < 500$ nM (23). The more recent analysis confirmed this observation, supporting this historic threshold (24). It was further found that different alleles are associated with different affinity distributions (24), leading to the recommendation that allele-specific thresholds are preferred when class I binding predictions are used to predict immunogenicity.

In the case of class II, a 1,000-nM threshold was suggested, but not extensively validated over large datasets. To address this point, we generated curves capturing percent of epitopes retrieved from the IEDB restricted by different HLA class II molecules, or we generated a higher quality of data, restricting the data considered to be those associated with positive tetramer assays. The results shown in **Figure 2A** demonstrate that when alleles for at least 50 epitopes have been described with defined restriction, 83.3% epitopes bind at <1,000 nM (3,579 out of 4,297 epitope/allele combinations). As noted in the case of HLA class I, a significant spread exists from one allele to the next. Similarly, when only tetramer data are considered, we plotted data from 15 alleles with at least 20 epitopes (**Figure 2B**). We found that 80.1% epitopes bind at the <1,000-nM threshold (1,353 out of 1,690 epitope/allele combinations). **Table 2** shows the affinity at which 50 and 90% of epitopes are retrieved for each of the HLA alleles described in **Figure 2**. It is noted that the DRB1*01:03 allele has only one epitope at the <1,000-nM level and appears to be an outlier. Whether this reflects a problem with the dataset, or rather the algorithm, or a peculiarity of this rather infrequent allele remains to be investigated.

Rigorous benchmarking of epitopes in a single well-defined system where the epitopes are mapped to different HLA class II molecules is not currently available. The above-referenced study of Croft et al. (19), in addition to studying eluted peptides and measuring binding affinities, also measured the epitopes recognized following VACV infection, also in the

TABLE 2 | Binding affinity at which 50 and 90% of epitopes are retrieved for each HLA class II allele on the basis of epitope or tetramer data available in IEDB.

	Epitopes		Tetramers	
	Affinity at 50%	Affinity at 90%	Affinity at 50%	Affinity at 90%
DPA1*01:03/DPB1*01:01	135	4,220	NA	NA
DPA1*01:03/DPB1*04:01	104	2,609	NA	NA
DPA1*01:03/DPB1*04:02	439	3,015	NA	NA
DQA1*01:01/DQB1*05:01	2,294	9,706	NA	NA
DQA1*01:01/DQB1*06:02	6,180	1,813	1,141	204
DQA1*01:02/DQB1*06:02	300	2,439	943	186
DQA1*03:01/DQB1*03:02	1,091	6,001	NA	NA
DRB1*01:01	26	234	171	87
DRB1*01:02	553	966	NA	NA
DRB1*01:03	NA	NA	3,944.14	1,718.14
DRB1*03:01	196	1,823	2,026	7,107
DRB1*04:01	204	8,896	146	5,300
DRB1*04:03	69	321	NA	NA
DRB1*04:04	NA	NA	313.25	225.87
DRB1*04:05	1,163	6,678	NA	NA
DRB1*04:07	123	696	NA	NA
DRB1*07:01	65	565	54	206
DRB1*08:01	NA	NA	686.2	1,563.99
DRB1*08:02	110	1,673	NA	NA
DRB1*08:03	1,009	986	NA	NA
DRB1*09:01	66	678	14	194
DRB1*10:01	19	46	NA	NA
DRB1*11:01	43	582	212	179
DRB1*11:04	NA	NA	922.29	145.13
DRB1*12:02	119	361	NA	NA
DRB1*13:01	44	243	NA	NA
DRB1*14:01	NA	NA	380	423
DRB1*14:02	423	547	NA	NA
DRB1*14:04	222	247	NA	NA
DRB1*14:06	47	135	NA	NA
DRB1*15:01	115	1,843	382	1,233
DRB1*15:06	231	1,528	NA	NA
DRB3*02:02	1,233	1,788	NA	NA
DRB5*01:01	60	2,811	505	107

NA, not applicable; HLA, human leukocyte antigen; IEDB, Immune Epitope Database and Analysis Resource.

context of previously detailed immunogenicity studies (25). The benchmarking study of Paul et al. (12) provides a benchmarking analysis of these data. It was found that the top 1–2% of binding predictions captured 90% of the epitopes or of the total response and that the top 0.03–0.04% of the predicted binders accounted for 50% of the total epitopes and response. The analysis, however, also further underlined how binding predictions are very sensitive predictors but are associated with relatively low specificity. In other words, in the case of murine class I, when 90% of the epitopes are binders, only about 1% of the binders are epitopes. In conclusion, the lack of comprehensive benchmarking of binding prediction and HLA class II-restricted actual immunogenicity is a major knowledge gap, and generating suitable datasets should be considered a priority.

HOW EFFECTIVE IS LIGAND ELUTION AS A PREDICTOR OF IMMUNOGENICITY?

Few studies have benchmarked how effectively eluted ligand data can be used in terms of prediction of HLA class II immunogenicity. A study by Mutschlechner et al. (26) compared elution data and T-cell immunogenicity in a case of patients allergic to the known major birch pollen allergens. These authors found that, in general, elution data overlapped with immunogenicity data but missed one of two major T-cell immunogenic sites (around positions 77–93 of the Bet v1 protein).

It is reasonable to assume that all “true” HLA class II epitopes are naturally processed, but it is unclear how many

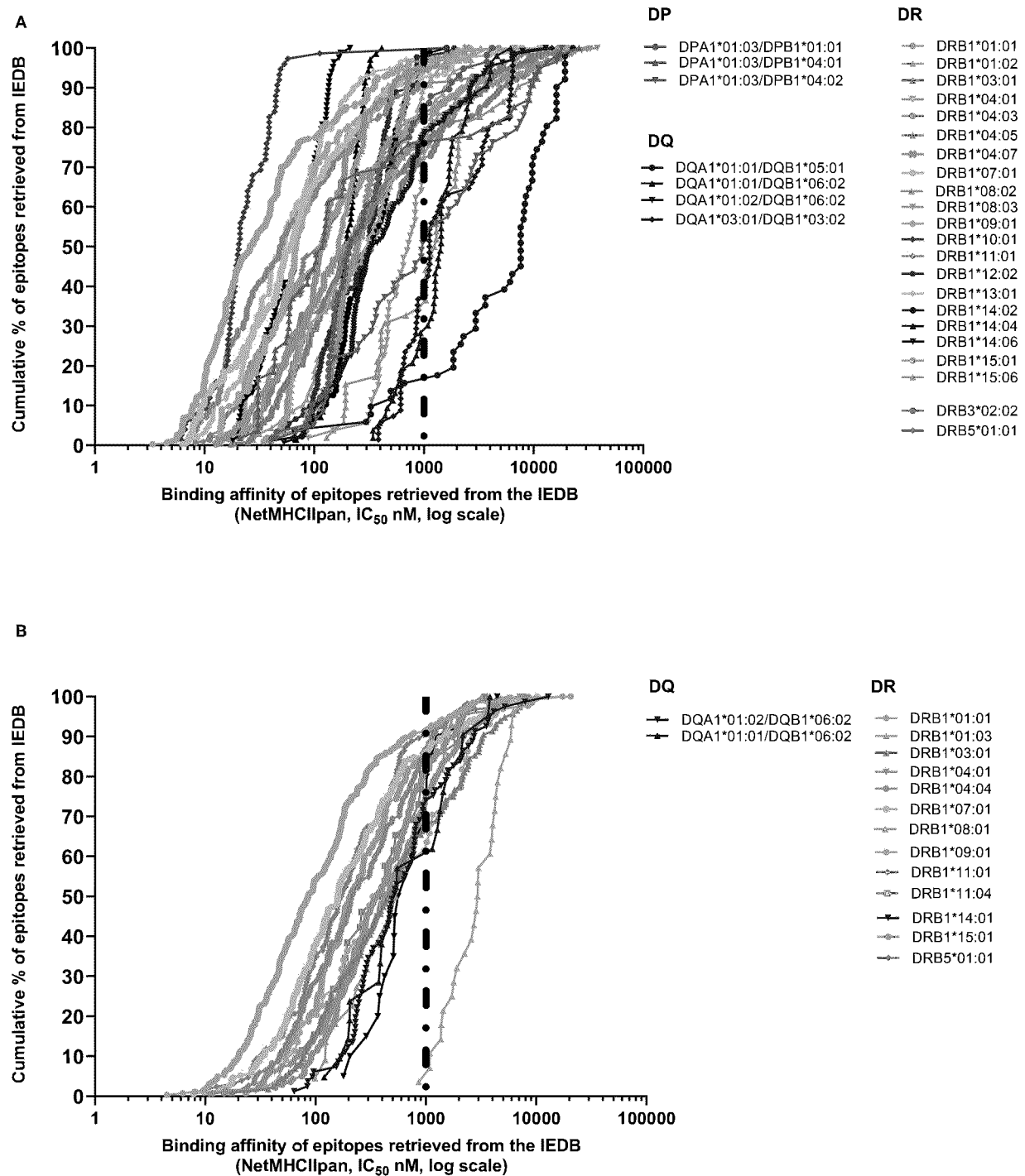


FIGURE 2 | Extrapolation of binding affinity threshold for human leukocyte antigen (HLA) class II prediction. Binding affinity retrieved by NetMHCIIpan by plotting IC_{50} predicted values for each HLA class II allele on the basis of the cumulative percentage of epitopes derived from the Immune Epitope Database and Analysis Resource (IEDB) (A) or based only on tetramer data available in IEDB (B).

are detected vs. missed given the limits of sensitivity of the assays. High abundance can compensate for low MHC affinity, but it is unknown how immunogenic these types

of ligands are. Conversely, a low abundance of ligand that binds with high affinity may be less easily detected but more strongly immunogenic.

TABLE 3 | Benchmarking summary of HLA class I molecules in the murine VACV system.

		Predicted binder or not		Total
		Binder	Non-binder	
Eluted or not	Eluted	60	5	65
	Non-eluted	15	2	17
	Total	75	7	82

HLA, human leukocyte antigen; VACV, vaccinia virus.

WHAT IS THE RELATIVE VALUE OF BINDING VS. ELUTION PREDICTIONS TO PREDICT IMMUNOGENICITY?

As mentioned above, Nielsen, Jurtz, Garde, and associates developed a methodology where binding data, elution data, or both can be used to train NNs, and they generated as output the likelihood that a given sequence will be an HLA class II binder or an eluted ligand. The question that is key in light of application to T-cell immunogenicity is which training is optimal for T-cell epitope predictions. And which output is optimal? The results of this analysis have been recently published (15) and show that training in both ligand and binding datasets is the most effective and that the optimal output is the prediction of eluted ligands. These results have been confirmed by three independent studies (27–29).

Although a formal benchmarking for HLA class II molecules in a controlled experimental system is to date lacking, these results are in strong agreement with the results of the murine class I study of Tscharke in the VACV system (19). In that study, it was found that of a total of 82 epitopes, 60 were both found to be binders in actual binding assays and also experimentally identified as eluted ligands (Table 3) (21). Fifteen epitopes were binders not identified as eluted ligands, whereas five eluted ligands were not experimentally found to be 500-nM binders. However, only two of the peptides experimentally determined to be epitopes were found to be neither binders nor eluted ligands. These data provide compelling evidence that a combination of both predicted binding and elution data should be considered for the purpose of epitope identification.

THE IMPACT OF HUMAN LEUKOCYTE ANTIGEN POLYMORPHISM ON BINDING VS. IMMUNOGENICITY PREDICTIONS

HLA polymorphism is an important issue to be considered in evaluating the performance of HLA binding or eluted ligand predictions as a predictor of immunogenicity. HLA class II predictions are by definition allele-specific. However, in real-life drug immunogenicity scenarios, this has to be reconciled with the fact that HLA class II molecules are remarkably polymorphic, encoded by seven different loci, and represented by thousands of different allelic variants.

At the level of individual patients, each human subject is typically heterozygote at four different HLA class II loci (DRB1, DRB3/4/5, DP, and DQ) and therefore expresses up to eight or more different HLA class II variants; this is because of the so-called heterozygous pairing of DP and DQ where both alpha and beta subunits are polymorphic and can form *trans* and *cis* pairings leading to an estimate of about 12 different molecules. And a patient population expresses hundreds of different variants, each represented in different frequencies, which also vary significantly across different ethnicities.

Human immunogenicity and clinical trials rarely determine the specific HLA class II molecule restricting the response, as this is considerably more complex and less clear-cut than in the case of HLA class I. As a result, actionable predictive strategies to target, not alleles, but individuals and populations are required.

Our group has defined a subset of 26 different DRB1, DRB3/4/5, DP, and DQ allelic variants (30) that afford 94.5% global coverage of general human populations. We have used promiscuity indexes (that is, predicting peptides binding to a majority of the most common alleles) as a way to identify peptides that correspond to the most dominant, most immunogenic peptides observed in real-life patient populations (30).

This approach was further optimized, utilizing datasets derived from peptide sets spanning entire proteins associated with measured immune responses in exposed humans to examine a) how many and b) which specific HLA class II variant predictions would be most effective, when combined, to predict immunogenicity in human populations. It was found that optimal results were found with a set of just seven variants, representative of common and dominant class II motif types (31).

PREDICTING IMMUNOGENICITY IN VIVO IN HUMAN POPULATIONS

The performance of the “seven-allele method” in predicting immunogenicity in patient populations was evaluated in a subsequent study (32). In the same study, we also considered an agnostic approach, where we used T-cell recognition data to directly train predictive algorithms. For this purpose, we used in-house data and IEDB-derived tetramer as training sets. The performance was evaluated using results from 57 different studies from other laboratories, which used overlapping peptides and exposed populations that contained 530 non-redundant dominant epitopes and 1,758 non-epitopes.

We observed that either the HLA class II binding predictions (seven alleles) or the T-cell immunogenicity tools were associated with overall AUC values of 0.7. Using the two methods in combination afforded modest gains, with AUC of 0.725. The relatively low overall AUC values should not be surprising, given the fact that what is predicted here is not an outcome linked to a given HLA but a population outcome, where the composition of the responding population is unknown and the restricting HLA molecules associated with each epitope are not determined.

PREDICTING IMMUNOGENICITY *IN VIVO* BY *IN VIVO* IMMUNOGENICITY ASSAYS

In vitro assays utilizing cells from naïve, non-exposed donors offer an obvious alternative to bioinformatic predictions. Primary immunogenicity can be measured *in vitro* by a variety of methods. These include immunizing with whole antigen or peptides, using dendritic cells or peripheral blood mononuclear cells (PBMCs) as APC, usually after a period of *in vitro* culture, followed by read-out assays that include proliferation, enzyme-linked immunosorbent spot (ELISPOT), and intracellular cytokine staining (ICS).

Despite their widespread use, benchmarking the performance of these assays as a predictor of *in vivo* immunogenicity is lacking. Rigorous benchmarking studies are required to establish whether these methods do actually predict *in vivo* immunogenicity and which method is most effective. Questions to be addressed include whether memory responses are detected in drug-treated subjects and whether HLA type predicts which subjects will develop memory T-cell responses. It is further unclear to what extent HLA binding, peptide elution, or *in vitro* immunogenicity assays or predictions actually predict which subjects will develop memory T-cell responses. Finally, benchmarking should address at the population level whether binding, elution, or immunogenicity assays or predictions actually identify which epitopes are dominant in *ex vivo* scenarios, with obvious implications for strategies aimed at protein de-immunization by removing T-cell immunogenic epitopes.

CONCLUSIONS

Do T-Cell Responses Correlate With ADA?

Surprisingly, this is still a very open question that rigorous benchmarking studies can help answer. This will require a global assessment of drug-specific memory T cells in drug-exposed individuals. We believe that the paper makes a clear and desperate plea for the need to generate more data and for honest and objective benchmarking, which are a necessary requisite for moving the field forward. Do the magnitude and/or specificity of memory T-cell responses correlate with ADA titers and/or neutralizing activity? Does immunogenicity (predicted or measured or in non-exposed subjects) predict immunogenicity in

exposed subjects? Are the same epitopes recognized as dominant in ADA+ and naïve subjects (with obvious implications for de-immunization) (33)?

It should be emphasized that this review does not address other variables that are appreciated to impact ADA and T-cell immunogenicity, such as induction of T-cell tolerance, self-similarity, protein-drug dosing and schedule, aggregation state, and general immune responsiveness of the drug recipient. In particular, the methods available to the scientific community are trained and derived for the most part on the basis of “strong” infectious diseases and allergy-derived epitopes (with a growing representation of autoimmune and cancer-derived epitopes). In the context of drug immunogenicity and design, it is possible that epitope prediction thresholds might need to be adjusted. This issue can be objectively addressed only when a sufficient amount of epitope data from protein drugs will be accumulated and made public. Ideally, these data could also be utilized to develop algorithms specific to the prediction of drug immunogenicity.

Answering these questions will ultimately require the coming together of bioinformaticians, cellular immunologists, and clinical scientists, applying rigorous and transparent methodologies and datasets. And ultimately, it will require prospective evaluations of immunogenicity including *in vitro* immunogenicity assay pre-exposure, HLA typing, and post-exposure immunogenicity and ADA measures to generate the datasets in which benchmarking can be applied. Ultimately, how can we predict immunogenicity outcome if all we do is run predictions and not test them in a prospective fashion if the immunogenicity assays predicted immunogenicity and ADA outcomes?

AUTHOR CONTRIBUTIONS

SP performed specific database queries, generated tables, and wrote the manuscript. AG generated the figures and wrote the manuscript. BP critically reviewed the review. AS conceived, wrote, and critically reviewed the review.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The Irony of Humanization: Alemtuzumab, the First, But One of the Most Immunogenic, Humanized Monoclonal Antibodies

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Alemtuzumab was designed to reduce the immunogenicity of the parent CD52-specific rat immunoglobulin. Although originally marketed for use in cancer (Mabcampath®), alemtuzumab is currently licensed and formulated for the treatment of relapsing multiple sclerosis (Lemtrada®). Perhaps due to its history as the first humanized antibody, the potential of immunogenicity of the molecule has been considered inconsequential, and anti-drug antibodies (ADA) responses were similarly reported as being clinically insignificant. Nonetheless, despite humanization and depletion of peripheral T and B cells, alemtuzumab probably generates the highest frequency of binding and neutralizing ADA of all humanized antibodies currently in clinical use, and they occur rapidly in a large majority of people with MS (pwMS) on alemtuzumab treatment. These ADA appear to be an inherent issue of the biology of the molecule—and more importantly, the target—such that avoidance of immunogenicity-related effects has been facilitated by the dosing schedule used in clinical practice. At the population level this enables the drug to work in most pwMS, but in some individuals, as we show here, antibody neutralization appears to be sufficiently severe to reduce efficacy and allow disease breakthrough. It is therefore imperative that efficacy of lymphocyte depletion and the anti-drug response is monitored in people requiring additional cycles of treatment, notably following disease breakthrough. This may help inform whether to re-treat or to switch to another disease-modifying treatment.

Keywords: anti-drug antibodies, CD52, humanized, immunoglobulin, immunogenicity, multiple sclerosis, neutralizing antibodies

INTRODUCTION

Alemtuzumab is a monoclonal antibody that is specific for the 21–28 kDa lymphocyte cell surface CD52 glycoprotein (1, 2). This was the first example of a humanized monoclonal antibody (mAb) (3). The initial formulation (Mabcampath® 1,033 mg over 12 weeks) was used to treat CD52+ T and B cell cancers, notably chronic lymphocytic leukemias, and other

lymphocyte-mediated conditions (1, 2, 4). However, it is now formulated (Lemtrada® 36–60 mg over 3–5 days over 13 months) and licensed for the treatment of relapsing multiple sclerosis (MS), which is a demyelinating, probable autoimmune disease of the central nervous system (5, 6).

Humanization was a process designed to reduce the immunogenicity of therapeutic monoclonal antibodies that had been generated in rodents (1–3). Although removing rodent constant regions and grafting the complementarity-determining regions onto human framework regions clearly reduced immunogenicity (1), it was soon recognized that alemtuzumab could generate anti-idiotypic responses that could prevent therapeutic benefit (4, 7). Subsequently, perhaps in recognition of the problem of antibody neutralization (8), strategies were developed to limit anti-globulin responses to alemtuzumab (8–10). The occurrence of binding antibodies (BAbS) received limited mention and notably neutralizing antibodies (NABs) were not discussed in the published reports (5, 6, 11) of the pivotal trials leading to the licensing and commercial development in MS. The first mention of neutralizing antibodies did not occur until we reported on them in 2017 (12, 13). They were described as “inhibitory antibodies” within the regulatory submissions (5, 6). According to the Food and Drug Administration (FDA), their effect on the clinical efficacy and safety profile was of unclear clinical significance. We are concerned that the effect of alemtuzumab anti-drug antibodies (ADA) on efficacy has yet to be adequately addressed (12, 14, 15), and may have safety implications (6, 13, 16, 17). Although ADA against alemtuzumab have been reported as being without clinical significance (14, 15), the dosing at intervals of 12 months or longer may have aided development of alemtuzumab by allowing ADA to subside (Table 1).

Alemtuzumab and Anti-drug Antibody Responses

While alemtuzumab (CAMPATH-1H) was originally designed to reduce the immunogenicity of the parent CAMPATH-1G rat immunoglobulin (1–3, 21) with alemtuzumab, ironically this appears not the case, as seen in this comparison among antibody therapies used in MS (Table 2). Moreover, alemtuzumab (36–60 mg Q52W) induces binding ADA in about 85% of cases within 24 months ($n = 811$), and about 92% of those develop neutralizing ADA (12, 13). In the phase III studies, it was evident that, despite substantial lymphocyte depletion, over 60% of pwMS developed ADA within the first month of infusion (12, 13). Furthermore, in the phase II extension study (Maximum $n = 232$), with three cycles of alemtuzumab administered, nearly all of the pwMS eventually developed ADA (8) (Table 1). Even chimeric CD20-depleting antibody (500–1,000 mg rituximab, Q48W) induced ADA in only about 25–37% of pwMS (22, 25). By contrast, humanized ocrelizumab (anti-CD20, 600 mg Q48W) induced ADA in only 0.4% of people with relapsing MS, with <0.1% of people exhibit neutralizing ADA within 2 years of treatment (23). This low level may not simply be due to the humanization process, as this is in part dose-dependent as lower ocrelizumab doses (20 mg) induce ADA in about 20% of pwMS

(26). While this dose induced comparable peripheral blood depletion to the 600 mg dose, repopulation was quicker (26), and possibly allows sparing of B cells within lymphoid tissues that can generate the ADA response. Humanized, natalizumab (anti-CD49d-CD29, 300 mg Q4W) induces ADA in about 5–9% of people with MS (24). These are all significantly less than that of alemtuzumab treatment of pwMS (12) (Table 2).

Biology Supporting the Generation of Anti-drug Antibodies

The antibody humanization process has been refined since alemtuzumab was first invented, as it may be possible to reduce the immunogenicity of anti-CD52 antibody compared to alemtuzumab (27, 28). However, high frequency of ADA following alemtuzumab infusion may be due to its particular biology, which probably relates to the pattern of CD52 antigen tissue-expression and the depletion/repopulation kinetics of immune cells. Alemtuzumab is (a) given as an effective bolus (5). As CD52 antigen has a wide distribution outside the circulation, the CD52 receptors on leucocytes outside in tissues can absorb the antibody, and this can lead to the relatively short, peripheral blood half-life of alemtuzumab and rapid clearance from the circulation (15). Thus, the cells that escape the initial depletion event are not targeted again until the next cycle of treatment ~12 months later. This is unlike cladribine and ocrelizumab that are administered again 2–4 weeks after the initial dose (23, 29). As such, pwMS who do not deplete lymphocytes effectively after the first dose are more likely to subsequently develop ADA (13, 15); (b) alemtuzumab targets antigen-presenting cells, which include dendritic cells, and B cells, due to their expression of CD52 (Figure 1). Although transiently depleted, monocytes repopulate within a month while circulating antibody is probably still present, and so could rapidly present antigen to remaining antigen-specific lymphocytes, as could any antigen presenting cell that escaped depletion (15, 34). Similar to T cells, surviving B cells (35), could exhibit homeostatic expansion (36), following alemtuzumab-induced depletion and 1 month after treatment memory B cells remain a significant proportion of the B cell pool (37, 38). These cells can be efficient at presenting antigens, notably their specific antigen (39, 40), and could as a result complement the rapid generation of ADA, probably stimulated by professional antigen-presenting cells and supported by the activity of surviving T cells (41); (c) ancillary molecules are needed for the lytic action of alemtuzumab. These include the need for the complement cascade for complement-fixation or cells for antibody-dependent cellular cytotoxicity to co-localize and enter the target tissue (42). This may explain why it appears that alemtuzumab does not purge the lymphoid tissue and bone marrow effectively, as seen in humanized CD52 transgenic mice (43). As such, sequestration of lymphocytes into lymphoid tissue (and possibly the bone marrow) by fingolimod, due to sphingosine-1-phosphate receptor modulation, appears to inhibit the activity of alemtuzumab in some individuals (44); (d) this may allow the B cell niche in the bone marrow to survive and could account for the rapid hyper-repopulation of immature/transitional B cells and naïve/mature B cells that may

TABLE 1 | Alemtuzumab dosing schedule can limit anti-drug antibody activity.

Dosing schedule	Observed effects (CARE-MS-Trials)	Biology that avoids ADA effects
First infusion cycle*	ADA develop in most pwMS.	Primary antibody response usually takes at least 6 days to generate#,
Five daily 6 h, 12 mg alemtuzumab infusions	(BAbs 62%, NAbs 54% in 1 month)**	Influence of NAbs avoided.
Second infusion cycle*	ADA develop in most pwMS.	Secondary antibody responses often take 3–4 days to generate#.
Three daily 6 h, 12 mg alemtuzumab infusions	(BAbs 83%, NAbs 79 % in 1 month)**	Influence of NAbs avoided.
Repeat infusion cycles*	ADA slowly subside with time.	Repeat dosing during high titers of BAbs and NAbs avoided.
A minimum 12 month intervals	Pre-cycle 1. BAbs 0.9% NAbs 0%**	Influence of ADA avoided.
Retreatment after disease activity	Pre-cycle 2. BAbs 29% NAbs 0.6%**	
At least 12 months from last dose		
Original schedule 2012–2017	ADA may become more persistent	ADA levels wane before next cycle.
Two cycles*	Pre-cycle 3. BAbs 75% NAbs 31%** (Results at 23 months)	Influence of NAbs avoided.
New schedule 2017 onwards,		Pre-dose NAbs may be more
≤ four cycles (EU), ≤ three cycles (UK)		problematic for some pwMS.
Prophylactic anaphylactoid treatment*	Reduction of infusion reactions/cytokine	Masks anaphylactoid responses,
Anti-histamines, paracetamol, steroids	release syndrome.	which occur rarely*

Dosing schedule of alemtuzumab * (5), the occurrence of anti-drug antibodies (ADA), binding (BAbs), and neutralizing (NAbs) and adverse effects in people with multiple sclerosis (pwMS) following the treatment cycles in the pivotal CARE-MS I and II trials ** (12, 13) and the biology, such as the kinetics of antibody formation (18, 19), which could influence the generation and/or action of ADA. European Union (EU), United Kingdom (UK). Pre-cycle refers to the results obtained 1 month before the next infusion cycle, unless otherwise stated as cycle 3 may be ≥24 months (20). Bold letter within the table in column 1 indicates the dosing schedule and in column 3 indicates the influence of NAbs.

form the precursors for ADA formation (12); (e) this occurs at a time when CD52 depletion appears to block immune-tolerance induction (12, 45). While it has been reported that the proportions of CD4 T regulatory cells increase compared to CD4 T helper cells (35, 46), in terms of absolute numbers they are dramatically decreased, especially in relation to hyperpopulating immature B cells (12, 37). However, CD8 T cells may control this response, and this subset is markedly depleted by alemtuzumab (12, 45). This perhaps creates an environment for ADA generation that occurs with high frequency within the first month of infusion (8, 12, 15). Whether this represents T cell-independent extrafollicular zone directed immune response, as suggested for the formation of ADA to other antibodies (47), is currently unknown. Regulatory cells recover faster than potentially pathogenic memory T and B cells, allowing for control of MS (12). However, this early loss of immune tolerance may also allow the generation of antibody-mediated secondary autoimmunity to develop, which occurs at a high frequency (~40–50%) in pwMS within 5 years from infusion (5, 20, 48). This problem occurs in MS at a higher frequency compared to that observed in cancer following alemtuzumab use (49). Similarly, only 4/211 (1.9%) of people treated for cancer developed ADA (50). This suggests a dose-related difference, or that perhaps the genetics of people with MS and other potential autoimmunities (7) may also predispose them for generating immune responses that may contribute to generating ADA responses; (f) Lastly, since peripheral B cell niches may not be effectively purged, and CD52 is only weakly expressed by plasmablasts and plasma cells (Figure 1) (51, 52), alemtuzumab may not particularly target antibody-forming cells. The low expression of CD52 on plasma cells suggests that once formed, antibody responses (including ADA responses) will persist. Consistent with this view, vaccine responses to common virus and recall antigens persist following alemtuzumab treatment, and the ability to mount responses to novel antigens is retained

TABLE 2 | A high frequency of anti-drug antibodies develops following alemtuzumab treatment in people with MS.

Antibody	Target	Dosing	Frequency of ADA	References
Rituximab	CD20	1,000 mg Q26W	BAbs 24–37%	(22)
Ocrelizumab	CD20	600 mg Q26W	BAbs 0.4%, NAbs <0.1%	(23)
Natalizumab	CD49d	300 mg Q4W	BAbs 5–9%	(24)
Alemtuzumab	CD52	36–60 mg Q52W	BAbs 85%, NAbs 78%	(12)

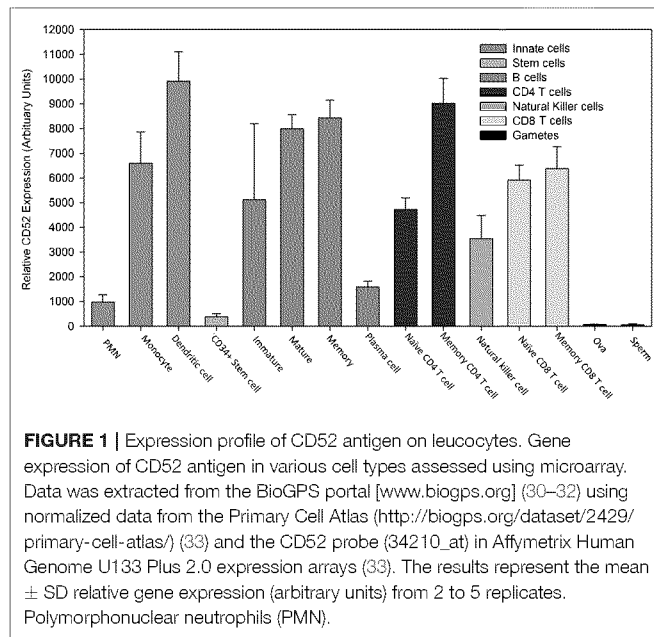
Reported frequency of anti-drug antibody (ADA) responses to various disease-modifying therapies (infusion dose and frequency are shown) during the first 2 years of use in major clinical trials for multiple sclerosis.

once the antibody has cleared (53). Thus, ADA titers are boosted with each infusion cycle (15), and this increases the risk of neutralization over time, as the number of treatment cycles increases.

Anti-drug Antibody Generation May Have Influenced the Treatment Protocol for Alemtuzumab

With the recognition that humanized forms of CAMPATH-1 could still elicit strong ADA responses (4, 7, 54, 55) with a reduction in therapeutic benefit (7, 56), strategies to inhibit ADA to alemtuzumab were investigated (8–10). As such, ADA might have been a consideration in the clinical dosing schedule developed for alemtuzumab in MS (Table 1). Dosing is limited to 5 days for the first treatment cycle and 3 days for the second and subsequent treatment cycles. Repeat dosing has to be 1 year after the last treatment cycle, rather than following disease breakthrough, and only two cycles of dosing were to be initially administered (5). Lastly, infusion of alemtuzumab occurs under anaphylactoid reaction prophylaxis (5, 11, 20).

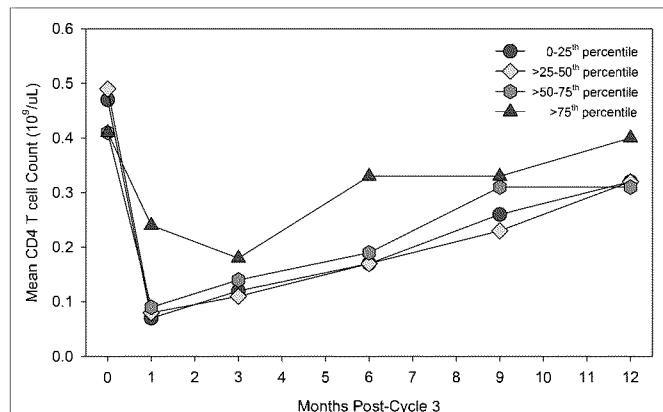
The dosing schedule may thus avoid potential influences of ADA response. As such, it takes at least 6 days for B cell



responses to be generated, with primary antibody levels peaking sometime after that, and it will take a few days to generate an effective secondary antibody response (18, 19, 57). These antibody responses appear to take about a year to subside to near background levels to allow re-dosing (12, 15). Importantly, while there were no pre-treatment NAb responses prior to treatment cycle 1, and only about 0.6% of people had NABs prior to cycle 2 in the phase III trials, about 31% of people had persistent NABs, which can limit activity at the end of the second treatment cycle, and over 75% of people had persistent BAb (12, 13, 15). Anaphylactoid reaction prophylaxis is largely being given to limit the problems of infusion reactions, which are common (>80%), especially during the first cycle of infusions. These are associated with the reactivation of symptoms that occur with pre-existing demyelinated lesions (5, 58). Infusion reactions are largely a product of the cytokine release syndrome, occurring following cell lysis, due to antibody-mediated attacks. The antihistamines and glucocorticosteroids would also mask any potential anti-globulin allergic response, which have not been a significant adverse event (5, 11, 20). While this dosing schedule may have served to avoid the potential issue of antibody immunogenicity, it also generated the concept of pulsed “immune reconstitution therapy” (IRT). This demonstrates that it is possible to get long-term benefit—and possibly long-term remission for some people—from a short treatment cycle, creating a new therapeutic paradigm that did not depend on continuous treatment (48, 59).

Neutralizing Anti-drug Antibodies Generation May Become More Problematic With Increasing Number of Treatment Cycles and Will Need Monitoring

While the frequency of ADA during two cycles is high, the titer generally drops sufficiently to allow effective re-treatment (12, 48). However, with time they may become more persistent



(8, 13). As such, ADA could be an issue for any pwMS receiving a third cycle of alemtuzumab, although they have not been a problem at the population level, as alemtuzumab continues to deplete (14, 15, 48, 60). While available data suggests that a lack of response after a third cycle of alemtuzumab is probably only in a minority of pwMS (14), it still appears that those with the highest titer ADA (binding and neutralizing) pre-cycle 3 (>75 percentile) exhibit the poorest lymphocyte depletion potential (Figure 2) (14). People with high-titer neutralizing ADA responses can fail to deplete. This can lead to disease breakthrough and accumulating disability (Figures 3, 4) (8, 13, 16, 17).

Initially, alemtuzumab had a liberal license in Europe, requiring only an active lesion on MRI for use (15). More recently, vascular side effects following infusion has moved alemtuzumab largely to a second-line status in the European Union, and it remains largely a third-line treatment in the United States, where it has remained ever since receiving FDA approval (15, 61). However, as the third and potentially fourth treatment cycle of alemtuzumab was approved by the European Medicines Agency (EMA) in 2017, and the National Health Service (NHS) in the United Kingdom in 2018 (15, 62), measurement of ADA would be more important to help inform re-treatment or drug-switching decisions for individual patients.

If using alemtuzumab, it is imperative that lymphocyte depletion is assessed. This also applies to any other lymphocyte-depleting agent, as people fail to reduce lymphocyte levels in response to treatment with a variety of agents, probably due to their genetics (13, 63). Although it has consistently been reported that total lymphocyte levels do not predict disease activity (15, 60), lack of depletion can be seen to be associated with disease breakthrough and treatment failure in individuals, necessitating a

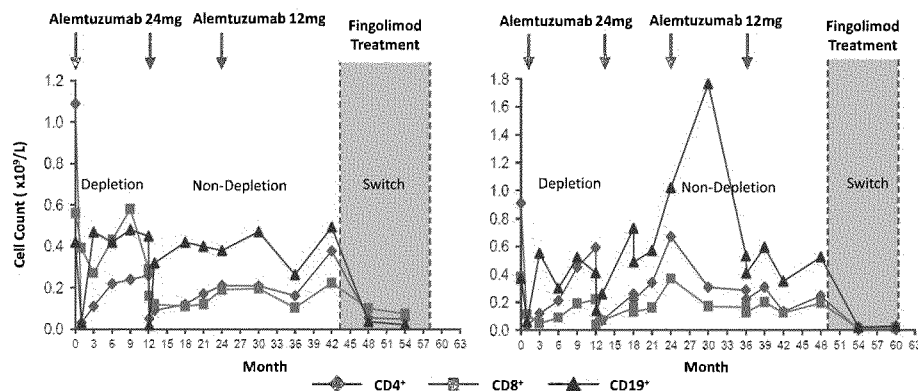


FIGURE 3 | Loss of lymphocyte-depleting function after three alemtuzumab cycles. Lymphocyte depletion following alemtuzumab treatment in people that switched to fingolimod. Evidence for loss of function after two or more cycles. Although the clinical course is not shown, additional treatments after two cycles or switching to another treat in an indicator for disease breakthrough in the form of new relapses or magnetic resonance imaging. Poster available <http://www.empireneuro.org/sitebuildercontent/sitebuilderfiles/ean2015poster.pdf> (accessed 5th December 2019). Reproduced with permission from Genzyme and D. J. Arnold.

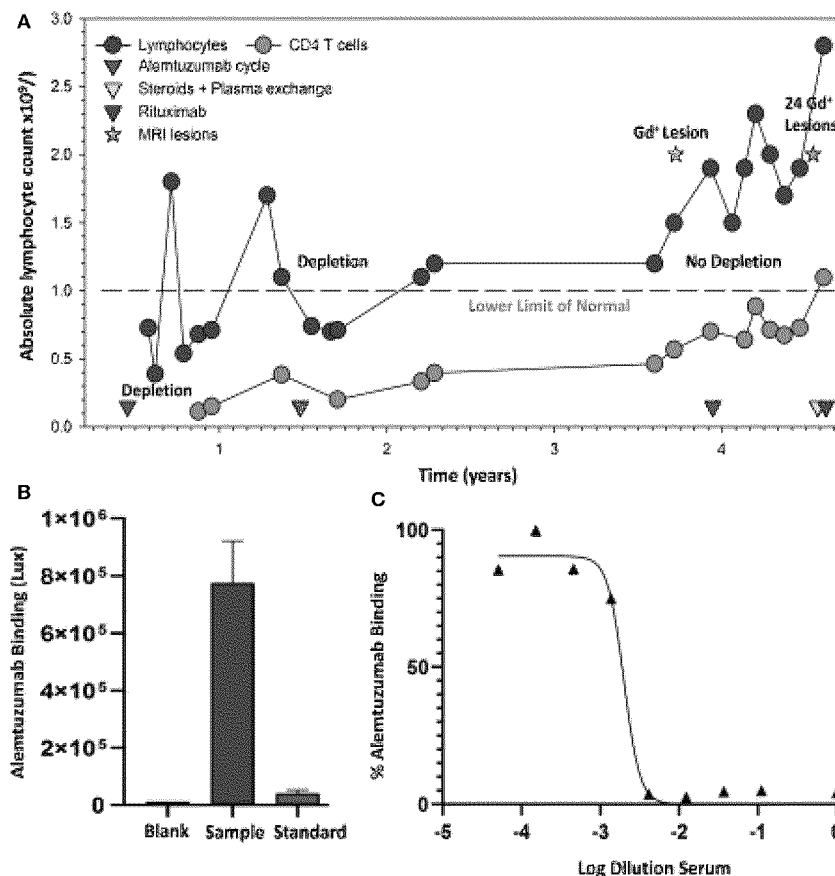


FIGURE 4 | Alemtuzumab neutralizing antibodies develop in a person failing treatment. **(A)** Lymphocyte (lower limit of normal is shown by a dashed line) and CD4 T cell levels were assessed in a person with clinically-definite multiple sclerosis who received the standard 12 mg/day dosing of alemtuzumab at 12-month intervals. Following detection of an active spinal cord lesion, detected by T1 gadolinium (Gd+) enhanced magnetic resonance imaging, an additional alemtuzumab infusion cycle was given. Lymphocyte depletion was limited. A magnetic resonance imaging scan subsequently detected 17 brain and 7 spinal cord gadolinium enhancing lesions, and prompted intravenous methyl prednisolone and plasma exchange, followed by an oral steroid taper. A serum sample (collected during routine sampling) following five cycles of plasma exchange was used following informed written consent and approval given for publication, consistent with institutional guidelines. These were assayed to conform with United Kingdom regulations. **(B)** Binding (Saxena et al., in press) and **(C)** neutralizing ADA (Ali et al., in press) levels taken prior to 1000mg rituximab therapy, which was given at 2-week intervals.

switch to alternative therapies (**Figure 3**). Such individuals could be found in meeting reports (8, 16, 17). However, the scale of the issue needs to be addressed, although presumably the frequency of total lack of efficacy is low (14, 48). Nonetheless, in one study, two out of six people switching to fingolimod could be seen to totally fail to deplete prior to switching (**Figure 3**) (16)—this suggested the presence of neutralizing ADA. Appreciation of this issue could possibly spare individuals from unnecessary disease activity and disability that untreated MS causes.

Alemtuzumab Screening Assays

At present, alemtuzumab ADA assays are neither routinely supplied by the manufacturer nor required by regulatory authorities. However, the reagents with which to construct an assay for alemtuzumab ADA and a neutralizing assay are commercially available. To support clinical use of alemtuzumab in our clinical practice—as it is a valuable treatment for many people with MS, and the possibility of third and fourth courses of treatment were available (5, 48)—we developed a novel assay to detect ADA against alemtuzumab, using a synthetic recombinant construct Alem GloBody (64). This consists of the alemtuzumab variable heavy (V_H) and light (V_L) domains held together by an engineered tandem nanoluciferase molecule, such that the V_H and V_L will pair up and retain antigen binding, and the dual luciferase activity is not compromised (64). In the presence of ADA, the Alem GloBody-IgG complexes form. Since the Alem GloBody lacks the IgG constant domains, it cannot bind to Protein G. However, the complexes can be captured via the Fc of the ADA, and the retained luciferase activity is proportional to the level of ADA in the sample. The assay is performed in <3 h and currently only requires 20 μ L of serum. Secondly, we have developed a stable adherent CHO cell line expressing human CD52 for use in a competition assay with sera and alemtuzumab conjugated with Alexa-488 (65). In the absence of neutralizing antibody, the alemtuzumab-Alexa-488 binds to the cells with maximum fluorescence. If neutralizing antibodies are present, they inhibit alemtuzumab-Alexa-488 binding to the cells and the signal is reduced. This reduction in signal can be titrated, and a value assigned to the dilution, requiring giving 50% inhibition (ID_{50}). This assay currently requires only 10 μ L of serum and takes 3 h (65). These two assays can be used to detect the development of binding and neutralizing alemtuzumab-specific ADA, as seen in an individual with MS (**Figure 4**) who stopped depleting lymphocytes and exhibited breakthrough disease activity as indicated by new lesion formation (**Figure 4A**). In comparison to untreated serum (baseline 1.22×10^4 Lux), blank, and a 50 μ g/mL anti-alemtuzumab standard (4.66×10^4 Lux), the serum had a very high titer ($>7.7 \times 10^5$ Lux, despite five cycles of plasma exchange) of binding (**Figure 4B**), and neutralization (**Figure 4C**) of ADA could be detected. Although this does not prove cause and effect, it is inconceivable that high titers of neutralizing antibodies are without any influence if there are pre-existing ADA. It has been suggested that ADA are without significant influence (5, 11, 14). While this may be the case at the population level (14, 60), this seems

not the case for certain individuals (13). This study indicates that neutralization of alemtuzumab occurs and appears to be clinically relevant in some individuals. Thus, the monitoring of ADA responses may be helpful in the decision to re-treat or switch treatments.

CONCLUSIONS

High-titer neutralizing ADA responses can be associated with a lack of clinical response (8, 13, 17) (**Figure 4**). These can become high-titer and persist for years (unpublished observations). However, it remains to be determined whether there is a pre-dose antibody-titer limit, above which further dosing is unlikely to work effectively. People within the phase III CARE-MS trial ($n = 712$), and extension studies ($n = 292$), had their ADA (binding and neutralizing) and lymphocyte levels monitored (14)—suggesting that the manufacturer could address this point. Based on our findings, it would seem important for 1-month pre-dose neutralizing ADA titers relating to post-dose lymphocyte depletion failure to be reported, in order to evaluate the concerns raised here. Further, ongoing studies on assay development and validation are in progress and are required to define prognostic ADA levels that may predict lymphocyte depletion and potential treatment failure, such that they can inform on re-treat or switching options. We believe pre-dose screening should be offered and adopted, and that the switching of treatments should be instigated where relevant, as it is important that further neurological disability is not accumulated because patients are being given an ineffective treatment. Here, we demonstrated the utility of GloBodyTM for alemtuzumab ADA detection. GloBody reagents based on other antibody-therapeutic binding sites may be generated (including for CART-cell), and the generic platform may be adopted to monitor ADA responses (64).

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AK and DB conceived the concept and drafted the initial manuscript. AK designed the GloBody molecule and assays. GS, LA, MJ, and GP developed the cell line, prepared the reagents, and developed and applied the assays. KM, LS, AG, SG, KS, and GG provided the clinical samples. All authors discussed the results and contributed to the final manuscript.

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Immunogenicity of TNF-Inhibitors

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Tumor necrosis factor inhibitors (TNFi) have significantly improved treatment outcome of rheumatic diseases since their incorporation into treatment protocols two decades ago. Nevertheless, a substantial fraction of patients experiences either primary or secondary failure to TNFi due to ineffectiveness of the drug or adverse reactions. Secondary failure and adverse events can be related to the development of anti-drug antibodies (ADA). The earliest studies that reported ADA toward TNFi mainly used drug-sensitive assays. Retrospectively, we recognize this has led to an underestimation of the amount of ADA produced due to drug interference. Drug-tolerant ADA assays also detect ADA in the presence of drug, which has contributed to the currently reported higher incidence of ADA development. Comprehension and awareness of the assay format used for ADA detection is thus essential to interpret ADA measurements correctly. In addition, a concurrent drug level measurement is informative as it may provide insight in the extent of underestimation of ADA levels and improves understanding the clinical consequences of ADA formation. The clinical effects are dependent on the ratio between the amount of drug that is neutralized by ADA and the amount of unbound drug. Pharmacokinetic modeling might be useful in this context. The ADA response generally gives rise to high affinity IgG antibodies, but this response will differ between patients. Some patients will not reach the phase of affinity maturation while others generate an enduring high titer high affinity IgG response. This response can be transient in some patients, indicating a mechanism of tolerance induction or B-cell anergy. In this review several different aspects of the ADA response toward TNFi will be discussed. It will highlight the ADA assays, characteristics and regulation of the ADA response, impact of immunogenicity on the pharmacokinetics of TNFi, clinical implications of ADA formation, and possible mitigation strategies.

Keywords: immunogenicity, antidrug antibodies, tumor necrosis factor inhibitors, drug levels, assay

INTRODUCTION—A BRIEF HISTORY OF TNF INHIBITOR DEVELOPMENT

During the last decades, recombinant therapeutic proteins (biologics) have revolutionized the treatment of a wide variety of diseases. Since the demonstrated clinical effectiveness and market approval of the first recombinant therapeutic protein (insulin, 1982), which was quickly followed by the first therapeutic monoclonal antibody (OKT3, 1985), the development of these therapeutics has expanded exponentially. Currently, recombinant therapeutic proteins are the fastest-growing sector in the pharmaceutical industry with an estimated value of around 150 billion dollars. Within

the field of rheumatology currently seven monoclonal antibodies, two fusion proteins, and one cytokine mimic are available that aim to meet the unmet needs of treatment with empirical medication such as methotrexate. Five of these biologics belong to the group of TNF inhibitors (TNFi) and include the monoclonal antibody-based proteins adalimumab, infliximab, golimumab, and certolizumab and the fusion-protein etanercept.

The first step toward the development of therapeutic monoclonal antibodies was set in 1975 by Kohler and Millstein who discovered how to generate monoclonal antibodies *in vitro* (1). Initially the monoclonal therapeutic antibodies were of murine origin which brought about several significant shortcomings such as the development of antidrug antibodies (ADA, termed human anti-murine antibodies or HAMA at the time) (2–4), a relatively short half-life due to weak binding to the Fc receptor (5, 6), and reduced efficacy due to poor stimulation of effector functions (6, 7). In order to overcome these drawbacks, the next generation of therapeutic monoclonal antibodies were chimeric antibodies in which the murine constant domains were replaced by their human counterpart. Although chimeric antibodies such as infliximab and rituximab (anti-CD20) are less immunogenic, they can still induce ADA formation (8, 9). With further advances in antibody engineering, humanized and fully human monoclonal antibodies became available. During the process of humanization, residual mouse-related epitopes in the variable domain are replaced by human sequences while retaining the target binding properties. Fully human antibodies can be derived from phage-display or be generated in xenogenic mice carrying the human humoral immune repertoire. Humanized and fully human monoclonal antibodies are less immunogenic and have better pharmacological properties compared to the earlier antibodies, but they still induce ADA formation (2, 9, 10).

In parallel with the advancements in antibody engineering, Brennen et al. described in 1989 that blocking of TNF inhibits the production of several important pro-inflammatory cytokines (11). This novel concept, in which TNF initiates a cascade of cytokine production, designated TNF as an interesting target for the treatment of inflammatory diseases like rheumatoid arthritis (12). Although at the time the rationale for anti-TNF therapy in rheumatoid arthritis was new and not widely accepted, several TNF-inhibitors were generated as a possible treatment for bacterial septic shock (13). After demonstrating the beneficial effect of TNFi in animal models of arthritis (14) it was shown that TNFi were also effective in patients with rheumatoid arthritis (15).

Currently, five TNFi are approved by FDA and EMA, which are infliximab, etanercept, adalimumab, golimumab, and certolizumab pegol. Adalimumab and golimumab are fully human IgG1 antibodies, infliximab is a chimeric IgG1 antibody, etanercept is a fusion-protein between a human IgG1 Fc-tail and the TNF-receptor type 2, and certolizumab pegol is a PEGylated Fab fragment of a humanized anti-TNF antibody. Despite the fact that TNFi have significantly improved the treatment of rheumatic diseases, a fraction of patients needs to discontinue treatment due to ineffectiveness or adverse reactions. Both can be the result of ADA development. The first studies that drew attention toward

the immunogenicity of TNFi showed a shorter drug survival in patients after subsequent doses of TNFi (16, 17). Later it was demonstrated that most TNFi induce formation of ADA (17, 18), mostly toward the idiotype of the antibody (19–21). The reported frequencies of ADA detection and ADA titers vary between studies, which can be explained by both patient- and treatment-related factors such as genetics, type of immune response, TNFi characteristics, dosing regimen and co-medication (17). In addition, the assay format used for the assessment of ADA affects the results (22). Measurements with drug-tolerant assays have shown that the majority of patients treated with a TNFi develop ADA (22). However, not all detectable ADA result in drug levels below the therapeutic window; the clinical consequence is dependent on the relative amount of drug and ADA. Even in the presence of ADA, drug levels can be sufficiently high and contribute to clinical remission. Recently published data suggests that concentrations of around 0.1–0.5 mg/L might be sufficient to control TNF (23). The foregoing emphasizes that for the assessment of the clinical relevance of immunogenicity adequate drug level measurements are essential. In this review these different aspects of immunogenicity of TNFi will be discussed in more detail.

ASSAYS USED FOR ADA DETECTION

Assessing the immunogenicity of TNFi is complex, amongst others due to potential interference of the drug with the assay, variable time course of the ADA response, and variability of the antibody characteristics such as affinities and isotypes. Drug interference complicates accurate quantification of ADA and thereby the assessment of its effect on PK of the TNFi and its clinical relevance (24). Information about which assay is used and familiarity with the most important characteristics of the assay are essential to interpret ADA measurement correctly.

The most important distinction that can be made between the available assays is the extent to which the assay is sensitive to the drug in the serum (either free or bound to the ADA), i.e., the drug-tolerance of the assay. When drug is present in the serum it will form complexes with ADA. Since detection of ADA in all assay formats is based on a labeled variant of the drug, this complex formation will shield binding of the detection reagent. This phenomenon is called drug interference and it will result in underestimation of the immunogenic potential of the TNFi. Early studies that focused on the immunogenicity of TNFi used assays with very low drug-tolerance (drug-sensitive assay), thereby underestimating the levels of immunogenicity. Although current assays are often more drug-tolerant, they still are affected by the level of the TNFi to a certain extent (24).

Drug-tolerant assays can, in contrast to drug-sensitive assays, also measure ADA that are bound to the TNFi. Nevertheless, even completely drug-tolerant assays will underestimate the true amount of ADA formation since ADA-drug-complexes may be cleared from the circulation more rapidly. Using the drug-tolerant assays it was discovered that the majority of patients treated with a TNFi develop an immune response toward these therapeutic proteins (22). Although drug-tolerant assays provide

a more accurate assessment of the presence of ADA compared to drug-sensitive assays, they are not necessarily more useful in clinical practice. This can be explained by the fact that drug-tolerant assays also detect ADA that would not have caused a clinically relevant decrease in drug level, while drug-sensitive assays will typically only detect ADA when drug levels are below the clinically effective threshold. Not surprisingly, the strongest associations between ADA and clinical impact were mainly established using drug-sensitive assays (24–26).

The different assay formats have been reviewed before and will be discussed only briefly in this review (24–27). Widely used formats include bridging immunoassays and antigen-binding tests (ABT). In general, both the enzyme-linked immunosorbent assay (ELISA) and the electrochemiluminescence (ECL) assay are often performed as bridging formats where (labeled) drug is used for capture as well as detection. This bridging format hinges on the multivalency of the ADA that is being detected (e.g., being able to bind at least two drug molecules). Since circulating IgG4 antibodies are largely monovalent, due to half-molecule exchange (28), ADA of the IgG4 isotype will not be appropriately detected in these bridging formats, which may result in an underestimation of the ADA response. The ABT is different in that it uses a capture ligand (generally protein A) to immobilize specific and non-specific immunoglobulins present in the sample, which is followed by specific detection of the ADA using radiolabeled (in case of the radioimmunoassay, RIA) TNFi. For all these assays, drug-tolerant formats have been developed by employing acid pretreatment which dissociates the drug-ADA complexes that may be present in the sample.

CHARACTERIZATION OF THE ANTI-DRUG ANTIBODY RESPONSE

Following antigen exposure, it may take up to a week or more for specific antibodies to become detectable in circulation (29). Initially these antibodies are expected to be of the IgM isotype. In case of proper B-cell stimulation by follicular T-helper cells, isotype switching can occur leading to the generation of antibodies of the IgG isotype. Detection of these antibodies is dependent on the sensitivity and the characteristics of the assay, which in the case of ADA are generally optimized to detect antibodies of the IgG isotype. Therefore, ADA are usually detected 2–4 weeks after the first administration (30), but may have been present at undetectable levels earlier. In the absence of proper T-cell help, ADA formation is expected to remain limited to a low titer transient IgM response with little clinical impact. Since none of the currently marketed TNFi have a mucosal route of administration, antibodies of the IgA isotype are not expected to be formed. Also, antibodies of the IgE isotype, that are associated with hypersensitivity reactions, are rarely detected (31). Previous studies demonstrated that enduring exposure to toxins leads to the formation of antibodies of the IgG4 isotype, which are associated with a tolerogenic phenotype due to the lack of Fc functionality (32). Similarly, long-term exposure to TNFi was demonstrated to result in substantial IgG4 ADA production (33).

To maintain tolerance to self, negative feedback loops are in place that prevent the generation of high affinity antibodies that recognize self-epitopes. Consistent with this concept, ADA formation to therapeutic antibodies such as the TNFi appears to be almost exclusively restricted to epitopes that are drug-specific, i.e., the idiotype. Depending on the TNFi, different drug-specific epitopes can be identified [reviewed in van Schie et al. (34)]. With the exception of etanercept, all TNFi are monoclonal antibody (-based) proteins that by definition contain complementary determining regions (CDR). These hypervariable loops form the largest part of the TNF binding region, which is unique for every antibody clone. Due to this unique amino acid sequence (e.g., not present in the natural Ig pool of the patient), and potentially aided by its natural property to prompt protein binding, the antigen-binding site forms the prime immunogenic region targeted by ADA. The TNF-receptor/Fc-tail fusion protein etanercept is unique in the sense that does not have an idiotype and only the fusion region between the domains contains non-endogenous epitopes that are potentially immunogenic, which may explain its overall low immunogenicity (35).

Drug Neutralization by ADA

When looking at the functional impact of ADA on drug level, two types of ADA can be distinguished. These are non-neutralizing ADA (or binding antibodies, BAB), that specifically bind the drug but do not affect the drug-target interaction, and neutralizing ADA (NAB), that directly (or in close proximity) bind the pharmacologically active site of the drug thereby physically interfering with the ability of the drug to bind its target. Where BAB may indirectly decrease the drug level by increasing drug clearance via immune complex formation, NAB may have a direct negative impact on functional drug level. NAB have demonstrated to be a major safety concern for enzyme replacement therapies, where cross-reactivity to and subsequent neutralization of the endogenous counterpart has led to life-threatening side effects [reviewed in Wang et al. (36)]. However, no specific safety concerns from NAB against monoclonal antibody therapeutics, including the TNFi, have been reported.

The value of specific NAB assessment for monoclonal antibody therapeutics may be questioned. Inconsistent NAB incidences are being reported for the same drug depending on the assay used for detection, which is exemplified by the market authorization reports on biosimilars (37). Further, reporting only NAB incidences easily results in misunderstanding of NAB data. Typically, ADA-positive samples are assessed in a NAB assay. When a certain degree of neutralization is observed, the sample is considered “NAB-positive,” which is easily interpreted as “in this patient, drug is inactive because it will be neutralized by NAB.” Conversely, ADA-positive samples where no neutralization was measured in the assay would be “NAB-negative.” This may be taken to mean “this sample contains non-neutralizing antibodies.” Both interpretations might be true, but not necessarily so. To interpret NAB data it is important to realize that functional neutralization depends on the relative concentrations and affinities of the ADA, the drug, the target, and the target’s respective target (the TNF receptor in case of the TNFi). Since the relative concentrations of

these components is different between patients and between the various compartments where the drug may act, e.g., blood vs. tissue, it is not possible to mimic the exact level of *in vivo* neutralization in a single functional *in vitro* assay. In general, the relative concentrations of these components used in NAb assays far exceeds the natural variation seen in the patient, thereby limiting the relevance of the NAb assay outcome. As a consequence, reported NAb positivity only indicates the presence of ADA that potentially could neutralize the drug, but does not inform whether this truly happens *in vivo*. Conversely, reported NAb negativity only demonstrates that neutralization was not detectable in that particular highly artificial *in vitro* assay format, but does not definitively excludes *in vivo* neutralization. It is further important to realize that NAb assays, especially cell-based NAb assays, are often less sensitive than ADA assays. As a result, samples with only low titers may be deemed positive in the ADA assay but negative in the NAb assay, thereby (wrongfully) suggesting that non-neutralizing ADA are present (25). In case of ADA to TNFi, a more precise interpretation would be presence of neutralizing antibodies in quantities insufficient (also in relation to their affinities) to neutralize a significant amount of drug.

ADA are mainly directed to epitopes in the antigen-binding site, which is why binding of ADA to the drug interferes with TNF binding. Evidence supporting this notion has been provided by serological studies that demonstrated virtually complete (90–97%) loss of binding between ADA and TNFi in the presence of excess TNF (21), demonstrating that TNF and ADA binding are mutually excluding. Recently, this was also demonstrated for other therapeutic antibodies, for example natalizumab (38). These ADA were investigated in more detail by several studies (20, 38–40). The study by Cassotta et al. on natalizumab demonstrated by crystallography that monoclonal ADA that were scored positive or negative (i.e., below an arbitrary cut-off, weak neutralization was observed) for *in vitro* neutralizing functionality both occupied the same physical space as the drug target, suggesting that, given high enough concentrations, both types of ADA would be neutralizing (39). Taken together, these studies suggest that whenever an ADA response to any of these therapeutic antibodies (and probably any therapeutic antibody) is detected, most if not all ADA will have the capacity to neutralize. Therefore, there is no additional information to be gained from NAb testing in this setting. Together with the lack of specific safety concerns related to NAb and the lack of *in vivo* relevance and inconsistency of reported NAb assay outcomes, NAb testing for monoclonal antibody therapeutics and their biosimilars could be omitted.

REGULATION OF IMMUNOGENICITY

Covariates Influencing Immunogenicity

Previous studies have identified several patient- and treatment-related factors that influence the immunogenic potential of TNFi. It is useful to have some insight in which covariates affect the immune response toward an exogenous protein since this might help to develop strategies which potentially reduce the immunogenicity of these compounds. Treatment-related factors affecting immunogenicity are related to the structure

and composition of the mAb, its use in terms of dosage, route of administration, and co-medication. The structure of a biologic will influence immunogenicity, including the primary amino acid sequence, glycosylation and other post-translational modifications. Furthermore, the formulation of the drug can impact both chemical and physical stability, such as the tendency to aggregate. For instance, a higher murine content may trigger more ADA formation, just like the presence of aggregates. The duration and dose of the treatment and the route of administration probably affect the amount of ADA that is being produced (41–43).

An important patient-related risk-factor is the genetic background of a patient. Several studies have focused on variability in HLA-type and HLA alleles have been described to be associated with ADA formation (44–46). Some HLA alleles are thought to be protective against ADA formation (HLA-DQB1*05, HLA-DRB1*01, and HLA-DRB1*07, with odds ratios (OR) of 0.4 95% CI [0.186–0.862], 0.25 95% CI [0.073–0.927], and 0.2895% CI [0.078–1.004], respectively), while others might increase the risk of ADA formation (HLA-DRB1*03 and HLA-DRB1*011, with OR of 2.52 95% CI [1.37–4.63] and 2.64 95% CI [1.240–4.045], respectively) (45). In a recently published study performed in 1240 Crohn's disease patients from the PANTS cohort the allele HLA-DQA1*05, which is carried by ~40% of the European population, was also associated with a significant higher rate of ADA development [hazard ratio 1.90 (1.60–2.25)] (46). The observation that some HLA alleles are associated with an increased risk for ADA formation against multiple TNFi is intriguing and has been suggested by some as supporting evidence for the role of HLA alleles in ADA development. However, no mechanism has yet been described that functionally explains this observation. In general, studies exploring the functional association between HLA alleles and ADA formation are highly desired.

Variability in IL-10 production is another patient-related factor that might affect ADA formation (47, 48). Vultaggio et al. described that patients exposed to infliximab may initiate an adaptive cellular response resulting in the production of infliximab-specific T-cells (49). Some of these T-cells produce IL-10 which contributes to downregulation of the immune response of the infliximab-specific T-effector cells (50). When the kinetics of IL-10 and IFN γ were analyzed by Pratesi et al. it was found that the absence of IL-10 production and a low IL-10/IFN γ ratio were associated with formation of ADA (48). These data are in line with the earlier study of Bartelds et al. which described that ADA formation against adalimumab is associated with IL-10 gene polymorphisms (47). Some reservation toward these results needs to be maintained as they are based on small groups of patients. Polymorphisms in other immune response genes such as TNF and cytotoxic T-lymphocyte associated antigen 4 (CTLA-4) have been identified as risk factors toward other biological therapies (51).

Besides genetic factors, other patient-related factors that are thought to increase the risk of ADA formation are a longer disease duration, a higher baseline disease activity, and disease status. For example, patients with an activated immune system are more likely to develop ADA compared to healthy controls

or immunosuppressed patients (41–43). Another risk factor that has been described is not being naïve to TNF treatment, especially when ADA can be detected toward the previous TNFi. ADA could be detected more often in patients who developed a significant ADA response toward their first TNFi compared to anti-TNF naïve patients or patients without detectable ADA toward their first TNFi (52, 53). Use of concomitant medication also affects ADA formation. If methotrexate is not used in combination with the TNFi, it is more likely that clinically relevant ADA will develop (52, 54–56). ADA formation is also affected by serum concentration of the TNFi. Sufficiently high drug levels should be present in order to dampen the immune response toward the TNFi, especially in the first three months of treatment. In the past, TNFi were administered with irregular dosing intervals which resulted in lower drug levels and a higher percentage of ADA-positive patients. One last important patient-related factor that affects ADA formation negatively is induction of immune tolerance. In some patients the production of ADA toward TNFi can decrease over time (42).

Induction of Immune Tolerance

In some patients ADA responses are transient, which suggests a mechanism of immune tolerance. Immune tolerance refers to the absence of a measurable antibody response, skewing of the immune response to a less inflammatory phenotype, or exhaustion of the immune response to a particular immunogenic antigen. This physiological phenomenon is essential to prevent excessive immune responses to harmless antigens such as dietary antigens, allergens, and commensal microbiotics. The mechanisms contributing to immune tolerance have not yet been fully elucidated.

During treatment with TNFi, immune tolerance is mostly observed as a decrease in ADA titers over time. For example, van Schouwenburg et al. described a transient ADA response in 17/53 ADA-positive RA patients treated with adalimumab (22), while Steenholdt et al. described transient detection of ADA in inflammatory bowel disease (IBD) patients treated with infliximab (57). Two other studies also observed disappearance of ADA over time in comparable patient populations (58, 59). In a group of multiple sclerosis (MS) patients treated with natalizumab similar results have been found; anti-natalizumab antibodies develop in ~60% of patients but are persistent only in 3.5–10% of the patients (60–62).

Peripheral tolerance is likely to be of importance in acquiring immune tolerance to foreign antigens by preventing auto-reactivity. Several mechanisms have been suggested to be involved in this process, and most of them are T-cell mediated. One refers to the presentation of self-antigens by dendritic cells (DC) to T-cells. In the absence of appropriate co-stimulation, or in the presence of co-expression of ligands for the inhibiting receptors CTLA4 and programmed cell death protein 1 (PD-1), DC fail to activate the T-cell which leads to T-cell anergy. Other mechanisms are the deletion of autoreactive T-cell clones due to repetitive activation and upregulation of Fas-ligand on the T-cell and the suppressive activity of regulatory T-cells. Development of anti-idiotypic antibodies to self-reactive antibodies is another mechanism to accomplish immune tolerance (51). Furthermore,

since a large part of the antibody molecule is autologous, there may exist regulatory T cells that confer a dampening effect on an evolving immune response upon being presented peptides derived from these constant domains, possibly including parts of the IgG molecule that have been designated “Tregitopes” (63).

Hemophilia A, a bleeding disorder resulting from a deficiency in factor VIII (FVIII), is the sole disease in which the downregulation of ADA formation by induction of immune tolerance has been described (64). Consequently, it provides a model to elucidate the mechanisms involved in the induction of tolerance. Depending on the severity of the FVIII deficiency, ADA toward FVIII (called inhibitors) can be detected in 5–88% of the patients, mostly within 9–12 days after exposure (65, 66). Production of these inhibitors follows the classic immune response paradigm. Important targets for the induction of immune tolerance thus include regulatory T-cells, memory B-cells, and plasma cells producing anti-FVIII antibodies. Hausl et al. demonstrated that high levels of FVIII prevent FVIII-specific memory B-cells from differentiating into anti-FVIII producing plasma cells, and instead induced apoptosis of these cells (67). In addition, enduring exposure to FVIII in the absence of co-stimulatory pro-inflammatory signals results in the induction of regulatory T-cells. These cells produce IL-10 and TGF-beta, which inhibit the formation and activation of FVIII-specific effector T-cells, again preventing the differentiation of FVIII-specific B-cells to plasma cells. Development of anti-idiotypic antibodies against anti-FVIII antibodies have also been hypothesized to contribute to immune tolerance induction via neutralization of FVIII-specific antibodies, inhibition of FVIII-specific B-cells, and induction of apoptosis of FVIII-specific B cells (51). These concepts described are probably applicable to all antibody-based drugs, including TNFi.

Although several mechanisms which potentially contribute to induction of immune tolerance toward therapeutic proteins have been proposed, it is still largely unknown how this tolerance develops. To our knowledge, detailed studies explaining the underlying mechanisms of immune tolerance have not been performed. More insight in these processes might create opportunities to optimize treatment of therapeutic proteins, treat allergies and autoimmune diseases, and improve transplant acceptance.

ADA AFFECTING THE PHARMACOKINETICS OF TNFi

General PK of TNFi

Some familiarity with the pharmacokinetics of TNFi is essential in order to understand the clinical consequences of ADA formation toward TNFi. It was already stated that not all TNFi have the same molecular structure, which is important when evaluating their PK. The pharmacokinetic properties of therapeutic monoclonal antibodies such as infliximab, adalimumab, and golimumab have been reviewed extensively in the past (41, 42, 68–71). The most important aspects of the PK of these compounds will be highlighted in this review. In general, etanercept and certolizumab pegol have comparable PK

characteristics, but relevant differences between the therapeutics will be discussed.

All approved mAbs are intravenously (iv) or subcutaneously (sc) administered immunoglobulins of the IgG family. These exogenous IgG molecules are generally eliminated by the same mechanisms as their endogenous counterparts; both target-mediated drug disposition (TMDD) and nonspecific pinocytosis and endocytosis have been described to contribute to the nonlinear and linear elimination of mAbs, respectively, eventually leading to proteolysis of the mAb. Pinocytosis and endocytosis result in internalization of IgG molecules by fluid endocytosis or FcγR-mediated uptake, respectively, and contribute to the linear component of mAb clearance (71). However, not all immunoglobulins will be degraded directly after they have been taken up by a cell; IgG molecules can be recycled via the neonatal Fc receptor (FcRn receptor). Once this intracellular receptor has bound to an IgG molecule it will prevent its degradation by transporting the immunoglobulin back to the cell surface. In FcRn knockout mice models IgG is eliminated 10–15 times faster, indicating that this recycling mechanism contributes to the relative long half-life of both endogenous and exogenous immunoglobulins (68). In the presence of high IgG levels saturation of the FcRn receptor has been described, resulting in nonlinear clearance. However, in general such high levels will not be reached; mAbs are administered at doses of <10 mg/kg and this will increase the total IgG level by <1–2% (71). The charge of the variable fragment of IgG antibodies also affects the clearance of mAbs via FcRn-mediated recycling. The charge can affect binding of the IgG molecule to the FcRn receptor and thus alters the half-life of the IgG molecules (72).

Binding of a mAbs to its target generally increases the clearance of a mAb nonlinearly, and this process is referred to as TMDD. In theory, higher amounts of target result in a faster clearance of mAbs. However, population pharmacokinetic modeling has not been able to demonstrate the effect of amount of target, i.e., TNF, on the clearance of TNFi. This might partly be explained by the fact that TNF levels are difficult to quantify (73), which is why disease status is regularly used as a proxy of amount of target. However, disease status is not very specific, and measurements are often subjective (74). Another possible explanation is that patients receive an excess of TNFi relative to the amount of TNF (74, 75). The effect of TMDD will only be observed when the concentration of the mAb is in the same concentration range as the target (41, 68). This is not the case for TNFi; serum concentrations of TNFi are, even at trough level, much higher than TNF levels in serum. Therefore, binding of a TNFi to its target will barely contribute to the clearance of TNFi (42, 73, 76). This is different for tocilizumab, which is a monoclonal antibody that targets the membrane-bound IL-6 receptor (IL-6R). It is the only bDMARD in which non-linear clearance has been detected. The PK of tocilizumab is mainly influenced by systemic IL-6 receptor (IL-6R) binding (76), for which CRP is commonly used as a surrogate marker (77). This can be explained by the fact that the liver can express a lot of IL-6R, resulting in a low tocilizumab concentration relative to the amount of IL-6R expression.

In contrast to target binding, binding of ADA to TNFi can alter elimination rates significantly. Increased clearance due to ADA may also be classified as target-mediated drug elimination. Ternant et al. specified that in the presence of detectable ADA, the clearance of adalimumab increases 5.5-fold at the population level (76). Berends et al. estimated an average 4-fold increase in clearance of adalimumab in the presence of detectable ADA (78). ADA detection thus seems to be one of the most important contributors to the pharmacokinetic variability seen for TNFi (42).

Compared to the pharmacokinetics of mAbs, some differences should be highlighted when evaluating TNFi with another structure. For example, certolizumab pegol is a PEGylated Fab' fragment that is derived from an anti-TNF humanized mAb. The conjugation of certolizumab to hydrophilic polyethylene glycol (PEG) chains increased the half-life of certolizumab pegol to around two weeks (76). Its clearance is somewhat different due to the absence of an Fc-tail, preventing FcRn-mediated recycling. In addition, renal excretion of the Fab' fragments has been described due to the relative small size of certolizumab (76). Certolizumab is, like the mAbs described earlier, an immunogenic molecule; anti-certolizumab antibodies can be detected in 37–65% of the patients. ADA detection is associated with lower drug levels over time, but high certolizumab levels (>10 µg/ml) could still be measured in most ADA-positive patients (79, 80).

Another bDMARD with a slightly different structure is etanercept, which is a dimeric fusion protein consisting of two p75 TNF receptors and an Fc part of an IgG molecule. Etanercept is the least immunogenic TNFi; most studies did not detect any relevant ADA toward the fusion protein (81–83). Dore et al. detected ADA toward etanercept in around 6% of the patients, but the association between ADA and PK was not investigated (84). It is therefore quite surprising that the half-life of etanercept is relatively short, with a mean ± standard deviation of 102 ± 30 h. One possible explanation is that etanercept has a lower affinity for the FcRn receptor, reducing FcRn-mediated recycling.

Population Pharmacokinetic Modeling

Population pharmacokinetic modeling allows us to identify sources of variability in PK within study populations and also provides us with the tools to quantify these effects (41). Regarding the covariates in population PK models, the result that is quite consistent among all studies is that the detection of ADA toward a TNFi significantly increases clearance rates of the TNFi, resulting in a decrease in drug levels. This has mainly been observed for infliximab, adalimumab, golimumab, and certolizumab pegol (76). Most PK studies have included ADA detection as a dichotomous variable, but due to the highly variable nature of ADA this will probably not correctly explain all variability in clearance due to ADA. However, since continuous ADA level measurements can also be difficult to interpret, other methods to quantify the effect of ADA on clearance might be useful.

An indirect method that can be used to quantify the effect of ADA detection on clearance of therapeutic antibodies is to compare the PK of endo- and exogenous IgG antibodies. In general, the PK of these molecules is quite similar, which is reflected in clearance rates. The clearance of endogenous

IgG is estimated to be 0.21 L/day, while several PK studies described a median clearance of therapeutic mAbs of 0.31 (0.066–0.535) L/day. The lowest clearance rates were described for denosumab, the highest for efalizumab, which can partly be explained by differences in their molecular structure and immunogenic potential (41). Antibody formation toward exogenous IgG molecules might explain why the clearance rates of these therapeutic proteins is higher compared to endogenous IgG molecules (41, 70). The difference in clearance rates of endo- and exogenous IgG molecules could then be used to quantify the effect of ADA formation on clearance of therapeutic proteins.

Another method that might be used to demonstrate the effect of ADA formation on serum drug levels is to compare data from patients that do and do not use concomitant methotrexate. It is known that use of concomitant methotrexate significantly increases drug levels of several TNFi (55), and this effect is probably mediated by a decrease in ADA formation. The difference in drug level between patients that do and do not use methotrexate therefore potentially reflects the effect of (undetectable) ADA formation on clearance of the TNFi.

Eventually, PK models might be able to estimate the amount of ADA that is being produced in a patient based on drug levels. If other covariates are known and corrected for, a PK model can potentially estimate the effect of ADA formation on parameters like clearance and volume of distribution. The PK of the therapeutic protein would then be used as a marker for immunogenicity.

CLINICAL IMPLICATIONS OF ADA: IMPAIRED RESPONSE AND ADVERSE EVENTS

All TNFi trigger an immune response, but this response will not always have clinical implications. Usually only those patients with high ADA titers will experience a diminished clinical response or adverse events. These clinical implications will be discussed.

Impaired Response

Previous studies have demonstrated a diminished clinical response of TNFi due to the development of ADA. Three meta-analyses have shown that the presence of ADA reduces the number of patients that reach clinical response (17, 18, 85). ADA reduce clinical efficacy by neutralizing the drug, preventing it from binding to TNF, and by enhancing the clearance rate due to formation of complexes. Many of the published studies investigating the clinical relevance of ADA used drug-sensitive assays, meaning that ADA can only be detected in the absence of drug, and such studies report strong associations with ADA and loss of response. In studies using drug-tolerant assays the association between the presence of ADA and clinical inefficacy is much weaker as ADA detection is independent of the drug concentration. This is demonstrated for RA patients treated with adalimumab, IBD patients treated with infliximab and for MS patients treated with natalizumab (25). The clinical effects of ADA formation are dependent on the amount of drug that is

neutralized by ADA, and the amount of free drug present. Drug-sensitive assays as a rule will correlate with the amount of free drug, whereas drug-tolerant assays will not. This is why it is important to report the quantity of ADA within patients, next to the percentage of patients that develop ADA.

The amount of administered drug correlates with serum drug levels and these drug levels correlate with the therapeutic effect. Several studies have explored the relationship between drug level and treatment response. These studies, focusing on several different autoimmune diseases, demonstrated that good responders in general have higher drug levels compared to non-responders or moderate responders (54, 86). Interestingly, there is evidence for an upper bound above which no additional clinical benefit is achieved; Pouw et al. described a concentration-effect curve of adalimumab which demonstrated that serum drug concentrations ranging between 5–8 mg/L are sufficient for an adequate clinical response in RA (87). Similar therapeutic ranges have been reported for psoriasis and IBD (88–90). The concentration-effect curves indicate that drug levels exceeding the upper limit of the therapeutic range do not lead to additional improvement of disease activity. Recently published data suggests that even very low drug levels (serum adalimumab concentrations around 0.1–0.5 mg/L) might be sufficient to control TNF (23). A possible explanation for the difference between the earlier mentioned therapeutic range of 5–8 mg/L and recently published data could be that higher drug levels are required during the initial phase of treatment compared to later treatment phases. This may be partly linked to the formation of a (transient) immune response in the early treatment phase. PK data from the pharmaceutical dose finding studies could help to get more insight in the relationship between drug levels and response during different treatment phases.

Even in the presence of ADA, drug levels can be sufficiently high to reach clinical remission. Clearance of the drug will be affected by these ADA since it becomes non-linear in the lower serum level ranges due to target-mediated drug disposition. Despite this decrease in half-life of the (unbound) TNFi, these lower levels could still contribute to clinical remission. Serum drug level measurements are thus crucial to get insight in the clinical relevance of ADA formation; ADA measurements cannot be interpreted correctly without the context of a drug level measurement.

Adverse Events

Besides their ability to reduce drug levels and contribute to clinical inefficacy, the presence of ADA is also associated with adverse events. Severe reactions have been described after the formation of ADA toward therapeutic proteins resembling endogenous proteins. This is exemplified by the formation of ADA toward recombinant human erythropoietin (rhEPO). ADA against rhEPO were demonstrated to cause pure red cell aplasia due to cross-reactivity with endogenous erythropoietin (91). Although TNFi share epitopes with endogenous IgG (in case of the antibody based TNFi) and the TNF-receptor (etanercept), no cross-reactivity toward the endogenous counterparts have been described so far. However, other adverse events related to TNFi have been described.

Infusion reactions are the most common ADA related adverse event described for TNFi. They are characterized by symptoms such as fever, pruritus, bronchospasms, or cardiovascular collapse during or within the first day after drug administration (62, 92, 93). The reported incidence rate of infusion-related reactions after administration of infliximab varies between 4–15% (31). The presence of ADA to infliximab is associated with a higher risk of infusion reactions (58, 94–96). A meta-analysis by Maneiro et al. showed a 2 to 4-fold higher risk of infusion reactions in ADA-positive patients compared to ADA-negative patients (18). Approximately the same numbers were described in the meta-analysis of Thomas et al. (17). This association has also been observed for natalizumab (62) and other therapeutic monoclonal antibodies applied in oncology (97). However, the fact that the majority of patients treated with monoclonal antibodies develop ADA and only a minority of these patients develop adverse events such as infusion related reactions, indicates that ADA often do not cause an infusion reaction. Even when the presence of ADA contributes to clinical inefficacy, these ADA do often not increase the risk of developing an infusion reaction. Factors that do seem to contribute to incidence of adverse events are size and shape of TNFi-ADA complexes. Several groups have studied the size of these complexes. The majority of the complexes are dimers (one therapeutic antibody bound to one TNFi) (40). However, under specific conditions larger complexes (tetramers, hexamers, etc.) can be found as demonstrated by Rojas et al. in monkeys treated with infliximab (98) and in study of van Schie et al. in sera of patients with anti-infliximab antibodies (40). The size of these TNFi-ADA complexes depends on the ADA titer and the drug/ADA ratio. Very large complexes are formed in presence of high ADA titers and when the drug/ADA ratio is around 1:1 (40).

Multimerization of antibodies can activate the complement cascade via C1. Antibody multimers, interacting through their Fc tails and forming complexes with their Fc tail close together and pointing inwards, serve as an optimal platform for C1 to bind to and to activate complement cascade (99, 100). ADA toward therapeutic antibodies are anti-idiotypic, so ring shaped complexes are formed in which the Fc-tails point outwards instead of toward each other. However, if very large, irregularly shaped complexes are formed, Fc-tails may get closer to each other, allowing to bind C1q and activate complement cascade (40). This might explain why large and irregular shaped complexes are able to activate complement cascade. A study of van der Laken et al. (101) support this finding. In this study radiolabeled infliximab was infused in three patients with ADA. Patients with small complexes did not experience any adverse events. In contrast, one patient who had developed large complexes experienced a severe infusion reaction (101). Luckily, usually only the minority of TNFi-ADA complexes are large and irregularly shaped; these tend to be formed only at high ADA concentration. This could be due to a more rapid and effective phagocytosis and clearance of larger complexes by macrophages. Internalization of small complexes are much less efficient, which is consistent with the previous observation that dimeric complexes persist in the circulation for an extended period of time. The low incidence of severe

infusion related reactions can be explained by the fact that the majority of TNFi-ADA complexes are small non-immune activating complexes (40).

Infusion related reactions can range from mild (e.g., rash, pruritus, dizziness, dyspnea) to severe (anaphylactic-like reactions such as hypotension and respiratory distress). As the latter group resembles a type 1 allergic reaction, it is thought to be IgE mediated. However, a study by van Schie et al. suggests that the majority of infusion related reactions may be IgG mediated (31). Only 11% of patients with infusion reactions had detectable IgE ADA (31). Similar results has been demonstrated in other studies (102, 103).

In addition to infusion reactions, one study describes association between thrombo-embolic events and presence of ADA to adalimumab (104). However, these findings are not validated in other studies. In this study patients with ADA had a more active disease status than patients without ADA. Thus, activation of coagulation cascade could be due to systemic inflammation instead of presence of ADA.

MITIGATION STRATEGIES

Concomitant Use of Immune Modulating Drugs

Immunosuppressive medication is often used in combination with TNFi to decrease ADA formation. Previous studies have demonstrated that concomitant use of immunosuppressant's reduces immunogenicity. Patients using concomitant methotrexate have lower rates of ADA development (17, 18, 85) and higher drug trough concentrations (56, 75, 105). These findings were validated in three meta-analyses. However, the reduction in risk to experience clinically relevant ADA development varies between the studies and different autoimmune diseases. The aforementioned could encourage coadministration of methotrexate in other autoimmune diseases, even when use of methotrexate is not part of the standard treatment regimen.

The mechanisms underlying the beneficial effect of methotrexate on immunogenicity and clinical response of most inflammatory diseases is still not fully elucidated. One hypothesis is that methotrexate reduces TNF levels and due to reduced target-mediated drug disposition, it contributes to higher TNFi concentrations and an improved clinical response (56, 76). However, given the high quantity of TNFi compared to TNF, a reduction in TMDD does not seem to be a plausible explanation for reduced clearance of TNFi. Like mentioned before, effect of TMDD is only noticeable when the ratio target/drug is low (64, 70, 81). A second hypothesis is that methotrexate might suppress early B- and T-cell responses leading to modulation of the immune response (69). Considering the significant role of T- and B- cells in the classical immune paradigm, this seems to be a more plausible explanation. A third hypothesis is that methotrexate reduces FcγR levels, which might result in reduced clearance of TNFi (41, 70).

Some observational studies performed in patients with inflammatory bowel disease showed that concomitant use of

azathioprine and glucocorticoids reduced the incidence of ADA detection (18). However, conflicting results have been found in other studies, which is why there is still some uncertainty about the effect of these drugs regarding reducing immunogenicity.

A therapeutic protein that could theoretically interfere with the immune response toward TNFi is abatacept. Abatacept prevents T-cell activation by binding to CD80/86. Just like T-cells and costimulatory signals, CD80/86 is required for differentiation of memory B cells to plasma cells and for an effective interaction between antigen presenting cells and T-cells. Therefore, blocking one of these processes might prevent alloimmunization to therapeutic proteins. However, trials focusing on concomitant use of abatacept and TNFi did not demonstrate an improved clinical response and led to a higher infection rate (106, 107).

In hemophilia A patients with inhibitors toward FVIII, intravascular administration of immunoglobulins (IVIg) results in the suppression of inhibitors because IVIg contains anti-idiotypic antibodies toward the inhibitors (108). Combination therapy with cyclophosphamide or rituximab have also been investigated to reduce immunogenicity in hemophilia A patients. Concomitant use of IVIg and cyclophosphamide can be challenging due to technical difficulties and potential toxicity. However, concomitant rituximab, which inhibits B-lymphocytes and interferes with IgG production, seem to be promising (109, 110). To our knowledge, concomitant use of rituximab in inflammatory disease has not been studied yet. Due to an increased risk of infections, this approach might be controversial.

Dose and Dosing Regimen

As early as in 1998, Maini et al. suggested that immunogenic tolerance can be induced by higher dosages of infliximab (111). Since then, this has been supported by several other studies that have been performed in rheumatoid arthritis and inflammatory bowel disease (17, 85). The rationale behind this is that this higher infliximab dose results in higher levels of free drug compared to the amount of drug that is neutralized by ADA. Higher infliximab doses might also accelerate ADA clearance due to formation of complexes (112). Yet, this approach is not generally applied due to the higher risk of adverse events and higher costs. Especially in the field of rheumatic diseases several alternative therapeutic proteins are available. Consequently, switching to another therapeutic protein is generally preferred over a dose increase.

In IBD the initial infliximab maintenance dosing was episodic rather than scheduled. However, soon thereafter a scheduled regimen was preferred over episodic treatment strategies, as scheduled regimens resulted in lower risk of ADA development and better response (16). Patients undergoing a scheduled treatment strategy have higher trough levels, which is associated with better response rates. Similar results have been seen in adalimumab - and certolizumab trials. Due to the higher rates of ADA formation in episodic treatment regimens, is the rate of infusion reactions higher after infliximab infusion (113–115).

The above-mentioned observations are in line with the studies that have focused on the induction of immune tolerance in hemophilia A patients. Higher levels of the therapeutic protein might inhibit the reactivation of memory B-cells and prevent

them to differentiate to ADA producing plasma cells. In addition, chronic exposure to the therapeutic proteins might lead to induction of T-regulatory cells. These cells are involved in peripheral tolerance and possibly also in evolution of the immune response (67).

Therapeutic Drug Monitoring

Considering the large variation in the natural course of inflammatory diseases and the pharmacokinetics of TNFi, a personalized treatment approach would be more appropriate than a “one size fits all” approach. Therapeutic drug monitoring (TDM) might be able to facilitate this. By combining clinical data regarding disease activity and the PK of the TNFi, the drug could be applied more efficiently. Especially in case of a diminished clinical response, TDM might be able to guide clinical decision making. For example, in RA, where TNFi have failed, two treatment approaches are available; switching to a second TNFi or switching to a therapeutic with a different mode of action. The EULAR recommendations for the management of RA advocate that any biologic agent, including a second TNF-inhibitor, can be used in case of refractory response to a previous TNFi (116). In clinical practice, switching to another therapeutic agent is often a random decision and based on clinician’s preference and local preference policy. TDM could improve this process by identifying subgroups of patients who would benefit more from either a second TNFi or a non-TNFi as subsequent treatment. It has been shown that a good response to a second TNFi can be anticipated in patients that lose response to their first TNFi due to ADA formation. However, loss of clinical response to the first TNFi in the absence of ADA predicts a less effective response to a second TNFi (43, 52, 53). In this group of patients drug levels are usually sufficient to control all TNF. Therefore, TNF might not be the main cytokine provoking or perpetuating disease activity and these patients might benefit more from a switch to a biological with a different mode of action. Thus TDM, or measurement of drug levels by itself, could help to make better treatment decisions, taking immunogenicity into account.

CONCLUSION

TNFi have significantly improved the management of various immunological disorders. Unfortunately, a substantial fraction of patients needs to discontinue treatment due to ineffectiveness of the drug or due to adverse reactions. Both can in part be related to the development of ADA.

Historically, the majority of studies focusing on immunogenicity used drug-sensitive assays which, in general, underestimate the amount of ADA present in the serum due to drug interference. Following the introduction of drug-tolerant assays (i.e., where presence of drug does not, or to a lesser extent, interfere with the measurement of ADA) it was found that many patients treated with TNFi, except for etanercept, develop ADA. Detection of ADA can be associated with a reduction in clinical efficacy, but this association is dependent on the type of ADA assay that is used. Importantly, reduction in clinical efficacy is primarily related to an inadequate drug level. ADA measurements should therefore

be interpreted in the context of the assay format that was used to measure ADA and a drug level measurement. In practice, the distinction between binding and neutralizing antibodies could be omitted since almost all ADA are neutralizing. Reported lower fractions of neutralizing antibodies can in most cases be attributed to lack of sensitivity of the assay used for detection.

Several patient- and treatment-related risk factors for ADA formation are being described in this review. One example being the presence of certain high-risk HLA-alleles as an important patient-related risk factor. ADA affect the PK of the drug by increasing clearance of TNFi. This becomes clinically relevant when drug levels are decreased to the point that no longer all TNF is bound. Another potential effect of ADA formation is the development of ADA-TNFi complexes which can induce hypersensitivity reactions.

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Engineering of Fc Multimers as a Protein Therapy for Autoimmune Disease

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The success of Intravenous Immunoglobulin in treating autoimmune and inflammatory processes such as immune thrombocytopenia purpura and Kawasaki disease has led to renewed interest in developing recombinant molecules capable of recapitulating these therapeutic effects. The anti-inflammatory properties of IVIG are, in part, due to the Fc region of the IgG molecule, which interacts with activating or inhibitory Fcγ receptors (FcγRs), the neonatal Fc Receptor, non-canonical FcRs expressed by immune cells and complement proteins. In most cases, Fc interactions with these cognate receptors are dependent upon avidity—avidity which naturally occurs when polyclonal antibodies recognize unique antigens on a given target. The functional consequences of these avid interactions include antibody dependent cell-mediated cytotoxicity, antibody dependent cell phagocytosis, degranulation, direct killing, and/or complement activation—all of which are associated with long-term immunomodulatory effects. Many of these immunologic effects can be recapitulated using recombinant or non-recombinant approaches to induce Fc multimerization, affording the potential to develop a new class of therapeutics. In this review, we discuss the history of tolerance induction by immune complexes that has led to the therapeutic development of artificial Fc bearing immune aggregates and recombinant Fc multimers. The contribution of structure, aggregation and N-glycosylation to human IgG: FcγR interactions and the functional effect(s) of these interactions are reviewed. Understanding the mechanisms by which Fc multimers induce tolerance and attempts to engineer Fc multimers to target specific FcγRs and/or specific effector functions in autoimmune disorders is explored in detail.

Keywords: IVIG—intravenous immunoglobulin, Fc multimer, autoimmune, FcγR, complement

INTRODUCTION

Immunoglobulin (IVIG) is approved as a therapeutic for chronic autoimmune and inflammatory processes such as immune thrombocytopenia purpura (ITP) and Kawasaki disease, among others (1). However, IVIG is expensive to produce, has blood borne pathogen risks, toxic side effects, and because it is pooled from plasma from thousands of human donors, there is both a lack of consistency among preparations (2–5) and intermittent supply shortages. There is a critical need to develop recombinant therapeutics that reproduce the anti-inflammatory effects of IVIG. One

of the major mechanisms by which IVIG exerts anti-inflammatory properties is through the Fc domain of the IgG molecule (6). The Fc fragment has important biological effector functions that are controlled by IgG isotype, aggregation, interactions with FcγRs and complement components. Identifying the mechanisms by which these factors contribute to the protective effect of IVIG is critical to the development of novel IVIG replacement therapies.

STRUCTURE OF IgG AND FC FRAGMENT

The immunoglobulin molecule (IgG) consists of two identical light chains and two identical heavy chains that can be divided into two proteolytic fragments; the antigen-binding fragment (Fab) and the Fc fragment consisting of the C_H2 and C_H3 regions of the heavy chain (**Figure 1**). The Fc fragment mediates effector functions of IgG such as antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC), through the binding of soluble and cell-surface proteins to distinct residues within the C_H2 and C_H3 domain. The classical FcγRs bind to residues near the hinge region in C_H2 and have a partial overlap with the site of C1q binding (12–17). Additional Fc binding proteins such as the neonatal Fc receptor (FcRn) and Tripartite motif-containing 21 (TRIM21) bind to residues within

both the C_H2 and C_H3 region (18, 19). There is considerable heterogeneity in Fc glycosylation and the amino acid sequences of hinge regions between different IgG isotypes, all of which affect the binding affinity of IgGs to FcγRs and consequent effector function.

Glycosylation

Within the C_H2 portion of the Fc fragment is a conserved N-linked glycosylation site at Asn-297, which consists of a mannose and N-acetylglucosamine (GlcNAc) core structure and varying glycan species including fucose and galactose that may have added sialic acid residues (20). The N-linked glycan, N297, is necessary for stabilization of the Fc fragment and binding to FcγRs and its removal abolishes Fc effector functions (7). Changes in the patterns of N-linked glycosylation are recognized to affect the biologic properties of antibodies (21–24). Particularly germane to this review, are studies suggesting that α 2,6 sialylated Fc fragments are responsible for all of the anti-inflammatory properties of IVIG through engagement of the C-type lectin DC-SIGN (25). In support of this theory, several reports now suggest that the degree of circulating sialylated antibodies correlates with the activity of specific autoimmune and inflammatory conditions in humans (26–28). However, a large cohort of other studies clearly refute the import of α 2,6 sialylated Fc fragments in

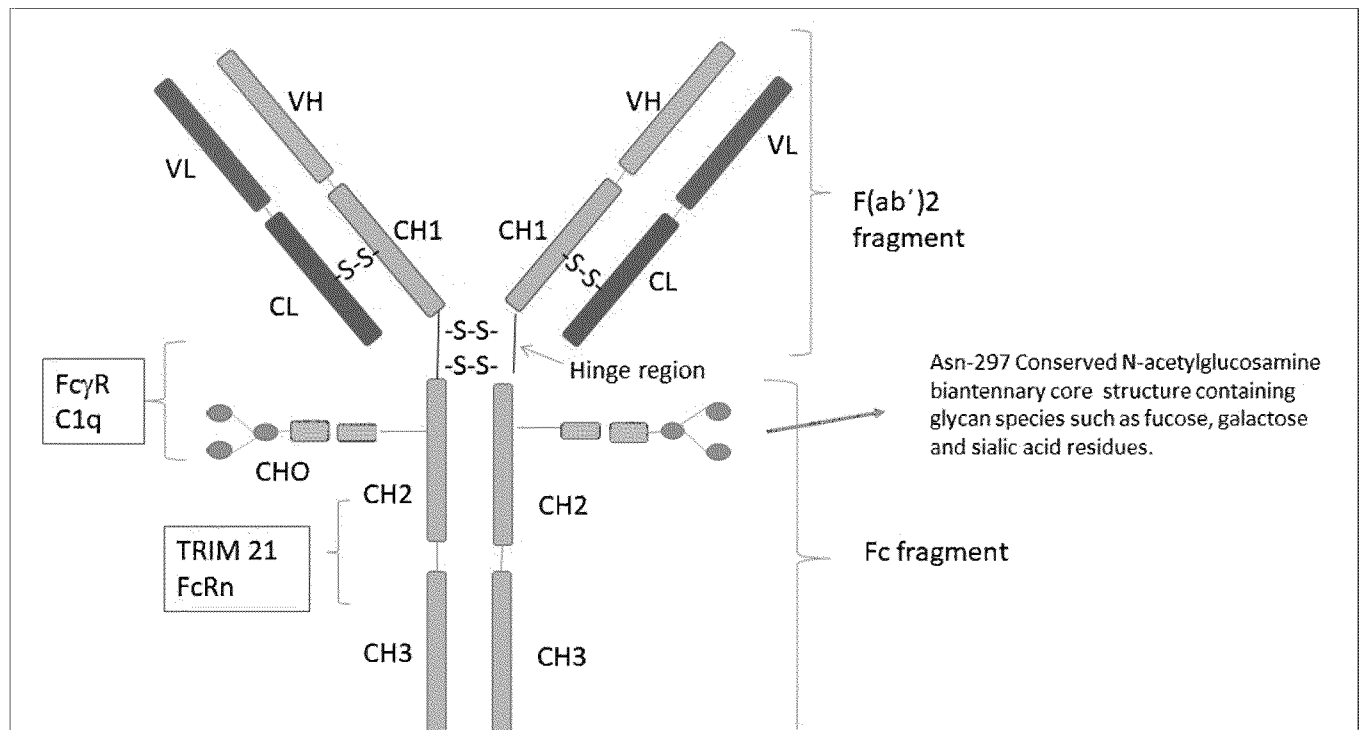


FIGURE 1 | Structure of IgG1. Human IgG consists of four subclasses (IgG1–IgG4) that differ in the number of disulfide bonds in the hinge region, amino acid residues throughout the constant chain as well as their glycosylation patterns. Although the conserved N-linked glycosylation site at Asn-297 is necessary for stabilization of the Fc fragment and its removal abolishes Fc effector functions of IgG (7–9), it is not absolutely required for the anti-inflammatory activity of Fc multimers. Residues near the lower hinge region, C_H2 and C_H3 are involved in binding to FcγRs and C1q. The FcRn and TRIM21 bind to residues within both the C_H2 and C_H3 region. The majority of studies with recombinant Fc proteins for use as replacement IVIG have focused on introducing sequences that allow for aggregation of the recombinant Fc molecules, for example addition of a IgG2 hinge region as a multimerization domain to the IgG1 Fc (10, 11).

the function of IVIG mediated tolerance, showing that these fragments do not bind DC-SIGN and that removal of α 2,6 sialylated Fcs does not impact the anti-inflammatory properties of IVIG (29–32). As such, the role of α 2,6 sialylated Fcs in the tolerogenic properties of IVIG remains the subject of ongoing scientific debate.

FC γ R'S

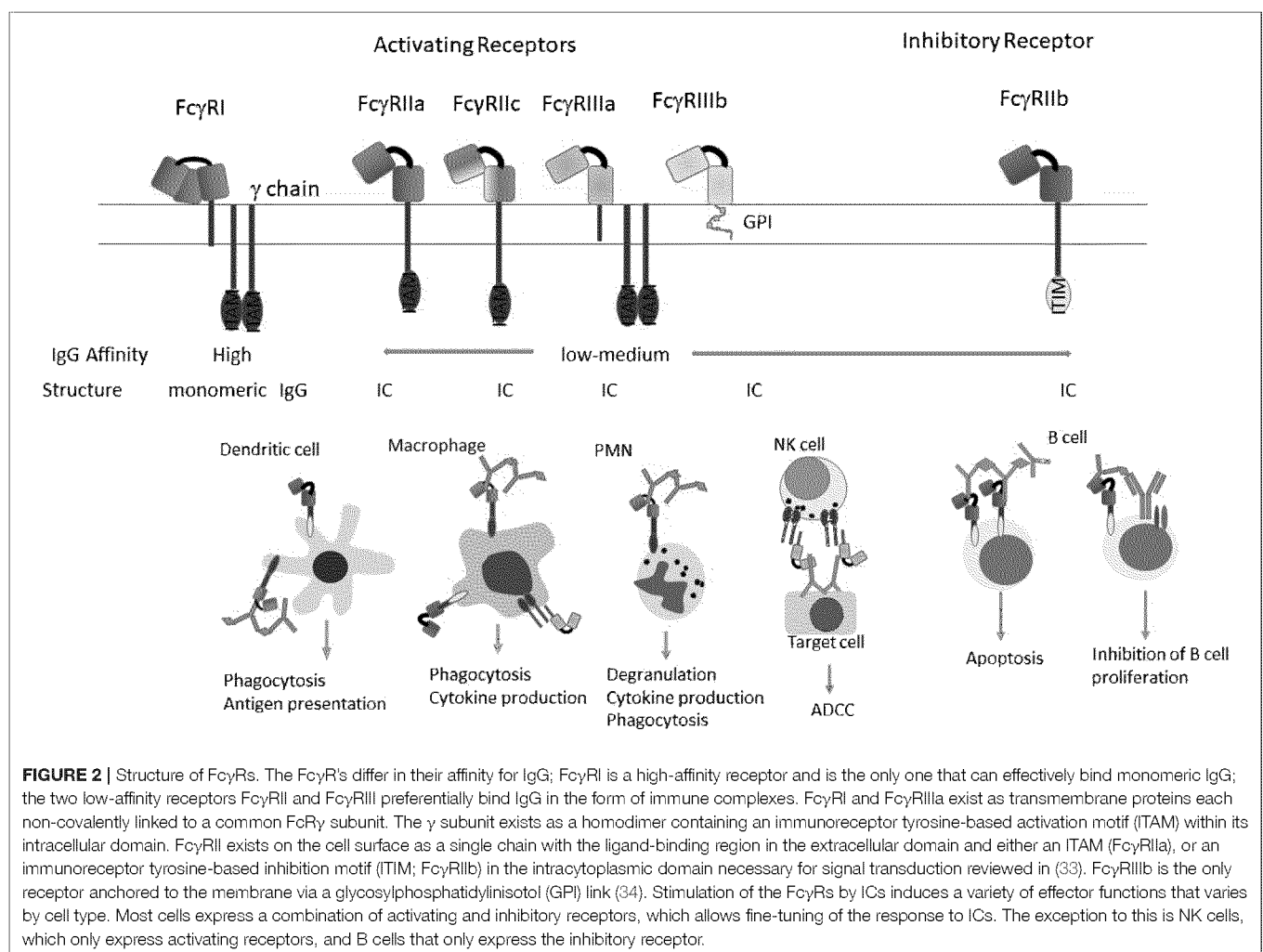
The classical Fc γ Rs are cell membrane associated proteins expressed on a variety of immune cells such as macrophages, dendritic cells (DCs), natural killer (NK) cells, neutrophils, and B cells. In humans, there are three types of Fc γ Rs: hFc γ RI/CD64, hFc γ RII/CD32, and hFc γ RIII/CD16, that are grouped based on structural homology (Figure 2). The Fc γ RII and Fc γ RIII subfamilies are further subdivided into Fc γ RIIa (CD32a), Fc γ RIIb (CD32b) and Fc γ RIIc (CD32c) and Fc γ RIIIa (CD16a) and Fc γ RIIIb (CD16b). From a conceptual perspective, the functional differences in these individual Fc γ Rs are based on their different affinities for the naturally occurring Fc fragment, their inducibility, their patterns of cellular expression, their ability to

mediate internalization of immune complexes and the pathways through which they signal [reviewed in (33, 35, 36)].

OTHER IgG FC BINDING LIGANDS

Complementing the classical Fc γ Rs, are a relatively diverse set of non-canonical Fc γ Rs which contribute to the functions of antibody homodimers and Fc bearing ICs. For instance, the neonatal FcR (FcRn) is an MHC Class I-like molecule that is associated with β 2-microglobulin and is responsible for IgG half-life as well as other functions such as transferring IgG from the mother across the placenta to the fetus [reviewed in (37–43)]. Similarly, intracellular receptor Tripartite motif-containing 21 (TRIM21) appears to play a role in neutralization of antibody decorated pathogens (19, 44–48)]. Finally, FcRL5 is a cell surface protein expressed on B cells and able to bind all IgG subclasses (49, 50). It has two ITIM and two ITAM motifs within its cytoplasmic domain suggesting that it can induce inhibitory or activating signals (51).

In addition to these cell-based receptors, soluble proteins such as complement can also engage multimerized/aggregated Fc. For



instance, C1q is a hexamer composed of two trimers containing an A, B, and C chain each with collagen-like stalk portions and globular heads resulting in the characteristic “bundle of six tulips” (52). Binding of C1q by ICs results in the engagement of C1r and C1s and subsequently activation of the classical complement cascade. (53–56). Importantly, and as discussed in later sections, unlike the scenario in which C1q binds an antibody decorated cell and induces C5b-9 pore formation, IC mediated activation of the classical cascade may occur away from the cell surface, resulting in a poorly defined series of immunomodulatory effects.

CONCEPTUAL BASIS FOR THE DEVELOPMENT OF FC MULTIMERS TO INDUCE TOLERANCE

The conceptual basis for developing Fc multimers as a tolerogenic therapy is based on the anti-inflammatory role of the Fc fragment in IVIG. However, there is historical evidence that ICs possess anti-inflammatory properties in addition to their well-described pro-inflammatory effects. Careful observational studies by Flexner in 1906, introduced the concept of tumor enhancement, where heated tumor emulsions administered 10 days prior to tumor implantation, augmented the growth of subsequent tumor implants in rats (57). Although any role of IgG in these earlier studies is unclear, the phenomenon of tumor enhancement has been observed following passive transfer of anti-serum prior to tumor inoculation (58). Studies by Kaliss et al. (59–61) demonstrated that transfer of serum from animals that had rejected a primary tumor to naïve animals potentiated subsequent tumor growth. The enhancing activity was found to be associated with the gamma-globulin fraction (62). Many mechanisms have been put forward to explain tumor enhancement by IgG including masking of tumor antigens by antibody (61, 63) or a shift in the Th1 to Th2 cytokine response favoring tumor growth (64), however FcγR dependent mechanisms such as inhibition of ADCC may also explain this phenomenon (63).

Clinical evidence supporting the potential therapeutic role of ICs in preventing disease is derived from the observation that treatment of ITP patients with anti-D antibodies results in an increase in platelet counts (65–67). The fact that treatment efficacy is dependent on patients being Rh⁺, suggests that these antibodies function by presenting aggregated Fc on the surface of Rh⁺ cells (68, 69). The observation that it requires at least 48 h for patients to respond—longer than the time necessary for saturation of the FcR—highlights the immunomodulatory nature of these effects (68).

The conceptual underpinnings for the idea that Fc multimers have anti-inflammatory properties are also founded on examples of naturally occurring multimeric Fc like proteins with tolerogenic properties. For instance, the short pentraxins are evolutionarily conserved precursors of existing antibodies, whose pentameric structure allows them to engage the low affinity FcγRs and complement (70). While initially recognized to bind and regulate the immune response to specific pathogens and apoptotic cells, recent data suggest that these proteins

also have profound anti-inflammatory activity [reviewed in (71)]. For example, serum amyloid P (SAP) inhibits many of the pro-inflammatory components of neutrophil function and also prevents the conversion of monocytes into fibrocytes, potentially mitigating fibrosis (72). Collectively, these studies provide both the historical context and biologic basis for employing recombinant multimerized Fc compounds to mimic the tolerogenic properties of the Fc portion of aggregates in IVIG. In addition, they force a re-evaluation of the concept that ICs only induce inflammation—suggesting that in some cases they may be induced in response to pro-inflammatory events as a means to restore immune homeostasis.

DEVELOPMENT OF RECOMBINANT FC MULTIMERS AS THERAPEUTICS

Based on the supposition that artificial Fc bearing immune complexes might induce tolerance, our laboratory in collaboration with Gliknik®, followed by several other groups, sought to develop fully recombinant IgG multimers for both clinical translation and mechanistic experiments. Specifically, we developed linked multimerization domain (MD) sequences from the hinge region of human IgG2 or the isoleucine zipper (ILZ) to the carboxy or amino termini of the murine IgG2a. The resultant stradomers™ contained both homodimers and highly ordered multimers of the Fc homodimers. One of these stradomers™, bearing the IgG2 hinge (M045), effectively binds to FcγRI, FcγRIIb and FcγRIII with significantly lower K_d values than control IgG2a Fc, inhibits the development of collagen-induced arthritis (CIA) and protects against platelet destruction. The fact that most of the therapeutic activity of this drug resides in the multimeric fraction, highlights the relative importance of avidity, rather than affinity, for its biologic activity (10). Collectively, these data provided the first evidence that recombinant immune complexes could induce tolerance.

Subsequent studies confirmed and extended these findings, demonstrating that stradomers™ can effectively inhibit development of experimental autoimmune neuritis model (73) and experimental autoimmune myasthenia gravis (EAMG) (74). Importantly, the studies in EAMG provided significant mechanistic insights, showing that daily administration of stradomers™ reduces Acetylcholine Receptor (AChR) antibody levels, decreases antigen specific T cell proliferation, down-modulates both B cell and DC maturation markers, up-regulates inhibitory FcγRIIb expression, and is associated with an increase in both Tregs and immunosuppressive cytokines such as IL-10 and IL-4 (74). We are attempting to distinguish the relative importance of the FcRs and complement on these biologics by employing complement preferential stradomers™ *in vitro* and *in vivo* (11, 75).

In order to translate our preclinical findings, we developed a human analog of these drugs, GL-2045, by joining the human IgG2 hinge region to the C-terminus of the human IgG1 Fc fragment (76). GL-2045 avidly binds human FcγRI, FcγRIIa, FcγRIIb and FcγRIIIa as well as to rat, mouse and cynomolgus monkey FcγRs, protects mice from platelet loss in a rodent ITP

model and inhibits CIA. Of perhaps greater import, GL-2045 infusion into healthy cynomolgus monkeys is well-tolerated and induces transient and highly ordered increases in IL-1RA and IL-10 as well as a temporary suppression of IL-8, without significant induction of proinflammatory cytokines (76).

Following our initial studies, several other groups reported that recombinant Fc multimers can ameliorate autoimmune disease, suggesting that some of the properties of these multimers might be generalizable. For instance, Mekhaie et al. (77) generated a hexameric Fc by joining the Fc portion of human IgG1 to an 18 amino acid sequence from the C-termini of the IgM μ -tailpiece with a leucine 309 to a cysteine mutation (78, 79). This compound exhibits greater affinity for the Fc γ R than IVIG and upon internalization, is associated with preferential degradation of the *activating* Fc γ Rs and protects mice from platelet loss for up to 3 days after dosing (80). Studies with analogous compounds demonstrate clinical efficacy in both CIA and in the K/BxN model of chronic arthritis (81). These data lend credence to the idea that structurally distinct ICs can have anti-inflammatory properties.

In order to better understand the relationship between IgG1 Fc valency/ structure on Fc γ R engagement/function, Ortiz et al. (82) evaluated the function of Fc multimers with increasing valency and observed that structures containing 2 and 3 Fc domains avidly bind Fc γ R, but unlike molecules containing 5 Fc domains, do not induce Syk phosphorylation or a calcium flux in macrophages. In addition, the larger structures are internalized along with Fc γ RII, whereas the smaller structures remain on the cell surface co-localized with Fc γ RII. Subsequent studies showed that the trivalent Fc (Fc3Y) competitively inhibits several IC mediated Fc γ R functions and protects mice from ITP (82). Importantly, given the valency of Fc3Y, the extent to which it can immunomodulate the complement cascade is uncertain. Collectively, these data support the idea that Fc bearing immune complexes may serve as a protective mechanism against inflammation and, as a corollary, that recombinant Fc multimers might have therapeutic value for the treatment of autoimmunity.

ANTI-INFLAMMATORY MECHANISMS OF RECOMBINANT FC MULTIMERS

The development of Fc multimers as a replacement for IVIG is a significant therapeutic advance. Importantly, like IVIG, these molecules likely function by numerous overlapping mechanisms, influenced by the number of IgG Fc domains presented to ligands, the IgG isotype, and the conformational flexibility of the aggregate or Fc multimers (76, 77, 82). Moreover, the relative activity is likely dependent upon the specific underlying disease, the state of maturation of immune cells in that disease and other prior treatments.

From a conceptual perspective, it is our hypothesis that recombinant ICs require and/or benefit from immune activation as a necessary precursor for the induction of tolerance. Specifically, given that multiple redundant immunologic pathways are in place to restore immune homeostasis, it is highly

possible, if not likely, that the initial inflammatory response induced by recombinant Fc multimers induces a compensatory response that restores immune homeostasis. In this regard, it is noteworthy that IVIG can be associated with initial fever, chills, headache, and transient release of pro-inflammatory cytokines (83, 84) and that hypotension with IVIG administration is related to the rate of infusion of aggregates (85). It is possible that patients receiving IVIG who experience these initial pro-inflammatory effects are the most likely to be subsequently induced into tolerance by the Fc aggregates in IVIG. While this hypothesis remains to be validated, it is conceptually helpful in understanding the potential links between the diverse immunologic alterations mediated by these drug candidates.

Biologic Decoys and Fc γ R Blockade

Immune complexes are associated with the development of autoimmune disease and resultant tissue damage. Because naturally occurring ICs are commonly generated around a pathogen or other type of foreign protein, it is difficult to separate the biologic effects of IC: FcR and/or complement engagement from those mediated by the target protein. Using ICs generated around single stranded DNA or RNA (ssDNA/RNA) as an example, the Fc:FcR interactions allow ssDNA/RNA access into the cell where they can drive TLR-mediated inflammation. Lacking ssDNA/RNA, recombinant Fc multimers competitively inhibit natural ICs from FcR engagement (76, 80, 86), preventing subsequent engagement of intracellular TLRs and potentially inducing other active inhibitory functions as a result of FcR stimulation in a non-inflammatory milieu.

Additionally, Fc γ R efficiently internalize ICs, which allows for processing and presentation of antigenic peptides to T cells, further amplifying an immune response. The lack of “core” antigen in the Fc multimer preparations is likely key to the ability of the multimers to block Fc γ R without inducing further inflammation.

FcRn Blocking

The FcRn has a documented role in antibody-mediated autoimmunity. Indeed, FcRn deficient mice are protected from serum transfer-induced arthritis (87). Doses of IVIG that resulted in saturation of the FcRn inhibited development of arthritis in mice lacking the inhibitory Fc γ RIIb (87). The FcRn plays a critical role in maintaining the plasma concentration of IgG such that high concentrations result in increased IgG clearance (88–91). Infusion of high dose IVIG results in a reduction of circulating autoantibody, which may be due in part to saturation of the FcRn and enhanced IgG catabolism (92–95), although it is likely other mechanisms such as the Fc γ RIIb engagement also play a role (96). It is likely that Fc multimer binding to FcRn may competitively inhibit engagement of circulating antibodies—pathogenic and otherwise—resulting in lysosomal degradation and non-specific decreases in antibody half-life. While many of the recombinant Fc multimers and hexamers bind FcRn *in vitro*, further studies are required to accurately characterize the role of the FcRn in mediating their anti-inflammatory activity.

Stimulation of the FcγRIIb Inhibitory Receptor

The FcγRIIb inhibitory receptor is the only FcγR expressed by B cells and crosslinking of the B cell receptor (BcR) and FcγRIIb suppresses B cell proliferation and activation (97, 98). In contrast, ligation of FcγRIIb alone may induce B cell apoptosis (99, 100). In addition to directly stimulating the inhibitory receptor on B cells, ICs and IVIG also induce its expression on both B cells and myeloid cells (25, 101, 102). Myeloid cells express both inhibitory and activating receptors and the ratio of inhibitory to activating receptor stimulation will dictate the outcome of signaling. Therefore, IVIG or Fc multimer induced FcγRIIb upregulation may favor inhibitory signaling pathways over the activating pathways.

Stimulation of Activating FcγRs

The suppressive effects of IVIG (and subsequently Fc multimers) may also be mediated by stimulating the activating receptors. For example, Park-Min et al. (103) demonstrated that IVIG suppresses IFNγ mediated phosphorylation of Stat1 and IFNγ-dependent gene expression in macrophages *in vitro*. *In vivo*, IVIG treatment of mice infected with *Listeria monocytogenes* results in increased bacterial burden and decreases expression of IFNγ-dependent genes IP-10 and MIG. This effect is FcγRIII dependent, as ICs do not inhibit IFNγ signaling in B cells (which only express FcγRIIb), inhibit signaling in NK cells and DCs that express FcγRIII, but fail to inhibit signaling in macrophages deficient in FcγRIII (103).

Stimulation of activating receptors may also modulate the production of anti- and pro-inflammatory cytokines. For instance, ICs induce the production of anti-inflammatory cytokines such as IL-10 (104) and suppress IL-12 production (105, 106). Ligation of the FcγR on macrophages during stimulation with LPS results in a decrease in IL-12 production and an increase in IL-10 compared to LPS stimulated controls, whereas other cytokines remain unchanged (106). The inhibition of IL-12 production is not due to the expression of IL-10 as IL-10^{-/-} macrophages also exhibit decreased IL-12 production following FcγR ligation and LPS stimulation. Taken together, these studies suggest that stimulation of the activating receptors by IVIG or Fc multimers, in the absence of pro-inflammatory signals, may result in induction of tolerance.

Expansion of Regulatory T Cells

Patients undergoing IVIG treatment exhibit an expansion in Tregs which may contribute to its therapeutic effects (107–109). Fc multimers are recognized to cause similar increases in Tregs, with both GL-2045 and IVIG inducing Treg expansion in the EAMG model (74). An intriguing mechanism by which IVIG (and Fc multimers) may induce Tregs is the presence of highly conserved Tregitopes in the C_H2 domain of the Fc fragment of human IgG (110). Upon uptake by antigen presenting cells, Tregitopes bind with high affinity to HLA molecules and cause the activation and expansion of Treg cells (111). Additional potential mechanisms for IC mediated Treg expansion include the induction of tolerogenic DCs (112–114). For instance, Trinith et al. (115) demonstrated that *in vitro*,

IVIG pre-treatment of DCs results in the differentiation and expansion of Tregs that is dependent on COX-2 induced PGE₂ production (115). Collectively, these data suggest that IVIG and ICs have the potential to induce Tregs through multiple independent pathways.

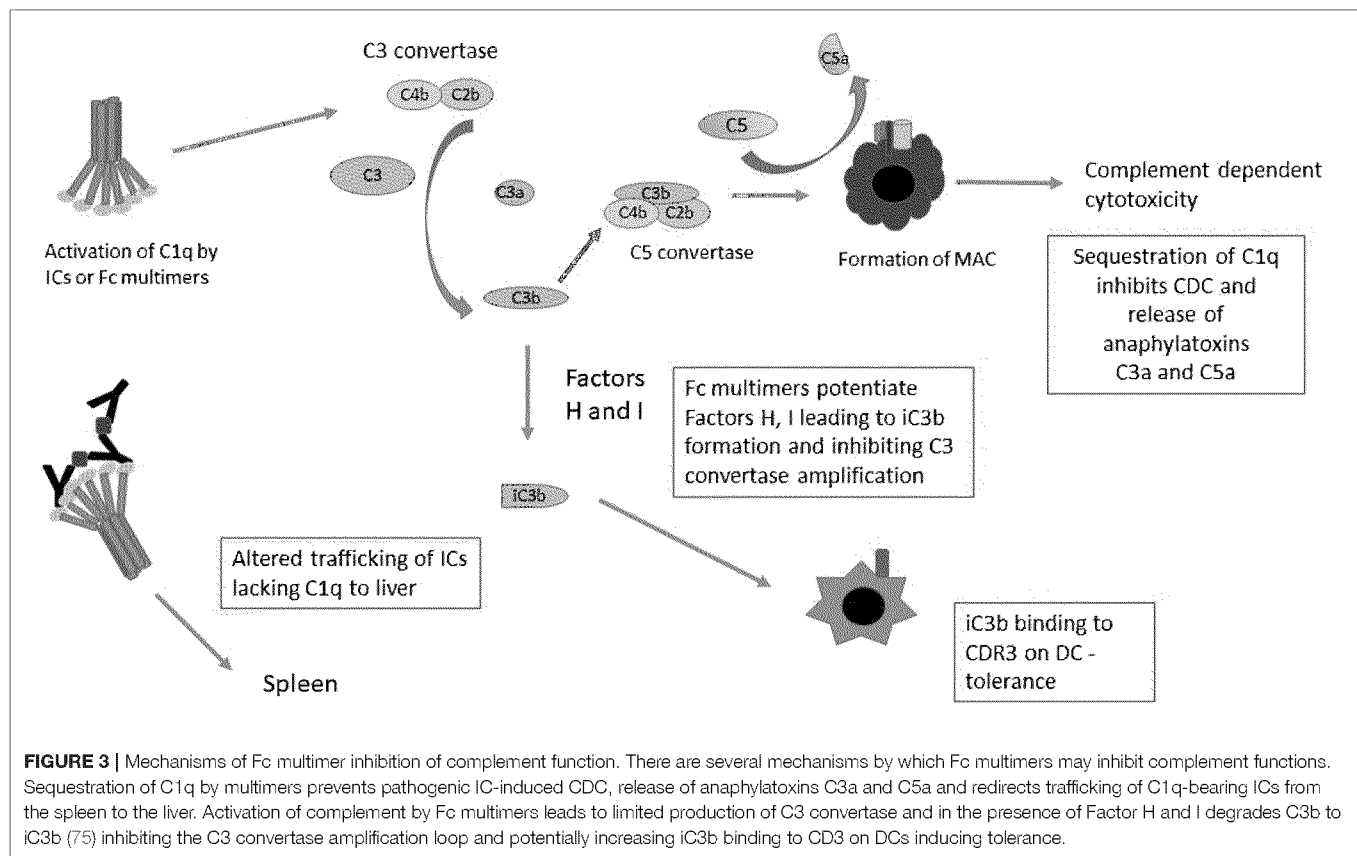
Complement Engagement

While the complement cascade has historically been viewed as pro-inflammatory—with the generation of “anaphylatoxins” such as C3a, more recent studies suggest that it be considered as immunomodulatory (116–118), with the ability to induce long-term tolerance following activation. For example, following cleavage, unbound C3b is very rapidly converted into iC3b; iC3b is capable of binding CR3 on dendritic cells and inducing long-term tolerance (119–121). Sohn et al. (117) demonstrated that iC3b binds to CR3 and modulates IL-10 and TGF-β₂, preventing delayed type hypersensitivity (DTH) in a rat ocular DTH model. The fact that stradomersTM that preferentially bind complement (e.g. G211 and likely others) effectively induce iC3b, provides a strong associative link between the generation of this and potentially other anti-inflammatory complement associated molecules and Fc multimer mediated tolerance (Figure 3) (11).

Similarly, indirect evidence suggests that Fc multimers interact with specific regulatory proteins to define the extent of complement activation. For instance, in human serum, G211 and others, mediate cleavage of C4 and to a lesser degree C3, but not significant amounts of C5, as evidenced by the generation of C4a, C3a, and the absence of C5a (11, 75). These effects are abrogated in Factor H deficient sera, suggesting that GL-211 potentiates factor H and/or co-factor I, limiting the generation of C5b-9 and associated cell death.

The classical arm of the complement cascade is generally activated on the cell surface as a result of antibody opsonization. Such activation mediates direct cell lysis through C5b-9 pore formation. In addition, these complement split products; decorating the cell surface, function as biologic bridges to engage complement receptors on other cells types. In contrast, hexameric C1q binding to IVIG or to recombinant Fc multimers, induces formation of C4 protease and cleavage of C4 away from the cell surface. C3 convertase is formed in solution, away from the cell surface, with cleavage of C3 to C3a and C3b which immediately degrades in solution to iC3b. These drugs thereby sequester complement substrates and competitively inhibit their ability to perform other biologic functions (75). Importantly, while this activation may result in soluble C5b-9 formation *in vivo*, soluble C5b-9 is not pore-forming, is not cell-bound, and is not associated with Complement Dependent Cytotoxicity (CDC). Indeed, the exact biologic functions of non-membrane bound C5b-9 remain unclear and may differ from the biology of cell-bound complement activation.

Finally, the act of binding of C1q and other complement products to aggregated immunoglobulins or to multimerized Fcs, alters both the ability of these compounds to engage FcRs and the C1q-mediated trafficking of ICs to liver and spleen. C1q-opsonized ICs bind CR1—located on RBCs in humans and on platelets in mice—where they are then preferentially transported to the spleen (122). When ICs are bound to C3b, they have a



limited ability to engage FcγRs, likely because of the partially overlapping binding sites between these moieties (123, 124). In the absence of C1q and/or in instances where the multimers have valency insufficient for C1q binding, they bypass the spleen and are preferentially transported to the liver (125). How/whether the changes in binding and trafficking mediated by complement engagement of Fc multimers alters their biology is an important subject for future study.

PRO-VS. ANTI-INFLAMMATORY EFFECTS OF IMMUNE COMPLEXES: A RESOLVABLE PARADOX?

Immune complexes can have pro-inflammatory activity—activity which may be conveniently classified in substrate dependent and substrate independent effects. Substrate dependent inflammation is induced by the partnership between FcR/complement engagement and the nidus of the IC. For example, in the case of ICs based on RNA viral substrates, FcR engagement may facilitate trafficking to intracellular TLR7, with resultant inflammation (126, 127). In contrast, substrate independent effects are induced independent of antigen, resulting from binding to the activating FcRs/complement with potential cytokine production, degranulation, cytotoxicity and/or complement activation [reviewed in (128)]. Because recombinant Fc multimers can engage FcRs and select complement fragments in the absence of an “antigenic core” they, by definition, competitively

inhibit substrate dependent, Fc mediated inflammation, while promoting substrate independent inflammation in a valency dependent fashion. The degree to which such substrate independent inflammation is required for the generation of tolerance is the subject of ongoing investigation.

Alternative Therapeutic Strategies for Targeting FcγRs

To our knowledge, there are no clinically relevant chemical based strategies to engineer Fc multimers. However, as an alternative to recombinant Fc multimers, investigators are employing antibodies to target specific FcRs with therapeutic intent. For instance, several groups have generated humanized monoclonal antibodies directed against the inhibitory FcγRIIb (129, 130) as well as the activating receptors FcγRI (131) and FcγRIIIa (132, 133) in an attempt to modulate disease activity. In addition, ongoing clinical trials are evaluating the potential of antibodies targeting the FcRn to reduce systemic antibody half-life and the absolute quantity of circulating antibodies (134, 135). These studies will likely define the precise roles of specific Fc receptors in different disease states.

ANTIBODIES AS CONTROLS

While not the primary focus of this manuscript, the creation of recombinant Fc multimers highlights biologic principles that must be considered when using non-specific IgG as controls

for antibody-based studies. Specifically, binding of multiple antibodies to their cognate targets on the surface of a cell, pathogen or other molecule enables their associated Fc fragments to engage low and intermediate Fc receptors and complement. Importantly, this engagement occurs at the site dictated by the location of the epitope recognized by the Fab. In contrast, because isotype specific antibody controls lack the ability to engage epitopes they will have a different distribution within the patient/animal as the experimental antibody and not engage similar Fc receptors or cell types as the experimental antibody. In addition, the IgG isotype controls will not form IC and therefore will likely engage higher affinity receptors rather than the low affinity Fc receptors engaged by ICs. These differences suggest that the isotype control antibody will lack the ability to induce correlate Fc functions to the experimental antibody. As such, IgG isotypes are probably not appropriate controls for antibody-based studies that employ functional Fc domains.

CLOSING THOUGHTS AND FUTURE DIRECTIONS

The development of recombinant Fc multimers as treatments for autoimmune and inflammatory conditions provides an opportunity for the scientific community to reconsider the

role of ICs in inflammation. Specifically, the data that ICs induce tolerance should prompt consideration of how/if fully competent monoclonal antibodies used for treatment of cancer and autoimmunity, may paradoxically mediate tolerogenic effects when decorating the target cell surface. Similarly, taken in concert, these data raise the possibility that the initial characterizations of ICs as pathogenic were perhaps oversimplified and that, in fact, the function of naturally occurring ICs are largely dependent on their antigenic core. Building on this theme, is it also possible, that some ICs are necessary sequelae of inflammatory events and that, under specific conditions, they play an important role in restoring immune homeostasis. Existing recombinant ICs, in combination with development of more specific agents—through altered N-glycosylation, sequence modifications, and valency specific selection—will help the scientific community address these basic questions that are fundamental to our understanding of inflammation.

AUTHOR CONTRIBUTIONS

EF, JW, and SS wrote sections of the manuscript. EF revised and edited the final document and generated figures. SS contributed critical input to revisions.

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A Machine Learning Approach for High-Dimensional Time-to-Event Prediction With Application to Immunogenicity of Biotherapies in the ABIRISK Cohort

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Predicting immunogenicity for biotherapies using patient and drug-related factors represents nowadays a challenging issue. With the growing ability to collect massive amount of data, machine learning algorithms can provide efficient predictive tools. From the bio-clinical data collected in the multi-cohort of autoimmune diseases treated with biotherapies from the ABIRISK consortium, we evaluated the predictive power of a custom-built random survival forest for predicting the occurrence of anti-drug antibodies. This procedure takes into account the existence of a population composed of immune-reactive and immune-tolerant subjects as well as the existence of a tiny expected proportion of relevant predictive variables. The practical application to the ABIRISK cohort shows that this approach provides a good predictive accuracy that outperforms the classical survival random forest procedure. Moreover, the individual predicted probabilities allow to separate high and low risk group of patients. To our best knowledge, this is the first study to evaluate the use of machine learning procedures to predict biotherapy immunogenicity based on bioclinical information. It seems that such approach may have potential to provide useful information for the clinical practice of stratifying patients before receiving a biotherapy.

Keywords: immunogenicity, biotherapy, machine learning, survival random forest, prediction

INTRODUCTION

In recent years, the introduction of biopharmaceuticals products (BPs) has opened a new area in the treatment of various cancer and auto-immune diseases. However, for some patients, the therapeutics induce an activation of the immune system, leading to the formation of anti-drug antibodies (ADA). These ADA may lead to partial or complete loss of efficacy of the drug (1). The mechanisms suspected for being involved in the immunogenicity of biotherapies process are patient-related (genetic background, immunological status, prior exposure, prior disease, co-administered drugs) or treatment-related (drug characteristics and formulations, route, dose, frequency of administration) but their relative contributions to the development of ADAs is not fully understood and remains to be deciphered for being used for predictive purpose (1, 2).

In this context, the IMI-funded ABIRISK consortium (3) had set up a real-world observational multicohort of patients suffering from various auto-immune diseases such as multiple sclerosis, rheumatoid arthritis, Crohn's disease, and ulcerative colitis. Participants recruited in the study were naive for the biotherapies they received during the study and were monitored during 12 months. ADA concentration was first measured at baseline and then at defined timepoints. The investigated BPs were TNF inhibitors, IFN β , anti-CD20, and anti-IL6R. For each subject, the time-to-occurrence of ADA provided a way to evaluate both the propensity to produce or not ADA (termed in the following as being an "immune-reactive" or "immune-tolerant" subject) and the dynamic of the ADA production (early/late) among the immune-reactive subjects. The main objective was to provide an estimate of the probabilistic susceptibility of an individual to produce ADA based on the drug received and the subject's clinical and genetic information.

With the production of high-dimensional datasets (so-called big data) there is nowadays a growing interest in using machine learning (ML) approaches for clinical prediction (4). Indeed, ML is particularly appealing for situations where complex non-linear relationships are expected to play a key role into the disease process such as in biotherapy immunogenicity. Random Forests (RF) as introduced in the seminal paper of Breiman (5) is one of the most effective ML approaches for prediction. Thus, RF and its variants are more and more frequently considered for delivering big-data-driven clinical prediction algorithms. Broadly speaking, the RF builds a series of decision trees from which a final prediction is obtained by combining the predictions from each individual tree. These latter tree-based learners are non-parametric approaches that partition recursively the space of predictor variables into disjoint sub-regions (so-called terminal nodes or leaves) that are homogeneous according to the outcome of interest. These partitions are obtained from a splitting criterion that either minimizes the within-node heterogeneity or maximizes the between-node heterogeneity (6). The well-known instability of each individual tree-based structure has been the main motivation to the development of RF, the main idea being that the combination of several survival tree predictors has better predicting power than each individual tree. In the original RF

procedure proposed by Breiman (5) each tree is built using a random set of individuals with replacement (bootstrapping) and each split of the tree is evaluated on a random small subset of predictor variables. The main goal of this process is to increase the diversity of the tree-based learners that are aggregated at the end. Among the key features of the random forests, the random choice of both the individuals and the features together with the splitting criterion play critical roles. Since the first introduction of RF, tree-based learners have been extended to censored data (termed survival trees) (7) and integrated in RF framework [termed as random survival forests (RSF)] (8, 9).

For this immunogenicity prediction study, we have been confronted to some specific issues that prompted us to consider a modified RSF approach. We first had to cope with a situation where we had collected a huge set of candidate predictors (clinical and genetic markers) but only a small number were expected to be relevant for prediction. Secondly, we studied a mixed population of subjects with both susceptibles (immune-reactive) and non-susceptibles (immune-tolerant) subjects for the outcome of interest (ADA occurrence). The first issue is linked to the RF procedure where at each split of a tree the recursive partitioning process is only applied to a random subset of all the predictors. Thus, when the number of relevant predictors is overwhelmingly small as compared to the size of the non-relevant ones (noise), the randomness in the variables selection leads to most of the subspaces having weak predictive accuracy and thus affects the final prediction of the RF. The second issue relates to the fact that our studied population is a mixture of immune-tolerant and immune-reactive subjects. The immune-tolerant subjects are those whose immune system is in a state of unresponsiveness to the exposure of the drug and thus will not produce ADA whereas the immune-reactive subjects are those who are able to produce detectable levels of antibodies. In such mixed population (10), the logrank statistic which is the commonly used splitting criterion for RSF does not take into account the dynamic of the ADA production which may decrease its discriminative performance.

In order to cope with these issues, we have considered a strategy which relies upon a particular splitting criterion and uses a modified RSF strategy with a random subspace sampling step. The splitting criterion is related to a previous work on heterogeneous population with non-susceptible patients (11). The random subspace sampling strategy follows the proposal of Panov and Dzeroski (12), that combines bagging (random subsamples with replacement) and random subsampling (random subspaces).

In this paper, we first present our modified RSF procedure and then apply this latter for predicting the occurrence of anti-drug antibodies from the ABIRISK cohort.

MATERIALS AND METHODS

Material

These data come from a real-world observational prospective multicenter cohort of patients suffering from various auto-immune diseases. Patients who had been prescribed a

biotherapy by a physician were followed for 12 months. The choice of the treatment was left to the physician.

Clinical data were recorded into an electronic Case Report Form. DNA samples and serum samples were collected for genetic analyses and ADA testing, respectively. Serum samples for ADA testing were collected at baseline before start of BP therapy and subsequently at each study visit after start of therapy. Anti-drug antibodies were detected by specific validated assays for each BP and analyzed in central ABIRISK laboratories.

Patients were followed for 12 months from the start of the therapy. The time-to-event (ADA positivity) was defined as the period of time from the date of first treatment to the time of first ADA positivity. Patients without ADA occurrence were censored at the date of their last follow-up (drop-out, drug switch), or administrative censoring (12 months). Patients with their consent for genetic testing, available high-quality blood DNA samples were selected for genotyping. Among the 609 recruited patients, 560 were eligible and 501 DNA samples were available.

Genotyping was performed with Infinium OmniExpress-24 v1.2 BeadChip. This array interrogated over 700,000 genetic markers (single nucleotide polymorphisms, SNPs) located throughout the genome. We imputed the missing SNPs using the Michigan Imputation Server with a European population panel as reference (13). After quality control procedures, we had 457 individuals and 495,792 SNPs. For the predictive analysis, in order to avoid highly imbalanced groups, we focused on common SNPs with intermediate minor allele frequencies (MAF > 25%). Thus, we retained 287,611 SNPs. Among these 457 patients, 114 were treated with Adalimumab, 76 with Infliximab, 64 with Etanercept, 27 with Rituximab, 35 with Tocilizumab, 64 with IFN β -1a sub-cutaneous, 40 with IFN β -1b sub-cutaneous, 37 with IFN β -1a intra-muscular. The clinical variables considered for the predictive analysis were biotherapy, disease, age, sex, tobacco smoking, body mass index, previous or concomitant medications. For the missing clinical variables, we considered a basic imputation strategy where we replaced the missing values by the mean of the non-missing values (continuous variable) or the most common class (binary variable).

For the predictive analysis, the clinical variables were included without recoding for binary variables (sex, tobacco smoking, previous or concomitant medications) and continuous variables (age, body mass index). For unordered categorical variable (disease), we considered the four partitions as candidate variables. For the treatment variable and based upon previous analyses, we considered three immunogenicity groups. The low immunogenicity group (Etanercept, IFN β -1a i.m.), the intermediate immunogenicity group (Tocilizumab, Infliximab and IFN β -1a s.c.) and the high immunogenicity group (Rituximab, Adalimumab, IFN β -1b s.c.). In practice, we included the treatment as an ordered variable (low/intermediate/high immunogenicity level) and also as dummy variables (one drug vs. the others). Genotyping data were considered as ordered variables (based on the number of alternative variants) such as the partition explored recessive and dominant genetic effects for the alternative variant.

Methods

Survival Model

Notations

Let denote T the time-to-ADA detection and C the censoring time. For each subject i ($i = 1, \dots, n$), $X_i = \min(T_i, C_i)$ is the observed time of follow-up and $\delta_i = 1_{(X_i=T_i)}$ the indicator of ADA detection (positivity). We also denote $Y_i(t) = 1_{(t \leq X_i)}$ the indicator of being at risk for the event at time t . Let $G1_i = (G1_{i1}, \dots, G1_{iq})$ be the q -dimensional vector encoding treatment and clinical variables. All these variables were binary or categorized variables. Let $G2_i = (G2_{i1}, \dots, G2_{ip})$ be a p -dimensional vector of genotypes for a patient i . Here, the genotype information relying upon p biallelic genetic markers (SNPs). The genotype of subject i is coded as an ordinal 0; 1; 2 variable where the values represent the number of alternative variants of the subject. Finally, let $G_i = (G1_i, G2_i)$. This m -dimensional ($m = p + q$) vector gathers information from the treatment, the clinic and the genotype of each subject.

When building each individual tree, at each node h , for each of the m variables of the G_i vector, the process searches for the best binary split.

Mixture Model

In this work, we take into account that the population under study is a mixture of immune-reactive and immune-tolerant patients. Here, the immune-reactive group is composed by those who are susceptible to produce detectable levels of antibodies within the 1-year window of monitoring. The immune-tolerant group is composed by those who are immune-tolerant to the BPs that is to say that they will not produce detectable levels of antibodies. As both immune-reactive and immune-tolerant subjects cannot be distinguished in the censored subset, we had to consider long-term survival models that explicitly consider the existence of a proportion of immune-tolerant subjects.

For modeling survival data with a proportion of non-susceptible individuals, there are broadly two main frameworks. The first one relies on two-component mixture models whereas the second one relies on defining the cumulative hazard as a bounded increasing positive function (10, 14). In this paper, we consider the latter framework since it has some interesting mechanistic interpretation of the biological mechanism of the occurrence of the event of interest. More precisely, we propose to model the distribution of the time-to-ADA detection through a simplified mechanistic model whereby each individual may or may not be able to produce ADA in response to the introduction of the biotherapy. This model is related to a previous work on long-term survival model with application to clinical oncology (11).

Here, we consider that ADA are produced by the activation of unobservable BP-specific (T-dependent) B-cell clones that emerge and become immunocompetent ADA-producing clones. Positivity occurs as soon as any one of the B-cell clones is able to produce levels of ADA of sufficient affinity and titre for being detected by the assay. Thus, the observed time-to-detection is the first time-to-detection associated with a competent B-cell clone. If no competent B-cell clone is produced by an individual,

then the patient is considered as immune-tolerant and his/her time-to-detection is considered, theoretically, as the infinity.

Since the B-cell clones are not directly observed for each individual, we cannot obviously specify the individual survival distribution. However, if we assume a particular distribution for the number of unobserved B-cell clones, we can specify the marginal or population (averaged over the population under study) survival function. Assuming a Poisson distribution for the number of B-cell clones, we can obtain the population survival distribution with bounded cumulative model that is used in this article and presented just below (11, 15).

At each node, for each binary split candidate variable $W_k = 0, 1$ ($k = 1, \dots, K$), we consider the following population survival distribution:

$$S(t|W_k = w) = \exp \{ -\Lambda(t|w) \} = \exp \{ -\theta(w) [1 - H(t|w)] \} \quad (1)$$

Here, $H(t|w)$ is an unspecified continuous positive function increasing from zero to infinity that is similar to a cumulative hazard risk function and $\theta(w)$ is a positive quantity. Thus, $S(t|w)$ shows a tail defect (related to the fraction of immune-tolerant subjects) with $S(t = \infty|w) = \exp[-\theta(w)] > 0$. The cumulative hazard function $\Lambda(t|w) = -\log(S(t|w))$ is bounded by $\theta(w)$.

In the following we will consider a classical multiplicative structure such as: $\theta(w) = \theta_0 e^{\alpha w}$ and $H(t|w) = H_0(t) e^{\beta w}$ where θ_0 and $H_0(t)$ are the baseline tail defect and pseudo-cumulative hazard function, respectively, and α, β are unknown parameters. Here, α quantifies the difference of the immune-tolerant fractions between the two groups and β quantifies the difference between the two groups in the dynamic of the production of ADA among the immune-reactive subjects. If $\alpha = 0$ there is no difference between the immune-tolerant fractions of the two groups. If $\beta = 0$ the dynamic of ADA production among immune-susceptible patients are identical for the two groups.

At any split, the hazard ratio between the two groups is such as:

$$\frac{\lambda(t, W_k = 1)}{\lambda(t, W_k = 0)} = e^{\alpha} e^{\beta} e^{-H_0(t)(e^{\beta} - 1)} \quad (2)$$

with $\lambda(t, W_k = 0)$ and $\lambda(t, W_k = 1)$, the instantaneous rates of ADA detection for group 0 and 1. As seen above, due to the existence of an immune-tolerant faction, the hazard ratio is not constant over time (non-proportional relationship).

Splitting Criterion

In the spirit of classical partial-likelihood-based splitting criteria (e.g., the logrank statistic), we propose to use a criterion which is based upon the score vector derived under the survival model presented above and computed under the null hypothesis of no difference between the two groups for both the immune-tolerant fraction and the dynamic of ADA production among immune-reactive patients. This criterion aims to identify the splitting candidate variable which maximizes the between-node heterogeneity. Maximizing the between-node heterogeneity leads to minimize the variability inside each node. In other words, it leads to more homogeneous groups with respect to the outcome

of interest. This criterion is identical to the global test proposed by Broët et al. (11)

Modified Random Survival Forests Procedure

In this section, we first recall the basic principles for building a survival tree and then we present the proposed procedure.

Building a survival tree

In practice, a survival tree is grown by first splitting the whole dataset (so-called the root node) into two so-called child nodes that maximize between-node heterogeneity. The same procedure is then repeated for each child node until each node reaches a predetermined stopping rule (e.g., minimum node size) or be homogeneous. A node that cannot be split any further is called a terminal node (or leave). Each terminal node is a distinct partition of the sample which is characterized by a unique combination of the predictors.

In **Figure 1**, we present an example of a survival tree. Here, we start with the whole population (top). Then, the procedure searches for the best splitting variable (which maximizes our splitting rule). Here, the process selects the variable X_{10} with threshold cutpoint a which splits the population in two sets of individuals, those with $X_{10} < a$ (left) and those with $X_{10} \geq a$ (right). For each of these two groups, the algorithm now searches for the next best splitting variables. On the left, the procedure selects the variable X_{20} with threshold b . On the right, the procedure selects the variable X_{15} with threshold c . For each of these four groups, the process continues to split in a binary fashion or stops if it has reached a predetermined stopping rule. As seen on the figure, on the left side, it stops. On the right side, it splits the far-right group in two new groups based upon the variable X_{40} with threshold d . Now, the algorithm stops as it reached a predetermined stopping rule.

We end up with a population with five more homogeneous groups (with their respective survival curves). These five groups are defined by a combination of the four splitting variables with their corresponding cutpoints. For each group, the Nelson–Aalen or Kaplan–Meier estimates of the survival function is calculated. It is worth noting that the use of the Nelson–Aalen or Fleming–Harrington estimator (16) (using the cumulative hazard) would be more suited for leaves with a small number of subjects rather than the Kaplan–Meier (product-limit) estimator. Here, the group in red has the worst prognosis whereas the group in blue has the best prognosis. The others have intermediate prognosis.

Building a modified Random Survival Forest with random subspace sampling

Our dataset (noted $\mathcal{L}$) consisting of n independent individuals with observed outcomes and predictor variables such as $\mathcal{L} = \{(X_i, \delta_i, G_i), i = 1, \dots, n\}$. The proposed procedure is based on the following algorithm.

We first draw with replacement n individuals of our dataset $\mathcal{L}$ and thus create a bootstrap sample (denoted $\mathcal{L}_b$ with $b = 1, \dots, B$) from the original dataset. This means that in average 63% of the original sample is included in the bootstrap sample and 37% are left out. At the same time, we randomly select a

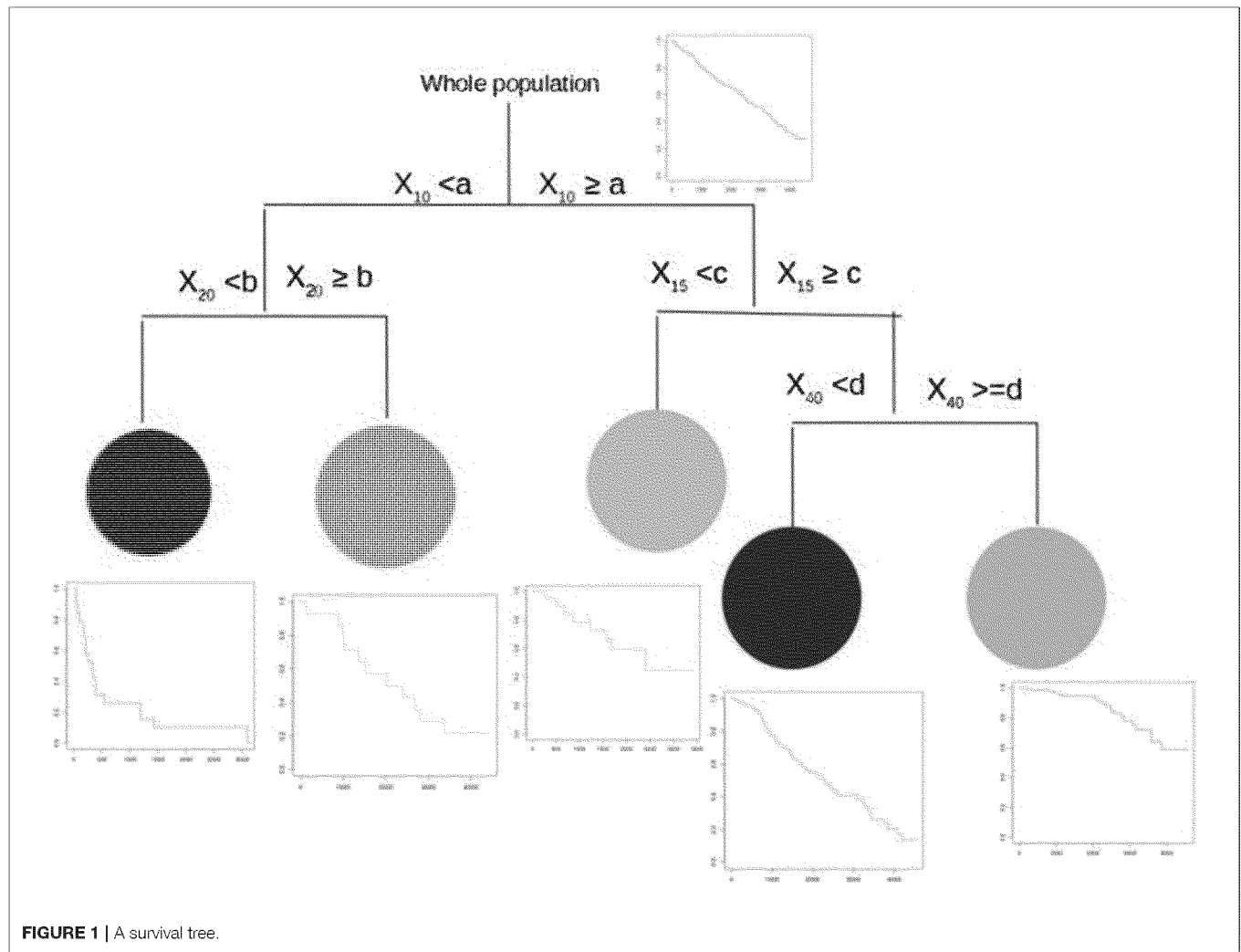


FIGURE 1 | A survival tree.

subspace sample of the predictors (denoted $\mathcal{P}_b$) of dimensionality p^* (with $p^* < m$).

Based on this subspace bootstrap sample $\mathcal{L}_b^{\mathcal{P}_b}$, we build a survival tree and repeat the process B times as described just below.

- Build a survival tree from $\mathcal{L}_b^{\mathcal{P}_b}$.
- * For each split candidate variable W_k , we compute the corresponding splitting criterion $S(W_k)$ presented above. We do the same procedure for all the split candidate variables.
- * Then, we find the best split S^* which is the one having the maximum value over all the candidates. Then, a new node is built and the observations are splitted accordingly.
- * We iterate the process until each node reaches a pre-defined minimum node size or be homogeneous.
- * We construct the final tree denoted $\mathcal{T}_b^{\mathcal{P}_b}(W_b)$ where $W_l^{(b)}$ ($l = 1, \dots, L(b)$) is a vector of indicator variables representing the $L(b)$ leaves of the tree such that $W_{il}^{(b)} = 1$ if the i^{th} observation belongs to the l^{th} terminal node of $\mathcal{T}_b^{\mathcal{P}_b}$, and 0, otherwise.

- Repeat the process B times.

At the end of the process, we have a series of B survival trees known as a random survival forest.

This procedure can also be seen as a bootstrapped RSF with random subsampling of the feature space. **Figure 2** illustrates this process with iterations of building survival trees from bagged samples and random subspace sample of the predictors.

Evaluation of the Predictive Accuracy

Here, each survival tree is built from a bootstrap sample of the data. The individuals who are in this sample are called “in-bag” individuals and those who are not are called “out-of-bag” individuals. Thus, we can compute the survival predictions of each “out-of-bag” individual. In practice, the split variables of the patient are dropped down the tree until it reaches a terminal node. Then, the patient’s survival prediction is the estimated survival of this terminal node. It is a valid prediction which is not optimistically biased since the survival is predicted using a tree that do not use this individual. The final prediction are given

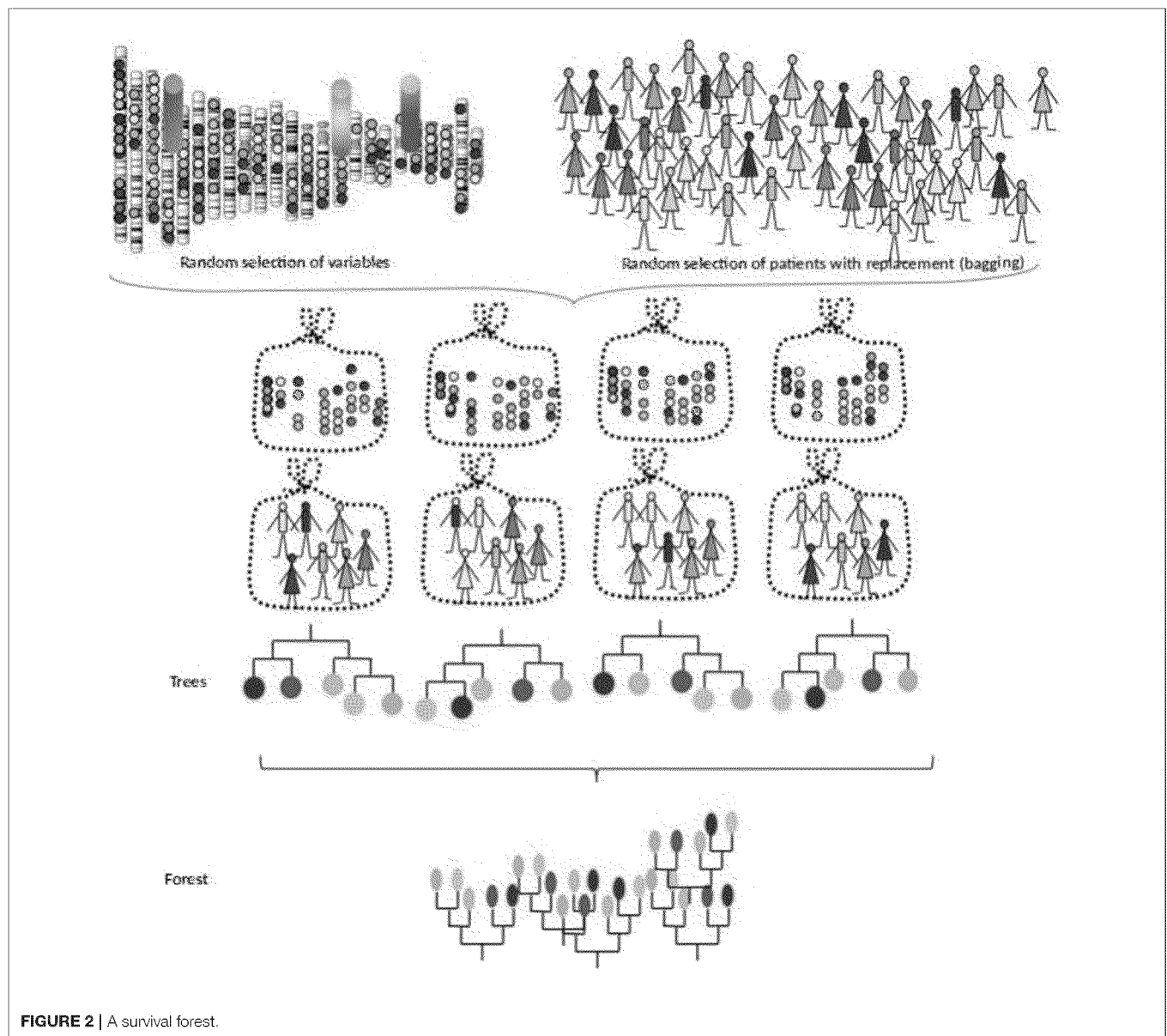


FIGURE 2 | A survival forest.

by averages overall the estimates. Then, we can compare survival predictions to what was really observed.

Figure 3 illustrates this process for the out-of-bag subject (in red).

To estimate the prediction error of our procedure, we use the Harrell's concordance index for survival data or C-index (17). This estimate is widely used in the literature as a way for assessing predictive accuracy in survival analysis. The Harrell's index estimates the probability that, in a randomly selected pair of cases, the case that fails first had a worst predicted cumulative hazard risk estimate. In the following, we reported the final error rate which is equal to one minus the C-index. If this value is 0.5 it corresponds to a procedure doing no better than random guessing, whereas a null value indicates perfect prediction.

Simulation Study

In order to evaluate the predictive performance of the proposed custom-built RSF procedure as compared to the classical RSF procedure, we conducted the simulation study presented just below. We used the classical Logrank statistic as the splitting criterion for both procedures.

In practice, we used the well-known *Primary Biliary Cirrhosis (PBC)* of the liver data set of the Mayo Clinic, which is publicly available in R through the package "randomForestSRC" (9). This data set is widely used in evaluating survival models and contains well-known explanatory variables. This dataset included 17 potential explanatory variables to which we added 50,000 random variables.

More precisely, we retained only the first 312 patients of the PBC data set who participated in the randomized trial

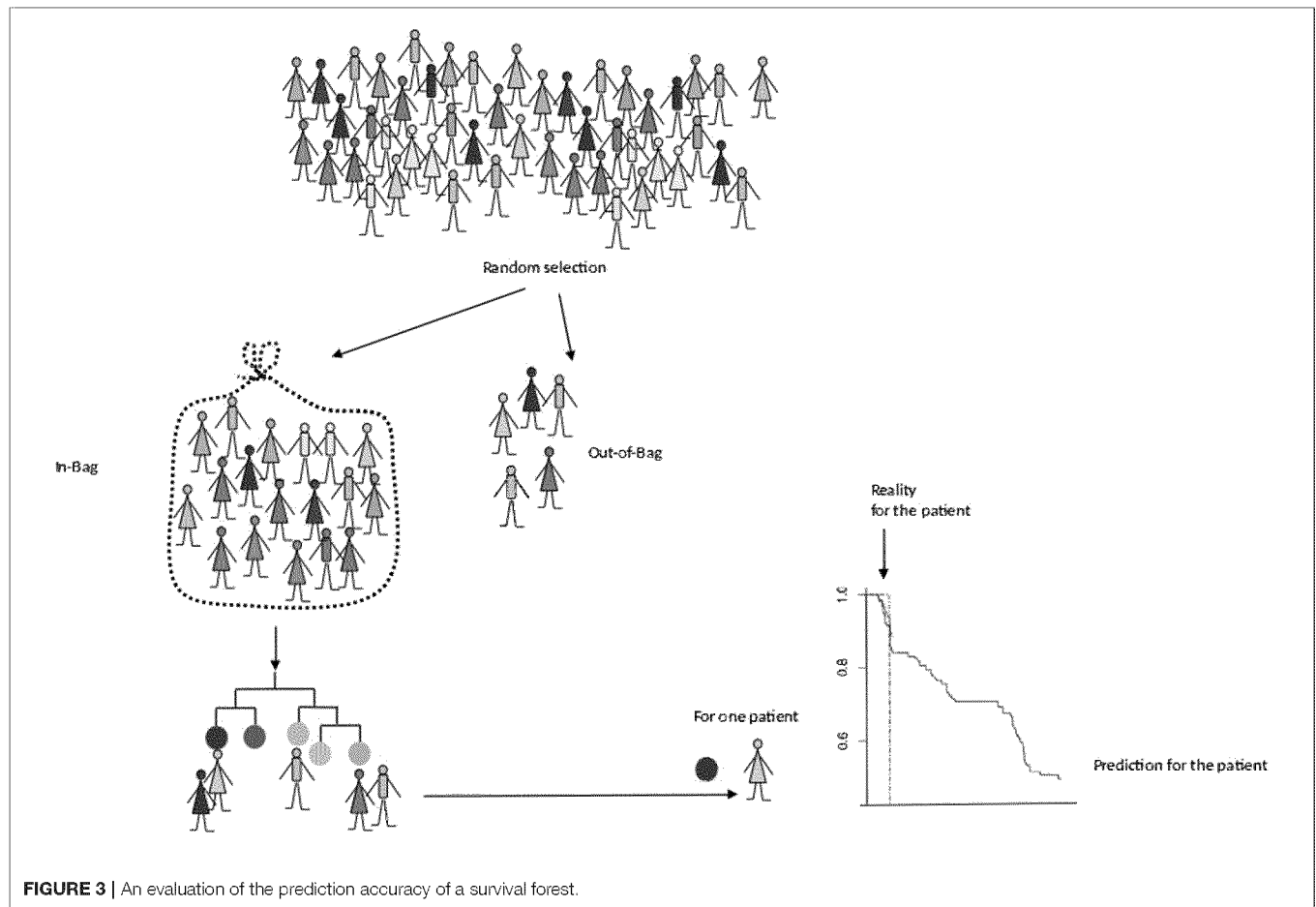


FIGURE 3 | An evaluation of the prediction accuracy of a survival forest.

and categorized continuous covariates using their quartiles. Then, we added 50,000 non-informative variables to the PBC dataset. The noise variables were 50,000 pseudo three-genotypes variables obtained from bi-allelic markers in Hardy-Weinberg equilibrium where each pseudo-minor allele is drawn from a uniform distribution ranging between 0.25 and 0.4.

The variable of interest was the time from the start of registration to death. Patients alive were censored at the date of their last follow-up visit. In total, we had 312 individuals with 50,017 candidate variables.

We compared the predictive performance of the classical RSF and the proposed custom-built RSF procedures with a random subsampling ranging from 1 to 100% and measured their error rates. We also computed the error rate obtained for a Bagging survival forest (a special case of RSF with all the covariates are considered as candidate) where the 17 variables were candidates.

In this study, we have considered the classical default parameters for generating trees. Thus, we put no constraint on the depth of the trees but there is a minimal number of unique cases in a terminal node which is of 15. For the RSF, the default parameter for the number of variables randomly selected as candidates for splitting a node is $\sqrt{n}$ where n equals the number of candidate variables.

In all cases, the size of the ensemble was fixed at 500 trees.

RESULTS

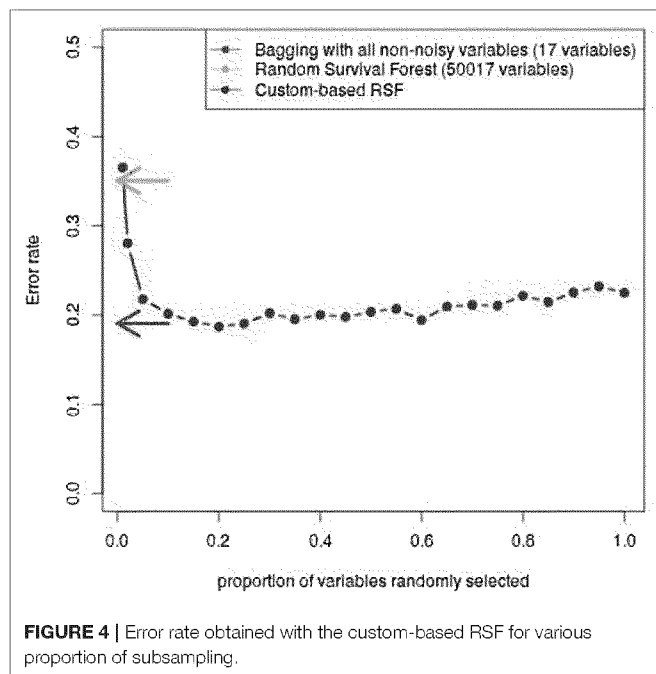
Simulation Results

As seen in Figure 4, We observe that the minimal values of error rate associated with the proposed custom-based RSF procedure is around 19.0%, which is obtained for a proportion of at least 20–25% for the sub-sampling. This value is quite close to the value obtained with the Bagging procedure applied to the small data set containing only the 17 covariates potentially associated with the outcome (18.9%). In comparison, the classical RSF procedure leads to an error rate of 35.1%.

ABIRISK Cohort

The cohort analyzed in this work consists in 457 individuals with genotyping information that successfully passed the quality-control procedures and who are suffering from auto-immune diseases. In this multi-cohort, 132 patients (29%) suffered from inflammatory bowel diseases (Crohn's disease or ulcerative colitis), 141 (31%) from multiple sclerosis, and 184 (40%) from rheumatoid arthritis.

There were 310 women (68%) and 147 men (32%). Patients were aged from 18 to 87 years old and the mean age was 43.5 years old. Among the 450 patients for whom the measure was provided, the body mass index (BMI) ranged from 15 to 49 with a mean



BMI of 25. Nineteenth patients (4%) were underweighted (BMI < 18.5), 252 (56%) had a normal BMI (between 18.5 and 25), 102 (23%) were overweighted (BMI between 25 and 30) and 77 (17%) were obese (BMI > 30). Among the 455 patients with a provided tobacco smoking status, 257 (56%) were currently smoking or had quit smoking and 198 (44%) had never smoked. Among the 445 patients with the provided information, 208 (47%) were taking immunosuppressants during the study. Ninety-four (21%) of the 442 patients whose information was available were taking antibiotics during the study.

Eight biotherapies were used in the study, forming four classes of drugs : TNF-inhibitors (Adalimumab, Etanercept, Infliximab), IFN β (IFN β -1a subcutaneous, IFN β -1a intramuscular and IFN β -1b subcutaneous), anti-IL6R (Tocilizumab), and anti-CD20 (Rituximab). 254 patients (55%) were taking TNF-inhibitors, 141 (31%) IFN β , 35 (8%) anti-IL6R, and 27 (6%) anti-CD20.

In the entire cohort, the probability of producing ADA at 1 year was 27.5% [22.9–31.7%].

Prediction Results

We applied our proposed modified RSF procedure on the dataset from the ABIRISK cohort and reported the error rate estimate (one minus the C-index). We also reported the results obtained using the classical RSF procedure [implemented in the “randomForestSRC” package (9)] with default parameters. For both RSF procedures, we ran 500 survival trees. For the modified RSF procedure, each tree-based classifier was grown from random subspaces composed of a subsampling of 75% of the candidate variables.

The predictive accuracy of our procedure leads to an global error rate of 26.4% whereas the classical RSF leads to a higher

global error rate of 51%. From these two procedures, we can compute the out-of-bag individual predicted probabilities of ADA occurrence. **Figure 5** displays the predicted cumulative density function for ADA occurrence for the 457 patients obtained using an out-of-bag estimator from both procedures. From this figure, we can see that the modified RSF procedure leads to a clear separation between two groups of individuals regarding the risk of ADA (**Figure 5A**). Individuals with a predicted probability of occurrence of ADA at 1 year greater than 50% can be considered at high risk whereas those lower than 50% can be considered at low risk. In contrast, the classical RSF procedure was unable to separate individuals regarding the risk of ADA (**Figure 5B**).

DISCUSSION

The main contribution of this work is to propose and evaluate the practical use of a custom-built machine learning procedure for time-to-event prediction which accommodates high-dimensional predictors and mixed population of individuals. This was indeed a real issue for evaluating immunogenicity prediction using the bio-clinical data collected from the ABIRISK cohort. The proposed custom-built (or modified) RSF procedure uses a particular splitting criterion and considers random subsamplings of the candidate predictors.

When applying this procedure to the ABIRISK cohort, the modified RSF procedure leads to a much lower error rate than the classical RSF. Moreover, the individual predicted probabilities of ADA occurrence provide a way of discriminating between low and high-risk group of individuals. We can hypothesize that this gain in predictive performance is mainly due to the existence of a tiny proportion of pertinent variables that interact with each other. Indeed, our proposal borrows predictive strength from the tree-based structure while avoiding the restrictive sampling of the classical RSF. Moreover, the counter-performance of the classical RSF shows that there is no one-size-fits-all solution for complex clinical issue, therefore advocating for custom-based solution. In practice, with a random subspace sampling, we ensure that relevant predictors are given to the survival tree growing algorithm as iterations proceed more often than in the classical RSF method, and hence it decreases the chance to include useless survival trees in the forest. From these results, the use of the classical survival random forest should be avoided when few pertinent predictors are expected. Surprisingly, few works have pinpointed this problem that can be however of high concerns for clinical research that copes with so-called fat dataset (i.e., more predictive candidate variables than individuals). It is worth noting that the rationale for considering such multi-diseases/multi-drug predictive analysis was that even though these auto-immune diseases encompass a broad range of phenotypic manifestations, the way the patients respond to various biotherapies suggest that similar clinical and/or biological pathways might be involved and that they also might share some genetic markers. Thus, such cross diseases/drugs strategy should provide gains in predictive power as compared to separate

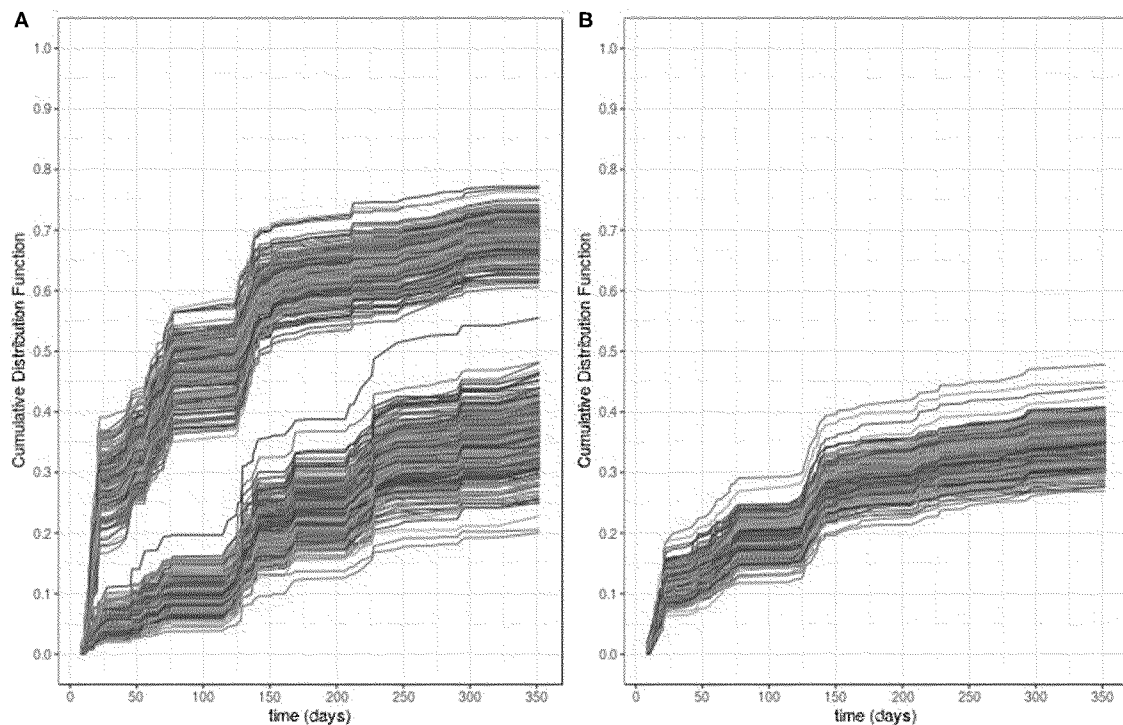


FIGURE 5 | Predicted survival probabilities obtained with our proposed random survival forest method (A) vs. those obtained by the classical random survival forest method (B).

analyses since it borrows information across several therapies and auto-immune diseases. However, if our assumption of common pathways is not correct, we should perform separate analyses (by disease or drugs) that require more individuals to ensure sufficient predictive power.

Despite these promising results, some limitations of the present exploratory predictive study should be considered. First, we should emphasize that our work is only a developmental study (model development and internal validation) which requires further validation studies. Moreover, even if the ABIRISK cohort includes hundreds of participants, it is still a small sample size and future studies with larger cohorts should be done to validate these results and generalize the use of this approach. Moreover, the definition of positivity relies upon the ADA detection methods that were harmonized but however different across the drugs. Second, it is difficult to identify the optimal percentage of subsampling. From our simulation study with fifty thousands of non-relevant variables, we have seen that sampling one-quarter of the feature space seems sufficient for obtaining a good predictive accuracy. For studies with dense genotyping data, we recommend building trees on more than half of the predictors for reaching competitive accuracy. More works should be however performed for providing practical guidance. It is worth noting that including all the predictors such as in the classical bagging strategy can lead to a small increase of the error rate due to the low diversity of the forest. Third, in this work, we use a particular splitting criterion

which takes into account the mixture population under study. The choice for this survival model stems from immunological as well as statistical considerations but other modelings could obviously be considered and evaluated. Fourth, this work is only a preliminary step to find new machine learning approaches and further works should be done to derive measures adapted to this approach for identifying the important predictors and decipher their interplay.

In conclusion and to our best knowledge, this is the first study to evaluate the use of machine learning procedures to predict biotherapy immunogenicity based on bioclinical information. We have showed that this custom-based machine learning approach provides a valuable tool for prediction. While the current approach obviously needs further improvement before its clinical practical use, it might have potential to provide useful information for the clinical practice of stratifying patients before giving them a biotherapy.

DATA AVAILABILITY STATEMENT

The data analyzed in this study were collected in the context of the ABIRISK project by ABIRISK partners. Access to the minimal dataset underlying the findings can be obtained by interested researchers upon request to the ABIRISK Sustainability Scientific Committee by submission of an analysis plan. The analysis plan should explain the purpose of the use of the data and

confirm the intention to use the data only for replication studies concerning anti-drug inhibitors, since this is the limitation of the ethical permission on how this data can be used. The contact person of the ABIRISK Sustainability Scientific Committee to whom the requests should be sent is Marc Pallardy (marc.pallardy@inserm.fr).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethics Committee of the General University Hospital in Prague (reference 125/12, Evropský grant 1.LF UK-CAGEKID) Institutional committee of Heinrich Heine University, Düsseldorf, Germany (protocol reference 4451) Ethikkommission der Fakultät für Medizin der Technischen Universität München, München, Germany (reference 335/13) Ethikkommission Nordwest- und Zentralschweiz, Basel (reference 305/13) Ethikkommission der Medizinischen Universität Innsbruck, Innsbruck (reference AN2013-0040 331/2.1) Comité Ético de Investigación Clínica de l'Hospital Universitari Vall d'Hebrón, Barcelona [reference EPA(AG)66/2013(3866)] Stockholm Regional Ethics Committee, Stockholm (reference Dnr. 2013/1034-31/3 and Dnr. 2015/749-32) Comité de Protection des Personnes Ile de France VII (reference 13-048) Medical Ethical Committee of the Academisch Medisch Centrum, Amsterdam (reference 2013-304#B20131074) Local Ethics Committee of AOU Careggi (reference Protocol N o 2012/0035982) NRES Committee London, City & East (reference 14/LO/0506) Comité de Protection des Personnes Ile de France IV (reference 2013/24) Comité d'éthique hospitalo-facultaire universitaire de Liège (reference 2015/55). The

patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JD and PB developed the proposed statistical procedure. PB coordinated the project and is JD's Ph.D. thesis advisor. JD and PB participated in writing the original draft. JD, SH, DB, and PB analyzed the data. SH, DB, MA, FD, AF-H, AG, SH-B-A, XM, MP, and PB participated in the collection of the data. MP coordinated the ABIRISK project. All authors read and approved the final manuscript.

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What Is the Clinical Relevance of TNF Inhibitor Immunogenicity in the Management of Patients With Rheumatoid Arthritis?

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Tumor necrosis factor- α inhibitors (TNFis) have revolutionized the management of rheumatoid arthritis (RA), however despite considerable progress, only a small proportion of patients maintain long-term clinical response. Selection of, and switching between, biologics is mainly empirical, experiential, and not evidence-based. Most biopharmaceutical proteins (BP) can induce an immune response against the foreign protein component. Immunogenicity and the development of anti-drug antibodies (ADAs) is considered one of the main reasons for loss of therapeutic efficacy (secondary failure). ADAs may neutralize and/or promote clearance of circulating BP with resultant low serum drug levels, loss of clinical response, poor drug survival and adverse events, such as infusion reactions. ADA identification is technically difficult and not standardized, making interpretation of immunogenicity data from published clinical studies challenging. Trough TNFi drug levels correlate with clinical outcomes, exhibiting a “concentration-response” relationship. Measurement of ADA and drug levels may improve patient care and improve cost-effectiveness of BP use. However, in the absence of clinically-validated, reliable assays and consensus guidelines, therapeutic drug monitoring (TDM) and immunogenicity testing have not been widely adopted in routine clinical practice in Rheumatology. Here we discuss the utility and relevance of TDM and immunogenicity testing of TNFis in RA (focusing on the most widely used TNFis globally, with the most available data, i.e., infliximab, adalimumab, and etanercept), the limitations of currently available assays and potential future immunopharmacological strategies to personalize disease management.

Keywords: immunogenicity, anti-drug antibodies, biopharmaceutical products, TNF-inhibitors, rheumatoid arthritis

INTRODUCTION

Biologic agents, such as TNF- α inhibitors (TNFis), have revolutionized the treatment of rheumatoid arthritis (RA), but despite this advance, not all patients respond favorably. Up to 40% of RA patients do not respond to the first biologic (primary failure) or lose response over time (secondary failure). Drug survival (the time to discontinuation of a drug) is influenced by many factors including lack or loss of efficacy, adverse events (AEs), and poor adherence. Immunogenicity is defined as the ability of biopharmaceutical products (BPs) to induce an immune response, resulting in

the generation of anti-drug antibodies (ADAs). ADAs are considered an important (albeit not the only) mechanism of secondary treatment failure and limited drug survival, due to effects on pharmacokinetics and bioavailability. ADAs are also implicated in treatment-related AEs, such as infusion and injection-site reactions (1). Immunogenicity testing is a mandatory, regulatory requirement for BP drug licensing, as part of the safety profile package required by both the US Food and Drug Administration (2) (FDA) and the European Medicines Agency (3) (EMA).

Therapeutic drug monitoring (TDM) and immunogenicity testing, using trough drug levels and ADAs, have the potential to improve clinical decision-making, by influencing drug selection, dose, and frequency of administration. This may allow clinicians to reduce under- and over- treatment for patients in clinical relapse or remission. There are currently no consensus guidelines recommending the use of BP drug levels and immunogenicity testing in RA, and as such, their use in clinical practice is widely variable.

TNFis (in combination with methotrexate and as monotherapy) are often selected as first-line biologic therapy in patients with RA who are refractory to non-biologic disease-modifying antirheumatic drugs (DMARDs), due to the availability of long-term data from clinical trials and extensive real world experience. Moreover, costs have recently lowered due to the advent of biosimilar TNFis. Infliximab, adalimumab, and etanercept (in bio-original and biosimilar forms) are the most frequently used TNFis, with the most available data. Here, we discuss the utility and clinical relevance of TDM and immunogenicity testing of TNFis in patients with RA, and potential future immunopharmacological strategies to personalize disease management.

IMMUNOGENICITY OF TNFIS IN RA

Consequences of Immunogenicity

Immunogenicity can impact both the efficacy and safety of BPs. ADAs may reduce the clinical efficacy of TNFis by competing with the cytokine binding site (neutralizing antibodies) or by accelerating drug clearance leading to subtherapeutic drug levels (non-neutralizing/binding antibodies; with formation of immune complexes), hence both neutralizing and non-neutralizing ADAs may be clinically relevant. Trough TNFi drug levels exhibit a “concentration-response” relationship (4) (an inverse correlation with clinical outcomes), which forms the basis for the rationale for TDM in RA. This has been observed in studies of the key TNFis used in clinical practice—including infliximab (5–10), adalimumab (11), etanercept (12, 13), golimumab (14), and certolizumab (15, 16).

ADAs are associated with low trough drug levels and loss of drug efficacy, although the association appears to be stronger for infliximab, adalimumab, and golimumab, than for etanercept and certolizumab (4). ADAs in isolation do not always correlate with poor clinical outcomes, as the antibody titer may be insufficient to reduce the active drug level below the therapeutic threshold. Furthermore, the risk of immunogenicity is not sufficient to predict loss of drug efficacy e.g., although adalimumab is more

immunogenic than etanercept, some studies report only a small difference in drug survival (17, 18).

ADAs have been linked to several AEs including infusion/injection site hypersensitivity reactions, serum sickness, and arthus reactions (1, 19). The pathogenic mechanisms are yet to be fully elucidated and may involve complement-mediated events, cytokine release, formation of immune complexes, and production of IgE antibodies. Reassuringly, switching from bio-original to biosimilar BP, has not been associated with greater AEs or immunogenicity concerns thus far (20).

Factors Influencing Immunogenicity

Historically, the foreign (murine) components of the drug were thought to be mainly culpable for the development of ADAs, which led to a drive to minimize non-human elements to reduce immunogenicity. It soon became apparent that even fully human BPs could provoke an immune response, due to TNF-binding idiotypes that are not part of the normal human antibody repertoire, and multiple factors influencing immunogenicity are now emerging. TNFis may be chimeric (e.g., infliximab), humanized (e.g., certolizumab), fully human (e.g., adalimumab and golimumab), or fusion proteins containing antibody fragments (e.g., etanercept). Infliximab is considered the most immunogenic TNFi, particularly when it is used without concomitant methotrexate (21, 22). ADAs have been reported in up to 53% of patients treated with infliximab within the first 6 months of treatment (5, 8, 23–25). By contrast, in the same timeframe, up to 19% of patients receiving adalimumab develop ADAs (8, 24, 26). Etanercept, a receptor construct, does not express idiotypes and thus is the least immunogenic out of the three; ADAs to etanercept are minimal, usually transient and non-neutralizing with a reported incidence of 0–7% (21, 27, 28).

Effective detection of ADAs is dependent upon several factors—the type of the assay used, the timing of the blood sample in relation to drug dosing (usually trough levels, taken before a scheduled dose) and the duration of treatment. In addition, assay results are affected by the relative amount of drug and antibody: excess serum drug levels can prevent the detection of free ADAs; equal drug and antibody levels can prevent measurement of both; and excess ADA usually permits only the detection of free antibodies (29).

Mechanisms leading to immunogenicity are complex and multifactorial; related to the drug (e.g., purity and aggregations) and its production process (e.g., contaminants), the patient and treatment (1, 30). Patient-related factors include genetic predisposition (31), disease activity (32), obesity (32), smoking (32), and indication (33) for biologic treatment. It is tempting to speculate that ADAs are more likely to be evoked in classical autoimmune diseases, where B-lymphocytes are implicated in disease pathogenesis, e.g., a trend toward higher frequency of ADAs is found in patients with RA compared with psoriasis, when treated with the same biologic (4). However, ADAs are clinically relevant in non-antibody-mediated rheumatic conditions e.g., axial spondyloarthritis (34) and are extensively described in inflammatory bowel disease (IBD). This concept was exemplified in a study of patients with spondyloarthritis ($n = 294$) and rheumatoid arthritis

($n = 276$) with secondary TNFi failure, where significantly more patients with spondyloarthritis (31.3%) had anti-infliximab antibodies, compared with those that had RA (21.1%; $p = 0.014$) (33). Treatment-related factors include the dose, frequency, route, and continuity of administration, prior drug exposures as well as concomitant immunomodulators (35). In general higher doses of the BP or a loading regimen (36) followed by continuous rather than episodic dosing (37), the intravenous (compared with subcutaneous) (38, 39) route of administration and concomitant immunosuppression (28, 40) are associated with a lower frequency of ADAs. However, there are some caveats—subcutaneous delivery (relatively more immunogenic and usually the preferred route of administration for most BPs) of tocilizumab (an anti-interleukin (IL)-6 receptor monoclonal antibody) is not more immunogenic than its intravenous administration (41) and whilst concomitant immunosuppressants reduce immunogenicity in RA and Crohns disease (28, 40), evidence for this strategy is not valid across all indications e.g., methotrexate co-prescription does not significantly influence drug survival of TNFis in psoriatic arthritis populations (42).

Limitations of Immunogenicity Testing

The clinical application and interpretation of immunogenicity data is challenging as studies of TNFis show wide variation in the prevalence of ADAs, as well as their impact on serum drug concentrations and clinical outcomes. These observations may be due to heterogeneous patient populations and differences in study design, duration of follow-up, drug dosage, use of concurrent DMARDs and timing of blood sampling. Comparisons between publications are difficult due to inter-laboratory variability and inconsistent (and occasionally absent) reporting of assay methods and characteristics. Furthermore, it is very difficult to make comparisons between different assays for different BPs, due to the reliance of each method on the specific positive control used (43).

Even if detection methods are reliable, most available assays do not evaluate the *in vivo* functionality of drug and ADAs, i.e., the amount of active circulating drug or the neutralizing capability of the ADA, which could limit the clinical application of the results.

ADA detection involves either a bridging ELISA (most commonly), or a radioimmunoassay (RIA). Available RAIs include the antigen binding test (radiolabelled therapeutic TNFi antibodies bind to free ADAs in serum samples) or pulldown assays (ADAs are coupled to a high-capacity solid substrate). Both ELISAs and RIAs are only able to detect free ADAs; therefore, high drug levels, with formation of ADA-drug complexes, can lead to false negative results. This is known as “drug interference/tolerance,” where ADAs are only detected if their amount exceeds the level of the circulating drug. ELISAs can further underestimate the presence of ADAs, as they do not identify IgG4 ADAs [which are more likely to be neutralizing (44)] and are less drug-tolerant than RIAs. RIAs are more specific than bridging ELISA, are less prone to interference by drug and rheumatoid factor and can capture clinically relevant IgG1 and IgG4 ADA. RIAs are more sensitive than ELISAs when using random blood samples [with better concordance between the

assays when ADA titres are high (45)], which would be more convenient for patients, however their widespread use is limited by the cost and complexity associated with radioisotopes.

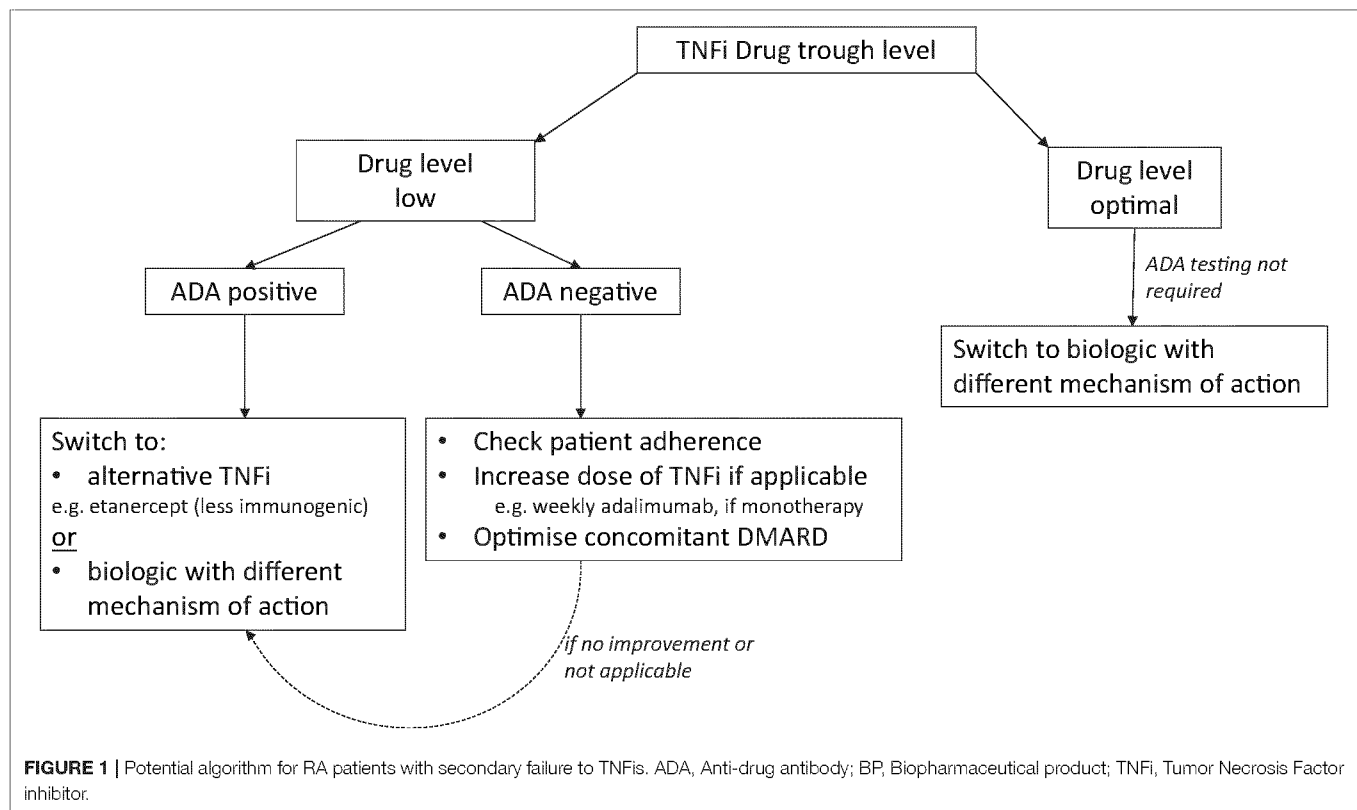
From a practical perspective, TDM and immunogenicity testing can be difficult. Ease of access to tests is variable, and it may be difficult to obtain accurately timed blood samples for trough drug levels. Newer drug-tolerant assays that measure both free and complexed ADAs, including the pH-shift anti-idiotypic binding tests (PIA), may be more suited to random blood sampling, but these tests are expensive, may only be available in specialized centers and have as yet, undetermined clinical utility (46).

CURRENT CLINICAL PRACTICE

Current options for managing TNFi failures in RA include cycling within class, i.e., to an alternative TNFi, or switching between class i.e., to a drug with a different mechanism of action. Published recommendations provide little guidance to determine the best strategy (47, 48). Both options are supported by data from randomized controlled trials and the real world, therefore the decision is generally empirical and based on physician discretion. This dilemma was summarized in a recent review (29). In the open-label, 52 weeks randomized Rotation or Change (ROC) trial, the treating physician selected between a second TNFi and a non-TNFi in patients with primary TNFi failure (49). The ROC trial results concluded that the reasons for improved drug survival when switching to a second TNFi was better efficacy, and with switching to a non-TNFi was reduced AEs. Further evidence from a prospective study, suggests better outcomes can be achieved using an algorithm based on trough drug levels and ADAs, compared with “empirical switching” (50).

Current treatment recommendations for RA endorse combination therapy with a biologic and DMARD (47, 48), which is consistently more effective than biologic monotherapy, possibly due to effects on immunogenicity. Methotrexate significantly increases adalimumab trough concentrations (51, 52), and in a dose-dependent manner, reduces immunogenicity (51), and improves clinical outcomes in early disease (53).

Given the limitations regarding assay diversity and data interpretation, and the lack of conclusive support for cost-effectiveness, routine use of TDM and ADA testing has not been widely adopted in British Rheumatology practice (54). There are exceptions, with local management algorithms for RA incorporating these tests (55, 56), but overall the use and interpretation of TDM and ADAs is inconsistent. By contrast, The British Society for Gastroenterology guidelines for the management of IBD includes clear, algorithmic recommendations for measurement of drug levels ($\pm$ ADA) (57). In IBD, clinical decision making using drug levels and ADAs in secondary non-responders is more cost-effective when compared to empirical drug escalation (58, 59). The recent prospective, observational personalized anti-TNF therapy in Crohn's disease study (PANTS), demonstrated that low concentrations of adalimumab and infliximab at week 14



were associated with primary non-response, non-remission at week 54 and the development of ADAs (32). ADAs predicted subsequent low drug levels and concomitant immunomodulators (thiopurine or methotrexate) mitigated the risk of developing ADAs (32).

POTENTIAL IMMUNOPHARMACOLOGICAL ALGORITHM

In time, readily available, accurate assays to measure drug levels and ADA titer, will hopefully arm clinicians with powerful tools to optimize the management of RA, especially in patients with secondary loss of response. A potential algorithm that could be used in future management strategies is shown in **Figure 1**.

Measurement of trough drug levels is the most valuable test in the first instance to identify patients with low or optimal (therapeutic) circulating drug. Using ADAs for the first branching in the algorithm is probably inappropriate, as ADAs are not always clinically relevant (especially if present at low-titer) if there is sufficient circulating drug. In cases of treatment failure, supplementary knowledge of ADAs (and perhaps the titer) may be helpful in determining the etiology of suboptimal drug levels. Low drug levels without ADAs may be due to factors such as poor adherence to therapy (as most biologics are self-administered injections), a higher BMI and/or faster drug metabolism, which would require different strategies compared with those for patients with detectable ADAs. To overcome this

problem, optimizing the dose of biologic by reducing the interval of administration, e.g., changing adalimumab monotherapy from fortnightly to weekly [as permitted by the National Institute of Health and Care Excellence (NICE) in the U.K. (60)], or optimizing dose of concomitant immunosuppressants may recapture a response (61, 62). Emerging evidence suggests that efficacy can be re-established in ADA positive patients with secondary failure, by addition of methotrexate to infliximab treatment in IBD (63), although there is limited support for this approach in the RA literature. If these strategies are unsuccessful or not applicable, switching BP should be considered.

If ADAs are detected in the context of a low drug level, switching to a less immunogenic drug within the same class (e.g., etanercept) could be beneficial, especially if the patient has previously responded to a TNFi. Switching to a second TNFi may be successful due to differences in drug molecular structure, immunological action, immunogenicity, and pharmacokinetics, as well as different underlying disease pathogenesis (24). There is an argument however, to switch to a biologic with a different mechanism of action, as although ADAs are not cross-reactive, patients with ADAs to the first failed TNFi are more likely to seroconvert and produce ADAs with subsequent TNFis (64–67) and are thus less likely to respond to a second TNFi, especially if this is a monoclonal antibody (64, 66). Of note, ADAs to bio-originals are reactive to the corresponding biosimilars, and therefore after detection of ADA, switching a bio-original to its biosimilar version would not be recommended (68). It is plausible to suggest that a patient with ADAs, refractory to

multiple biologics, may benefit from a treatment with a less or minimally immunogenic drug, e.g., a receptor fusion protein e.g., abatacept (69) or a small molecule [JAK inhibitor (70)]. In the case of non-responders with optimal drug levels, the presence/absence of ADAs is unlikely to influence subsequent management. These patients have a lower probability of response to an agent within the same class and therefore we would postulate that they are most likely to benefit from switching to a drug with a different mechanism of action (64).

Given the high cost and potential AEs associated with biologic therapies, strategies have been proposed to taper biologics (by reducing drug doses or increasing dosing intervals) in patients with sustained clinical remission, thereby reducing risks and costs overtreatment. In some studies, correlation between DAS28 (disease activity score; a composite measure of disease activity in RA) improvement and serum drug trough levels has been verified up to a threshold of drug level, above which no significant DAS28 changes occur (71). A recent study using certolizumab found that a drug level above a defined threshold was not associated with any additional clinical benefit, and therefore it may be possible in the future to use TDM to titrate treatment (15). Withdrawal of treatment in disease quiescence is an area of active research and currently there is insufficient evidence to draw meaningful conclusions about the role of TDM and immunogenicity testing. Data from ongoing, randomized controlled trials (72) using TDM or ADA to guide withdrawal strategies may inform future practice. It is reasonable to hypothesize that drug withdrawal may be possible in patients with inactive disease and undetectable drug levels or high ADA titres, as remission is probably not being maintained by treatment with the BP.

FUTURE DIRECTIONS AND UNANSWERED QUESTIONS

The increasing and earlier use of BPs in RA is likely to lead to a greater proportion of patients receiving these therapies. Efforts

are expanding to predict, reduce and reverse BP immunogenicity to mitigate the impact on drug development, which was summarized in a recent review (73). Strategies to reduce the immunogenic potential of BPs include “de-immunizing” approaches through protein engineering e.g., rational amino acid substitutions and/or addition of epitope-masking moieties, as well as induction of peripheral tolerance (73). There are emerging concerns that immunogenicity may limit the development of newer investigational medicinal products such as the bispecific antibodies.

Despite long-standing interest and accrual of data, we are still unable to predict responses to TNFi. Prospective, longitudinal studies of BP-naïve patients may provide mechanistic information and address a critical unanswered question—why BPs are immunogenic in some patients, but tolerogenic in others. Prediction of immunogenicity may allow mitigation and management strategies to be implemented to prevent or minimize the generation of ADAs (73). Other strategies to personalize biologic selection, include pharmacogenetic testing to identify genetic factors that may predict lack of response to, or toxicities from, TNFi (74).

Further research is needed to develop standardized, clinically-validated assays for both drug and ADA testing. These tests could then be incorporated into evidence-based guidelines to optimize treatment decisions along the patient pathway: for patients with active disease about to start treatment, not responding to treatment (primary or secondary failure) or for those in remission, to permit drug tapering strategies. Taken together this may help to improve the long-term efficacy, safety profile and cost-effectiveness of BPs.

AUTHOR CONTRIBUTIONS

PM and JM co-wrote this manuscript and both authors approved the final version.

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Applying MAPPs Assays to Assess Drug Immunogenicity

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Immunogenicity against biotherapeutic proteins (BPs) and the potential outcome for the patient are difficult to predict. *In vitro* assays that can help to assess the immunogenic potential of BPs are not yet used routinely during drug development. MAPPs (MHC-associated peptide proteomics) is one of the assays best characterized regarding its value for immunogenicity potential assessment. This review is focusing on recent studies that have employed human HLA class II-MAPPs assays to rank biotherapeutic candidates, investigate clinical immunogenicity, and understand mechanistic root causes of immunogenicity. Advantages and challenges of the technology are discussed as well as the different areas of application.

Keywords: MAPPs, immunogenicity, HLA, proteomics, dendritic cells, drug development, ABIRISK, biotherapeutics

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INTRODUCTION

Anti-drug antibodies (ADAs) have been shown to have broad and diverse effects ranging from any clinical significance to loss of efficacy of the BP, impact on PK/PD profile, hypersensitivity responses, or in the worst case the cross-reactivity and neutralization of the endogenous counterpart of the BP. Understanding and predicting immunogenicity against BPs is a major challenge. There is a trend in using *in vitro* assays to assess the immunogenic potential of biotherapeutics, but they are not used routinely during drug development. For several years, MAPPs (MHC associated peptide proteomics) has been applied on many different types of immunological questions (Table 1) and can be considered as the assay best characterized regarding its value for immunogenicity potential assessment. While the use of the MAPPs technology for the identification of immunogenic hotspots of biotherapeutics has been reviewed previously (1), this review is focusing on recent studies that have employed human HLA class II-MAPPs assays to rank biotherapeutic candidates, investigate clinical immunogenicity, and understand mechanistic root causes of immunogenicity.

T cell immunity is a key element in the development of immunogenicity against proteins. Specific T cell receptor-mediated recognition of HLA class II associated peptides presented by professional antigen presenting cells (APCs), as well as co-signaling via co-stimulatory molecules and cytokines are required to activate CD4+ T cells. For the development of an adaptive immune response, help by CD4+ T cells is required to mount an efficient B cell response and the production of high-affinity IgG class antibodies (Figure 1). Naturally presented HLA class II-associated peptides, which are the prerequisite for a specific T cell response, can be determined via MAPPs.

The MAPPs assay consists of multiple steps, involving human primary antigen presenting cell culture, peptide isolation, and peptide identification via liquid chromatography-mass spectrometry (LC-MS). Professional antigen presenting cells are loaded with proteins of interest, which are internalized and enzymatically processed to peptides in the endolysosomal compartment of the cells. The binding to HLA class II molecules is dependent on the properties of the amino acid side

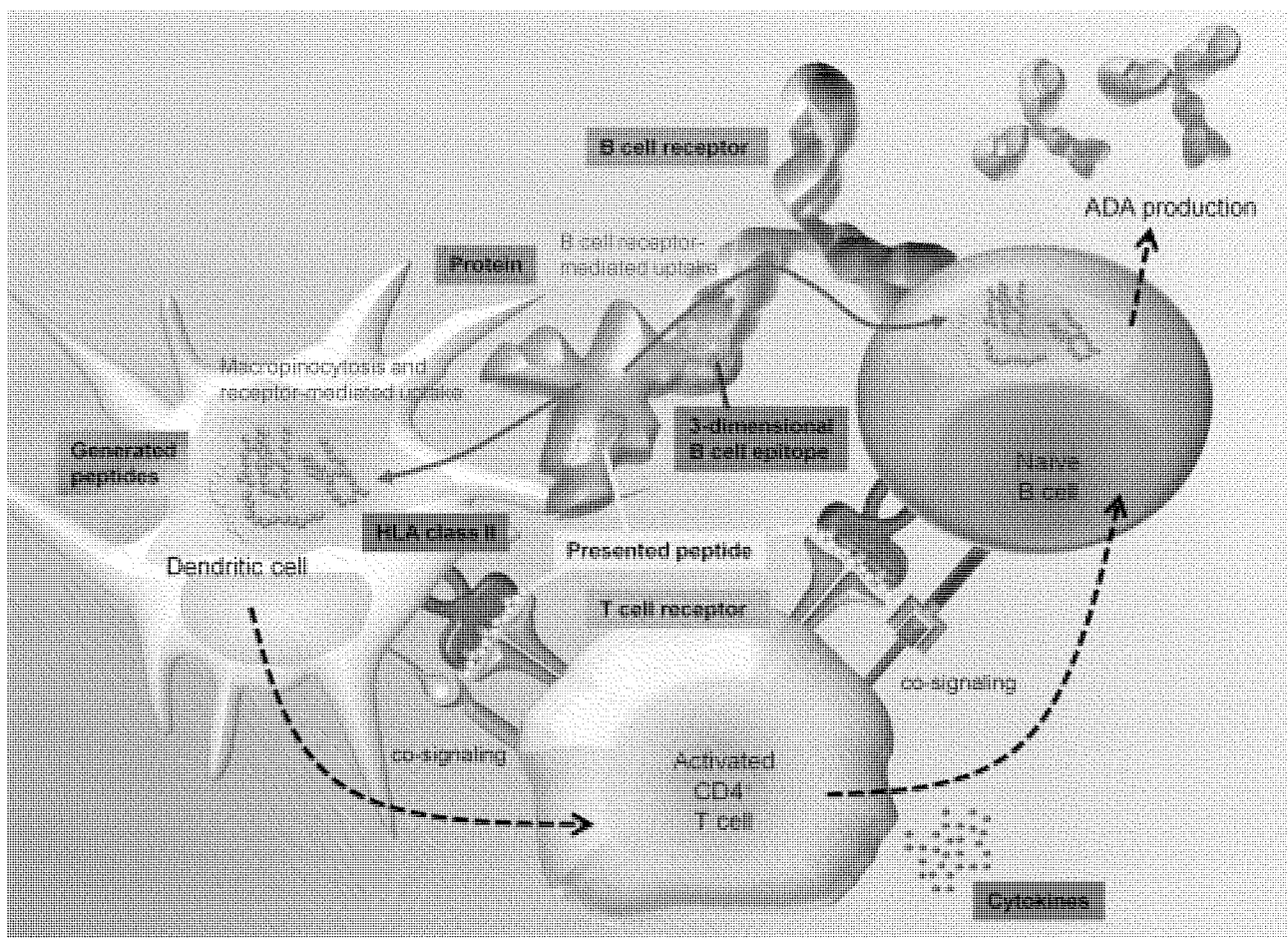
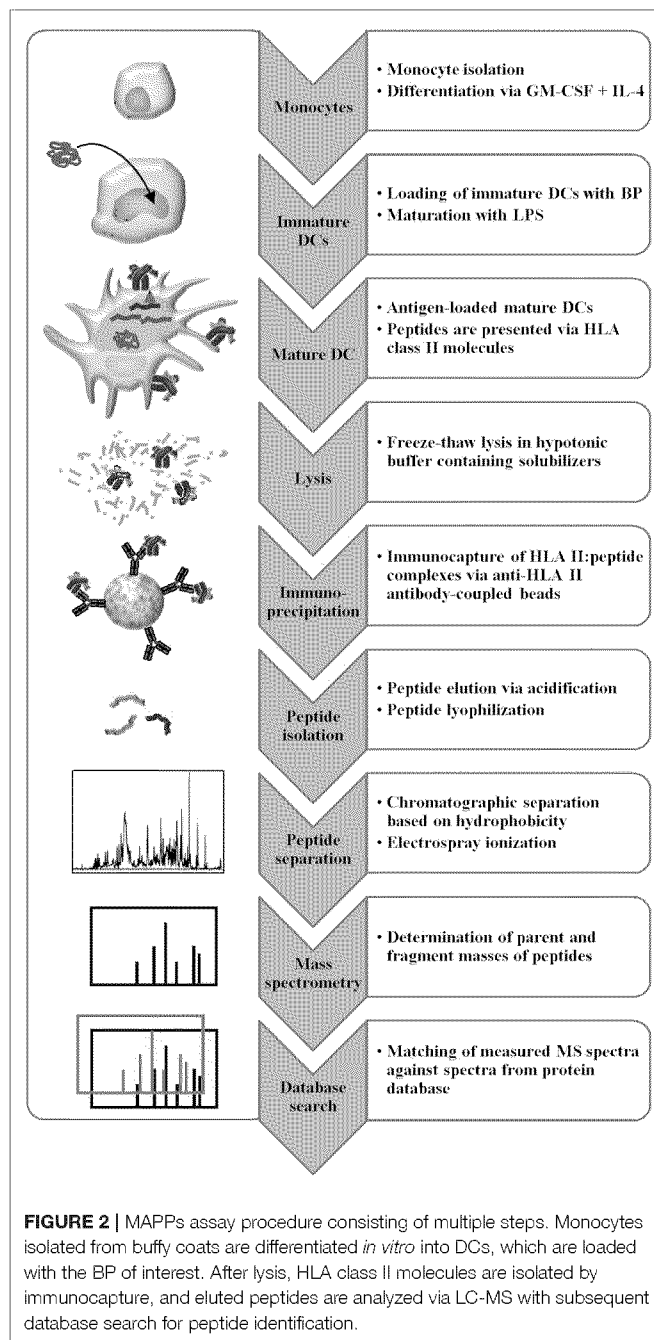


FIGURE 1 | Interplay between DCs, T cells, and B cells. Dendritic cells sample antigens mainly via macropinocytosis and receptor-mediated uptake and have access to a broad range of extracellular proteins. In contrast, B cells mainly take up antigens via specific recognition of structures on an antigen via their surface B cell receptor. T cells that have become activated by specific recognition of an antigen-derived HLA class II-associated peptide on DCs via their T cell receptor, can in turn activate B cells that are presenting the same sequences.

chains of a given peptide and the binding pockets of a given HLA class II molecule (2). HLA class II molecules are highly polymorphic and their peptide binding groove is open at both ends enabling the binding of different peptide length variants (3). Despite the high polymorphic diversity, the peptide binding repertoire of HLA molecules is not unlimited and there is an overlap in peptide binding specificities between HLA types (4). Following incubation with the protein of interest, the naturally processed and presented peptides are retrieved from the APCs by cell lysis, followed by membrane solubilization. Then, HLA

class II: peptide complexes are isolated by immunocapture using anti-HLA II antibody-coated beads. In the following wash steps, solubilizers are removed before the peptides are eluted from the HLA class II binding groove by pH shift and are further analyzed via immunopeptidomics, which is defined as the characterization of peptide ligands bound to HLA and related molecules. Peptides are separated according to their hydrophobicity via liquid chromatography prior to electrospray ionization and mass spectrometric analysis. The determination of the full mass and fragment masses of the peptides enables sequence identification via database search by comparing the measured mass spectra to calculated mass spectra generated from peptide sequences derived from a large protein database. The identified peptides are then mapped to the sequence of the BP (Figure 2). Identified peptides often accumulate in several sequence regions of the BP, referred to as “clusters” (5, 6). Since different donors will only present certain sequence regions/clusters based on their HLA class II alleles, the analysis of about 20 donors is required to cover most of the presentable sequence regions.

Abbreviations: PK/PD, pharmacokinetics/pharmacodynamics; mAb, monoclonal antibody; DC, dendritic cell; APC, antigen presenting cell; BP, biotherapeutic protein; HLA, human leukocyte antigen; mDC, myeloid dendritic cell; moDC, monocyte-derived dendritic cell; pDC, plasmacytoid dendritic cell; ADA, anti-drug antibodies; MAPPs, MHC associated peptide proteomics; PTM, post-translational modification; MS, mass spectrometry; LC, liquid chromatography; CDR, complementary determining region; FR, framework region; HCDR, heavy chain complementary determining region; LCDR, light chain complementary determining region; HFR, heavy chain framework region; LFR, light chain framework region.



MAPPs ASSAY APPLICATIONS

Due to the challenges with immunogenicity against biotherapeutics, methods that could potentially predict immunogenicity in human beings would be a game changer for drug development. One of the major caveats is the high variability of the published clinical immunogenicity data. Reported immunogenicity incidences are influenced by a multitude of aspects including patient-, treatment-, and sampling-related factors. Furthermore, they are impacted by the sensitivity, drug tolerance, and type of immunogenicity

assay used to measure anti-drug antibody (ADA) responses in patients. A meaningful correlation between *in vitro* data and clinical immunogenicity of different marketed biotherapeutics would require the generation of clinical immunogenicity data with harmonized assays and sampling in clinical trials, which is practically utopian to achieve.

The development of immunogenicity in an individual subject is dependent on multiple factors such as the presentation of BP-derived peptides via HLA class II molecules, the recognition of these peptides as well as co-stimulatory signals by T cells, the precursor frequency of responsive T cells, the recognition of the BP by B cells via the B cell receptor, the precursor frequency of such B cells, the efficiency of cell interaction in the lymph node and resulting affinity maturation, immune status, HLA haplotype, and the target biology of the BP, just to name a few. The MAPPs assay is covering one of the key contributing factors, the natural presentation of protein-derived peptides to T cells, which is the prerequisite for the development of a specific IgG-type immune response. The ability of the peptides to trigger T cell responses has to be addressed via subsequently applied T cell assays. Since the development of immunogenicity and the incidence of ADA in the patient population are depending on many more factors, the MAPPs assay data should never be considered as a direct prediction of immunogenicity incidence in human beings. Instead, MAPPs should be understood as a useful and relevant tool to: (1) rank similar protein variants regarding their immunogenicity potential and support candidate selection, (2) identify root causes for clinical immunogenicity, (3) confirm the sequences predicted by *in silico* algorithms to characterize and further improve them, and (4) improve the mechanistic understanding of principles of antigen presentation as well as factors that are contributing to the development of immunogenicity.

Selection of Biotherapeutic Candidates by MAPPs Assay Ranking

Due to the abovementioned caveats, absolute immunogenicity incidence rates between marketed BPs cannot be directly compared. Reported ADA incidences for a given BP can also vary significantly across studies depending on indication, co-medication, and assay format. Still, it becomes apparent that some BPs seem to have relatively low reported immunogenicity rates across many studies and indications, while other BPs seem to consistently show higher immunogenicity incidence rates. We have previously applied MAPPs and T cell activation assays on a panel of marketed monoclonal antibodies, secukinumab, adalimumab, ustekinumab, infliximab, and rituximab (6). In this study, molecules that showed on average a rather low clinical immunogenicity, also showed lower numbers of presented sequence regions and low T cell response rates. In contrast, monoclonal antibodies with elevated clinical immunogenicity rates also showed increased numbers of presented sequence regions and increased T-cell response rates in T-cell activation assays, indicating an approximate correlation between *in vitro* assay results and clinical immunogenicity incidence (6). This study indicates, that the number of presented sequence regions

may be a useful information to rank similar BP candidates during drug development. Since differences in the amino acid sequences of BPs will impact on the type and number of presented peptides, the MAPPs assay should best be applied for the ranking of similar BP variants as typically generated during the candidate selection phase in drug development.

Interrogation of Clinical Immunogenicity

The ABIRISK Project (www.abirisk.eu) of the European Innovative Medicines Initiative (IMI) investigated the correlation between patient, clinical factors and the incidence of immunogenicity and examined the underlying mechanisms of immunogenicity. As part of this project, the MAPPs technology was applied to identify naturally presented sequence regions of several biotherapeutics.

Infliximab

In the scope of the ABIRISK project, infliximab, a chimeric monoclonal anti-TNF α antibody, was examined via MAPPs and T cell epitope mapping assays using different sets of healthy donors as well as patients with inflammatory bowel disease or rheumatoid arthritis that had developed immunogenicity against infliximab. Despite the use of different donors, most of the identified T cell epitopes were also identified as naturally presented peptides by MAPPs. As expected, not all presented sequence regions identified in the 34 healthy donors via MAPPs were identified as true T cell epitopes in the sets of 16 healthy drug-naïve donors and 6 ADA positive treated patients. This can partially be explained by lower patient numbers and smaller HLA coverage in the T cell assay. Moreover, the T cell repertoire is shaped by central tolerance and peripheral tolerance, which are both impacting on the T cells precursor rates against different peptide sequences, and which are ultimately determining whether a donor will or will not respond. An independent study also employed MAPPs on the determination of presented sequence regions of infliximab on 9 donors (7). The comparison of both independent datasets reveals a high degree of similarity in terms of the location of presented sequence regions. In both studies, clusters in sequence regions were detected that were matching confirmed T cell epitope hotspots in patients showing immunogenicity (8), specifically: (1) a region slightly upstream of and spanning across the HCDR2 and (2) a region slightly upstream and spanning across HCDR3 and (3) a framework region in LFR1. Additional presentation hotspots were identified in both studies in HFR3 as well as in LFR3. Only one prominently presented sequence region in LCDR3 was only detected in the study by Hamze et al., likely due to the larger set of tested donors.

Adalimumab

Adalimumab, a human monoclonal anti-TNF α antibody, was tested in the MAPPs assay as part of the ABIRISK project (9) on 18 healthy donors. Eight clusters were identified in the VH region and four clusters in the VL region. Four of these presented regions, HCDR2, HFR3, HCDR3, and LCDR2 were also confirmed as T cell epitopes on an independent set of 14 healthy drug-naïve donors. In an unrelated study, Sekiguchi et al.

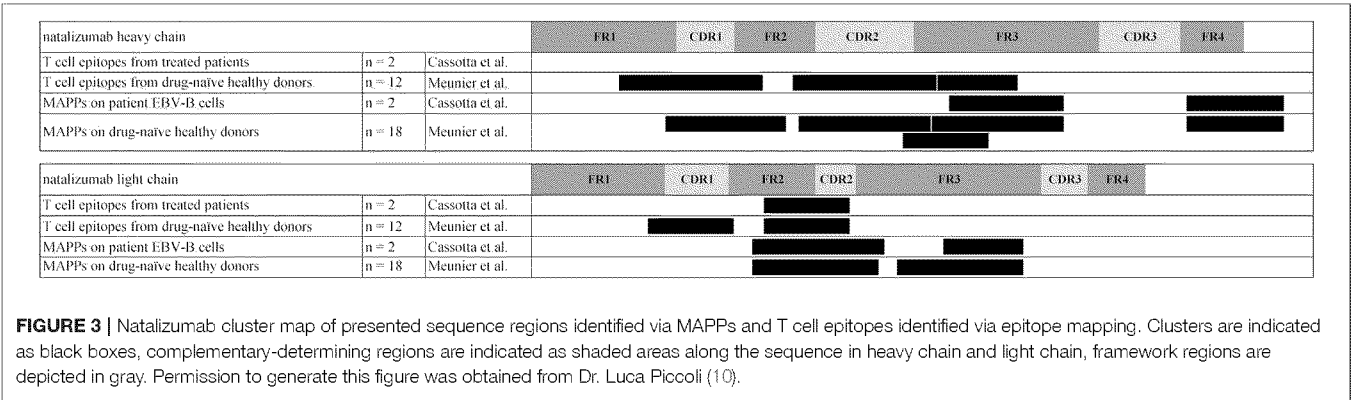
evaluated the presented sequence regions on a small set of 2 donors (7). Still, three presented sequence regions could be observed in VH and three in the VL region. Almost all of these clusters matched regions identified by Meunier et al., except for 1 cluster in LFR1 that was only identified by Sekiguchi et al. This discrepancy can be explained by the use of a different anti-HLA class II clones G46-6 and L243 in the two studies. Interestingly, this cluster in LFR1 was identified in our hands using anti-pan class II antibody clone IVA12 in an independent study (data not shown).

Natalizumab

Two groups, Cassotta et al. and Meunier et al. applied MAPPs to identify naturally presented peptides from natalizumab, a humanized monoclonal antibody directed against α 4-integrin. In the study of Meunier et al., which was performed as part of the ABIRISK project, T cell epitopes resided in HCDR1, HCDR2, and HFR3 as well as LCDR1 and at the interface between LFR2 and LCDR2 (9). Presented sequence regions identified via MAPPs were detected in the same regions, except LCDR1, likely due to the use of different donor sets. Additional presented regions resided in HFR4 and LFR3. In an independent study, Cassotta et al. applied MAPPs on natalizumab-specific EBV B cell clones that were isolated from two patients who had developed immunogenicity against natalizumab. The researchers could identify 3 naturally presented sequence regions that matched the regions identified in the ABIRISK study. Two of these regions were located in HFR3 and the HFR4, both of which did not induce specific T cell clones. The third presented region at the interface of LFR2 and CDR2 was also recognized by natalizumab-reactive T cell clones (10). This study shows, that natalizumab-specific B cells were able to recognize and internalize natalizumab via their BCR and subsequently present a natalizumab-derived peptide sequence that was verified as a natalizumab-specific T cell epitope. The interplay of linear HLA-DR associated peptide presentation by APCs, subsequent T cell response and T cell-mediated activation of B cells which are also presenting linear HLA-DR associated peptides upon BCR mediated antigen-specific protein uptake (**Figure 1**) are demonstrated in this study (10). A comparison of presented sequence regions and identified T cell epitopes is shown in **Figure 3**.

Ixekizumab

In a recent study (11), Spindeldreher et al. identified T cell epitopes of ixekizumab, a chimeric monoclonal antibody directed against IL-17a, from 31 healthy treatment-naïve donors via MAPPs-assisted T cell epitope mapping. All identified T cell epitopes resided in four main regions of the mAb, which were overlapping with the regions HCDR3, LCDR1, LCDR2, and LCDR3. For the epitope mapping approach, synthesized peptides from overlapping peptide libraries were used as well as peptides synthesized from presented sequences identified via MAPPs. We concluded, that the approach of MAPPs-assisted T cell epitope mapping appeared to be superior over “traditional” peptide-scanning approaches, and that the sequences identified by MAPPs represent antigenic regions which



underwent intracellular processing of the full-length protein and may thus present “authentic” T cell epitopes.

Human Factor VIII

The immune response to factor VIII is a significant complication in the treatment of patients with hemophilia A. In a recent study, MAPPs was applied to identify potential T cell epitopes of human factor VIII (12). A similar dataset was generated in our group in the scope of the ABIRISK project and a list of identified factor VIII-derived peptides is available in **Supplementary Data 1**. A comparison of the two datasets reveals a considerable similarity in presented sequence regions despite the use of completely independent healthy donor sets with potentially different HLA allele representation, as shown in **Figure 4**. Differences between the studies are observable in domains B, A3, C1, and C2. In the B domain, few presented regions were only identified by Jankowski et al., likely due to the higher number of tested donors. Additional sequence regions were identified in the ABIRISK study in domains A3, C1, and C2. Discrepancies between the 2 studies in the ability to detect certain sequences may be related to the HLA distribution and number of tested donors, type of tested factor VIII material and assay sensitivity.

Improving Mechanistic Understanding of Principles of Antigen Presentation

MAPPs is a powerful tool to interrogate the mechanistic principles of antigen presentation. We have previously generated data comparing the peptide presentation between monocytes, monocyte-derived DCs (moDCs), and B cells (20). Monocytes, B cells, and moDCs presented synthesized peptides to a comparable level, which can be explained by surface exchange. In contrast, moDCs were the most efficient cells in peptide presentation from full proteins such as ovalbumin, tetanus toxoid, and trastuzumab, likely due to higher uptake efficiency via macropinocytosis. In order to compare the pattern of presented peptides, two molecules were selected that were expressed by all three cell types and which were less influenced by the differences in antigen uptake processes between cell types. For the two selected intrinsic molecules (HLA-DRA chain and HLA-B), B cells and monocytes showed the same presentation pattern. MoDCs showed additional clusters, which could be due to a higher

HLA-DR expression on moDCs combined with the stronger membrane uptake via micropinocytosis, increasing the amount of presentable HLA-DRA and HLA-B derived peptides and boosting detection efficiency via MS. Alternatively, the additional clusters observed in moDCs may also be explained by differences in the composition of lysosomal proteases (21).

In another study focusing on the naturally presented peptide repertoire of APCs, Adamopoulou et al. applied the MAPPs technology to interrogate the HLA class I and class II human thymic peptide repertoire. They identified peptides eluted from whole thymus, from isolated thymic myeloid DCs (mDCs) and from mDC-depleted APCs containing pDCs, thymic epithelial cells, and other thymic APC (16). According to the authors, only about a third of the protein pool was shared between mDCs and non-mDC cells and the authors concluded that the differences in the presented peptide repertoire are supporting the idea that different thymic APCs present different peptides. The identified HLA class II peptide sequences provided in **Supplementary Data 1** of that study reveals, that within each dataset, several peptide families/clusters with shared core sequences were identified, each comprising several similar peptide lengths variants. Therefore, a comparison of peptide clusters rather than distinct peptide sequences is more meaningful, and shows that a presentation similarity between the mDC and mDC-depleted samples is actually much higher than postulated in the publication.

Another mechanistic study employed MAPPs to interrogate two potential modes of action of intravenous immunoglobulin (IVIg), which is commonly used in the clinic to treat autoimmune and severe inflammatory diseases (19). Numerous IgG-derived peptides were presented by moDCs along the antibody sequences, while surprisingly regulatory T cell epitopes 167 and 289 were not efficiently presented. Some sequences cannot be efficiently identified due to technical reasons such as strong hydrophobicity preventing efficient elution from the chromatography column, poor ionization and fragmentation in the mass spectrometer, or degradation of the peptides due to extensive protease activity during cell culture. To rule out that regulatory T cell epitopes 167 and 289 were potentially missed in the experiment due to technical challenges, the researchers confirmed efficient detection via mass spectrometry

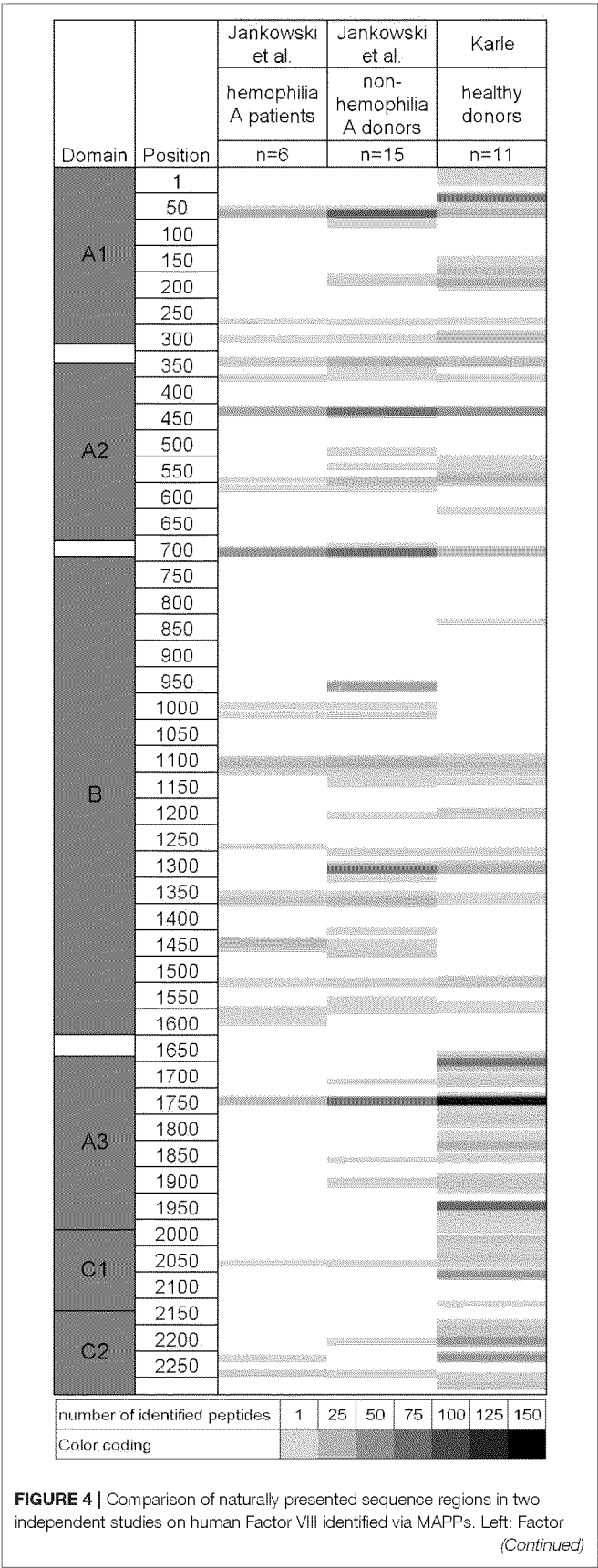


FIGURE 4 | VIII protein domains and amino acid positions. For each dataset, identified peptides are indicated as shaded areas along the amino acid sequence from top to bottom. The number of identified peptides per position is indicated by grayscale color coding. Raw data and permission to generate this figure was obtained from Dr. Zuben Sauna, Joseph McGill, and Dr. Wojciech Jankowski (12).

by direct infusion. They could also show that in DCs loaded with synthesized regulatory T cell epitopes 167 and 289, these sequences were efficiently presented and identified via MAPPs. The results suggest that regulatory T cell epitopes 167 and 289 can be efficiently presented by DCs and detected via MAPPs but only when provided to the cells as synthesized peptides, whereas they do not seem to be efficiently processed and presented as part of a full antibody protein. In another experiment the researchers could show that the simultaneous loading of moDCs with Ovalbumin and high concentrations of IVIg did not impair Ovalbumin-derived peptide presentation, indicating that a sufficient amount of empty HLA class II molecules are present in the endolysosome to bind all generated peptides. The absence of antigenic competition was confirmed by analyzing peptides derived from two endogenous self-proteins that are typically presented on HLA class II molecules, ANXA2 and MAN2B1, which also remained unaffected by co-loading of the cells with high doses of IVIg. The authors concluded that the clinical effects mediated by IVIg are neither caused by Tregitopes 167 and 289 nor by impaired antigen presentation.

Webster et al. investigated the potential of human IgG1 allotypes to stimulate CD4+ T cell responses in donors matched for homologous and heterologous IgG1 allotypes (18). No significant responses against allotypic variants of trastuzumab were observed in allotypic mismatched T cells. MAPPs was employed to investigate the lack of T-cell responses in relation to mismatched allotypes and revealed that no peptides from the sequence regions containing the allotypic variations were presented, and thus, no T cell response was to be expected. The authors concluded that allotypic differences in human IgG1 do not represent a significant risk for induction of immunogenicity.

Assessing the Impact of PTMs, Folding, and Aggregation on Antigen Presentation

Studies have shown that post-translational modifications (PTMs) or aggregation can affect peptide presentation by APCs (5, 15). Using the MAPPs assay, nitration of birch pollen allergen occurring in polluted air has been shown to result in enhanced presentation of allergen-derived HLA-DR-associated peptides. Both, the copy number of allergen-derived peptides as well as the number of presented sequence regions were increased for the nitrated allergen, likely due to changed processing in the endolysosome (15). Frequently recognized T cell-activating regions and peptides created by endolysosomal allergen degradation identified in a related study (22), corresponded to the birch pollen allergen derived peptides identified via MAPPs (15).

TABLE 1 | Summary of studies that employed MAPPs for candidate ranking, investigative, and mechanistic purposes.

References	MAPPs application	Purpose
Karle et al. (6)	Marketed monoclonal antibodies were compared regarding the number of naturally presented sequence regions.	Ranking of candidates
Xue et al. (13)	Determination of naturally presented sequences of an anti-IL-21R blocking antibody ATR-107.	Investigative
Lamberth et al. (14)	Determination of naturally presented peptides of vatreptacog alfa, a bioengineered analog of recombinant FVIIa.	Investigative
Hamze et al. (8)	Determination of naturally presented sequences of infliximab and rituximab from healthy donors. Determination of T cell epitopes in healthy drug-naïve donors as well as patients treated with infliximab or rituximab.	Investigative
Sekiguchi et al. (7)	Determination of naturally presented sequences of infliximab and adalimumab.	Investigative
Cassotta et al. (10)	Natalizumab-derived HLA-DR associated peptides identified from natalizumab-specific EBV-B clones generated from patients that had developed immunogenicity against natalizumab.	Investigative
Jankowski et al. (12)	MAPPs applied to investigate differences in peptide presentation between different FVIII therapeutics.	Investigative
This study	MAPPs applied to determine naturally presented peptides of human FVIII.	Investigative
Spindeldreher et al. (11)	MAPPs-assisted T cell epitope determination of ixekizumab.	Investigative
Meunier et al. (9)	Determination of naturally presented sequences and T cell epitopes of adalimumab and natalizumab in healthy donors.	Investigative
Karle et al. (15)	Changes in birch pollen allergen-related antigen presentation upon allergen nitration revealed by MAPPs.	Mechanistic
Adamopoulou et al. (16)	Identification of peptide repertoire eluted from whole thymus, isolated thymic mDCs and mDC-depleted APCs.	Mechanistic
Scally et al. (17)	T2 cell lines transfected with HLA DRB1*04:01, 04:02, and 04:04 analyzed by MAPPs, enabling an in-depth analysis of the repertoire of bound peptides and establishment of binding motifs.	Mechanistic
Rombach-Riegraf et al. (5)	Increased mAb-derived peptide presentation upon mAb aggregation via artificial heat stress.	Mechanistic
Webster et al. (18)	Comparison of naturally presented HLA class II peptide pattern of human IgG1 allotypes G1m3 and G1m1,17, presented by allotype matched and mismatched donors.	Mechanistic
Sorde et al. (19)	MAPPs assay on IVIg-loaded DCs reveals a broad repertoire of presented IgG-derived peptides.	Mechanistic

In another study we demonstrated that aggregation plays an important role in antigen presentation. Highly aggregated solutions of two model mAbs generated under exaggerated stress conditions induced strong changes in the pattern and quantity of mAb-derived HLA-DR associated peptides presented by DCs (5). An apparent linear relationship was established between the amount of protein contained within the subvisible particles and the increase in presentation. We postulated that the internalization of large densely compacted proteinaceous particles would rapidly increase the effective protein concentration in the endolysosome as compared to uptake of soluble monomeric material and thereby boost presentation.

Jankowski et al. have shown that folding, post-translational modifications or glycosylation patterns of different tested FVIII preparations may contribute to differential enzymatic processing and presentation (12). Plasma-derived FVIII showed fewer presented peptides compared to full length recombinant FVIII.

Since the MAPPs assay has been shown to be sensitive for aggregation, PTMs and alterations in protein folding, the assay can serve as a useful tool to further interrogate aggregation- and protein-structure-related mechanisms of immunogenicity.

ADVANTAGES AND CHALLENGES OF MAPPs

The major advantage of MAPPs is the identification of relevant peptides that have been naturally processed and presented by primary professional APCs. On the other hand, MAPPs is a

costly and time consuming assay and requires cutting edge instrumentation as well as a significant level of expertise to be able to generate high quality data. In our group, hands-on time and turnaround time could be reduced by (1) introducing automation during cell isolation via a robotic system, (2) implementation of a robotic system for bead-washing during peptide isolation, (3) automated LC-MS sample loading and dual column-switching in front of the mass spectrometer to increase throughput, (4) implementation of tailored in-house data processing softwares to significantly reduce the time required for data analysis and reporting. Still, turnaround times for the ranking of up to 10 BP candidates on 24 donors in the MAPPs assay are in a range of 8 weeks, which can sometimes pose a challenge to fit into the candidate selection phase. More importantly, the material amounts and purity required for the MAPPs assay can be a challenge during early phases of a project when BPs are produced in small scale.

MAPPs vs. *in silico* Algorithms

In silico approaches are often considered an alternative approach to MAPPs due to the short turnaround time, low resource requirements and the fact that no BP material is required. Recently, tools have been developed to predict hot spots at protein-protein interfaces (23, 24), but still most of the available tools are classical algorithms based on HLA class II binding data. Although these methods are more cost effective, less time consuming, and easier to perform, the MAPPs assay provides more meaningful and relevant information. The

advantages and limitations of these *in silico* tools for the identification of HLA class II binders have been discussed elsewhere in detail (25). Briefly, most algorithms are trained on data from HLA class II peptide binding experiments which are partially biased by selection, length and solubility of test peptides as well as the folding of the synthetic HLA molecules. Therefore, only a small fraction of the naturally occurring peptidome is represented in the training dataset. Moreover, resulting algorithms are typically not considering the binding contribution of flanking regions outside of the 9-mer binding core.

In contrast, the MAPPs assay enables the identification of relevant peptides that have been naturally processed and presented by primary professional APCs. These cells contain a complex mixture of enzymes that shape the peptide repertoire. While some generated peptides may be able to bind to HLA class II molecules, other sequences may be degraded and removed from the repertoire of presentable peptides.

MAPPs data also offers the opportunity to train new HLA class II binding prediction algorithms, with the potential to improve the predictive power of such new computational tools (manuscript in preparation). Moreover, MAPPs data generated on a multitude of antibodies may help to identify presented sequence stretches that are conserved across antibodies, and which are unlikely to contribute to the immunogenic potential of a given antibody.

MAPPs vs. HLA: Peptide Binding Assays

A previous study by Hamze et al. compared T cell epitope mapping data, MAPPs data, and HLA binding assay data (8). HLA-DR binders identified for the monoclonal antibodies rituximab and infliximab covered most of the variable antibody sequence, which did not allow for proper matching with T cell epitope data. In contrast, presented sequence regions identified via MAPPs were restricted to several distinct areas of the two molecules, which also excellently aligned with T cell epitopes identified from drug-naïve healthy donors as well as treated patients who had developed immunogenicity against rituximab or infliximab (8). While peptides with a fixed length or with poor solubility may give rise to false negative results in HLA-peptide binding assays, they may still be detected via MAPPs, since partially unfolded protein fragments can bind to HLA class II molecules with subsequent trimming by enzymes according to the “bind first, trim later” model (26). Moreover, the chaperone HLA-DM promotes the formation of stable HLA class II peptide complexes (27). This peptide repertoire-editing functionality of HLA-DM is not reflected in binding assays, but it is an integral part of the natural peptide processing in the cells used for the MAPPs assay.

MAPPs vs. T Cell Assays

APCs present a variety of potential T cell epitopes on their surface, and specific reactive T cells in the blood of an individual are required to induce a T cell response. As a result, not every presented sequence region in an individual subject is necessarily acting as a T cell epitope. The MAPPs assay is unable to

discriminate between sequences that will or will not induce a T cell response. T cell assays are often employed using full proteins, and the percentage of responders in a cohort of healthy drug-naïve donors is determined. While such assays yield a response rate, they are not useful in identifying the epitopes that triggered the immune response. Peptide-based T cell assays are suitable to identify T cell epitopes but epitopes with low specific T cell precursor frequencies may be missed. Also, T cell proliferation against immune-dominant epitopes will overgrow responses against weaker epitopes, so that the true number of T cell epitopes in a molecule can be underestimated. Ideally, MAPPs and T cell epitope mapping assays are used in conjunction: Presented sequences should be identified via MAPPs and the identified peptides of interest subsequently synthesized for down-stream T cell epitope mapping. An example of such a combined approach is detailed in the section on MAPPs assay applications in the paragraph on ixekizumab.

Impact of Assay Sensitivity on Assay Application

The sensitivity of the MAPPs assay is a crucial factor that requires adequate tuning for the different areas of application. While de-immunization approaches require a high sensitivity that enables the detection of all presented sequence regions within a molecule, ranking approaches should rather be tuned for a large dynamic range. A minimum number of copies of a given peptide is required to generate a decent fragment spectrum that will enable confident sequence identification via databases search. While too low amounts of available peptides will not consistently yield spectra qualities allowing for peptide identification, high amounts of injected peptides will lead to signal saturation resulting in plateauing peptide identifications due to speed limitations of the mass spectrometer. Signal saturation is advantageous for de-immunization approaches when all presented sequence regions need to be detected to be able to identify the sequences that should be de-immunized. For the ranking of different biotherapeutics, the differences in the amount of presented peptides are of relevance and thus, signal saturation should be avoided. In conclusion, MAPPs assays should be carefully tuned to the purpose rather than using one standard approach for all applications. The assay sensitivity is based on several key parameters: (1) Cell culture conditions: The amount of BP-derived peptides available for analysis is dependent on the differentiation and maturation status of the cell, the amount of expressed HLA class II molecules per cell, the number of cells used for peptide isolation, and the BP-concentration in the cell culture, the latter of which determines how readily the BP is taken up and processed by the APCs. (2) Peptide isolation methods: Different peptide isolation methods have been published using various detergents for membrane solubilization as well as different washing techniques to remove detergent and unrelated proteins after immunoprecipitation (5, 6, 28). All of these aspects can impact on the amount and purity of peptides that can be retrieved for analysis and on potential signal quenching by residual detergent in the peptide preparation as well as leachates from plastic surfaces.

(3) **Chromatography:** An efficient and robust chromatographic separation prior to MS analysis is required for the identification of naturally presented HLA class II peptides. The column chemistry, use or non-use of a pre-concentration column, applied solvents, gradient profile, flow rate, column length, as well as the amount of peptides loaded onto the column will impact on peak separation, peak sharpness, signal intensity and ultimately on peptide identification. (4) **Mass spectrometry:** The challenges of immuno-peptidomics, have been reviewed in detail by Faridi et al. (29). For MAPPs analyses, mass spectrometers with high-mass-accuracy are commonly applied (5–7, 28, 30). Measurement speed and mass accuracy are key parameters that enable identification of peptides in the highly complex HLA class II peptide samples. Often, a dynamic exclusion list is applied (30), which is preventing previously measured high intensity peaks from being triggered continuously, and which is enabling the detection of low abundant peaks. (5) For peptide identification based on the measured mass spectra, database search algorithms such as SEQUEST and MASCOT are commonly employed (5–7, 30). Settings are typically adjusted to limit false positive identification rates to a maximum of 1–2% (28, 30). One major challenge of the database search approach is that peptides with PTMs of unknown mass or glycosylated peptides will not be identified, as the measured masses will not match the expected masses.

SUMMARY AND CONCLUSION

Despite the fact that a clinical validation of the MAPPs assay will remain a challenge, a biological validation has been shown in several cases over the past years. Data generated as part of the ABIRISK project as well as independent studies show that presented sequence regions identified via MAPPs matched T cell epitopes identified from drug naive healthy donors and treated patients that had developed immunogenicity (7–12). Together, these studies show that MAPPs assays applied by independent research groups and performed on different donor sets yield comparable results. Sequences identified by MAPPs were shown to be meaningful and biologically relevant for the T cell responses observed clinically and can therefore help to understand immunogenicity against BPs. MAPPs data has also been shown to be more meaningful than soluble HLA class II peptide binding assay data (8). MAPPs assays have been shown to be a useful tool to interrogate clinical immunogenicity root causes by determining the natural presentation of peptides and confirming the relevance of T cell epitopes that have been identified via peptide library T cell epitope mapping approaches (7–11). Moreover, recent data suggests, that MAPPs-assisted T cell epitope mapping with MAPPs assay-derived peptide sequences could enable the identification of “authentic” T cell epitopes (11). Also, databases that compile previously identified T cell epitopes could be useful tools to be applied in conjunction with MAPPs. MAPPs has successfully been applied in a range of mechanistic studies, to interrogate the effect of PTMs (15), folding (12), and aggregation on antigen presentation (5). The technology has also been proven valuable for comparing the

antigen presentation across different APC cell types (16, 20), for determining naturally presented sequence regions of IVIg (19), and for evaluating the potential of human IgG1 allotypes to stimulate T cell responses in donors matched for homologous and heterologous IgG1 allotypes (18). As the MAPPs assay has shown the capacity to differentiate molecules regarding their content of potential T cell epitopes (6), the technology can also be useful to support candidate ranking and selection during early drug design and may contribute to the development of BPs with lower immunogenicity.

MATERIALS AND METHODS

Determination of Factor VIII-derived naturally presented HLA class II-associated peptides as part of ABIRISK project: Naturally presented HLA class II-associated peptides were identified via the MAPPs assay (6) from 11 healthy donors' monocyte-derived DCs exposed to recombinant human Factor VIII (Octocog alpha), kindly provided by Dr. med. Christoph Königs from Universitätsklinikum Frankfurt. CD14 positive mononuclear cells were purified from PBMC collected from consented healthy donors (Blood Donation Center Bern, Bern, Switzerland) and differentiated into immature DCs. Immature DCs were loaded with human Factor VIII at a final concentration of 0.6 nmol/ml in the cell culture, matured with LPS (1 µg/mL, Sigma) and incubated for 24 h at 37°C and 5% CO₂. HLA class II molecules were immunoprecipitated with anti-HLA-DR/DP/DQ monoclonal antibody IVA12-conjugated beads and peptides were eluted from HLA class II molecules by adding 0.1% trifluoroacetic acid (Fluka, Buchs, Switzerland) at 37°C. Lyophilized peptides were re-suspended in hydrophilic buffer containing 5% acetonitrile and 1.1% formic acid. Peptide composition was analyzed by liquid chromatography (nano capillary system, Dionex Corporation, Sunnyvale, California, USA) on a self-packed fused-silica C18 reversed-phase nano-high-performance liquid chromatography column connected to a mass spectrometer (Q-Exactive HF Biopharma, Thermo, California, USA) via electrospray ionization (LC-ESI-MS/MS). Peptides were identified via a database search approach using the SEQUEST algorithm as detailed previously (6).

AUTHOR CONTRIBUTIONS

AK wrote all sections of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.00698/full#supplementary-material>

Supplementary Data 1 | Complete list of Factor VIII-derived peptides per donor identified in the scope of the ABIRISK project.

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Immunogenicity of Bioproducts: Cellular Models to Evaluate the Impact of Therapeutic Antibody Aggregates

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Patients treated with bioproducts (BPs) frequently develop anti-drug antibodies (ADAs) with potential neutralizing capacities leading to loss of clinical response or potential hypersensitivity reactions. Many factors can influence BP immunogenicity and could be related to the patient, the treatment, as well as to the product itself. Among these latter factors, it is now well accepted that BP aggregation is associated with an increased potential for immunogenicity, as aggregates seem to be correlated with ADA development. Moreover, the presence of high-affinity ADAs suggests a CD4 T-cell dependent adaptive immune response and therefore a pivotal role for antigen-presenting cells (APCs), such as dendritic cells (DCs). In this review, we address the *in vitro* methods developed to evaluate how monoclonal antibodies could trigger the immunization process by focusing on the role of aggregated antibodies in the establishment of this response. In particular, we will present the different cell-based assays that have been used to assess the potential of antibodies and their aggregates to modulate cellular mechanisms leading to activation and the biological parameters (cellular activation markers, proliferation and secreted molecules) that can be measured to evaluate the different cell activation stages and their consequences in the propagation of the immune response. Indeed, the use of such strategies could help evaluate the risk of BP immunogenicity and their role in mitigating this risk.

Keywords: immunogenicity, monoclonal antibodies, aggregation, danger signal, antigen, cell-based models

INTRODUCTION

Bioproducts (BPs) such as recombinant proteins, including monoclonal antibodies, have proven to be effective in growing therapeutic areas and in particular for the treatment of chronic diseases. However, an ongoing concern while using these therapeutics is their immunogenicity, which results in the production of anti-drug antibodies (ADAs) in treated patients. ADAs detected in patients' sera are mainly IgG1 and IgG4, although IgE and transient IgM have also been evidenced in

patients developing hypersensitivity reactions (1, 2). Depending on the drug, ADA specificity is variable. For therapeutic antibodies, ADAs are mainly directed against epitopes recognized as foreign, e.g., mouse remaining epitopes in chimeric or humanized antibody paratopes, human allotopes, or human idiotopes (3). Moreover, some ADAs have a neutralizing activity, while others do not. For instance, in the case of anti-TNF antibodies, ADA characterization showed that 90% of anti-infliximab antibodies are neutralizing and more than 97% of anti-adalimumab antibodies are also neutralizing (4). Studies exploring peptide sequences targeted by these ADAs identified B-cell epitopes notably on infliximab and adalimumab variable regions, close to the paratope (5, 6). ADA development may lead to reduced BP serum concentrations due to the formation of immune complexes and a loss of efficacy (7) or adverse effects such as infusion reactions (8), cytokine release syndrome (9), or hypersensitivity reactions (2, 10). Immunogenicity is a growing issue that leads European and North American health authorities to regularly update recommendations in this area (11–13). Many factors can influence BP immunogenicity and they are related to the patient's immunological and genetic status, the followed therapeutic regimen, as well as BP inherent characteristics and quality (14). The latter include the presence of aggregated BPs upon administration as an increased risk that promotes immunogenicity. In fact, aggregates have been correlated with ADA production (mainly IgG1 and less frequently IgG2a, IgG2b, IgG3, and IgM) in mouse models, either in wild type (15–19) or transgenic animals (20–23). In particular, it has been shown that the break of tolerance induced by antibody aggregates was dependent on the chemical modifications induced by the aggregation process (23). Moreover, it has been shown that T-cell help is required for ADA production in response to aggregates in transgenic and wild-type mice given that CD4 T-cell depletion abolished the immune response to aggregates (21). Clinical evidence of aggregates involvement in BP immunogenicity is rare and has only been described for human gamma globulin preparations and recombinant forms of endogenous proteins, such as human growth hormone, erythropoietin, or interferons [reviewed by Moussa et al. (24)]. While direct clinical evidence has not been reported, a number of review articles have dealt with the immunological mechanisms that could lead to ADA production (24–26). The predominance of high-affinity IgG as patients' ADA main isotype suggests that ADA production arises from a T-cell-dependent immune response, in which BPs and/or aggregates could undergo APC uptake, to be processed and presented to T helper cells. In this context, the main professional APCs for peptide presentation are DCs that have a pivotal role in the efficient initiation of a specific immune response. It is therefore of high interest to focus on the mechanisms that underlie aggregates interactions with this innate/adaptive interface, to gain insight into BP immunogenicity.

Abbreviations: ADA, anti-drug antibody; APCs, antigen-presenting cell; BP, bioproduct; DC, dendritic cell; FcR, Fc receptor; IVIG, intravenous immunoglobulin G; MHC, major histocompatibility complex; moDC, monocyte-derived dendritic cell; PBMC, peripheral blood mononuclear cell; TLR, toll-like receptor.

In this review, we focus on cellular models that have been reported in the literature to assess the impact of aggregated monoclonal antibodies on the initiation of the immune response. As such, we will describe that antibody aggregates can behave as danger signals recognized by innate immune cells, but might also induce some alterations in the processing and presentation of antigens generated from the therapeutic antibody.

AGGREGATION PROCESS

The antibody aggregation process has been widely studied since the production of therapeutic antibodies at an industrial scale. Aggregation can occur at any stage of the manufacturing process, storage, transportation, or preparation for patient administration, under the influence of several critical environmental parameters (e.g., temperature, pH, ionic strength, shear forces, light, etc.). These stress conditions can alter the protein structure either by physical or by chemical damage and trigger the protein aggregation through different pathways. Thus, aggregation can occur as a result of the interaction of two monomers, either folded or unfolded, to gradually form reversible oligomers and then the initial irreversible aggregation nucleus, that is the starting point of aggregate growth. For monoclonal antibodies, the exposure of hydrophobic sequences representing the aggregation-prone regions (APRs) can promote oligomerization (27); these APRs are notably found in the complementarity-determining regions (CDRs) (28, 29) but can also be found in other positions on variable and constant regions of the antibodies (30). On the other hand, potential monomer self-association regions in Fab domains have also been identified (31). Moreover, a variety of high molecular complexes were evidenced when antibodies interact with their soluble target (32). Aggregation mechanisms are extensively described in two reviews (30, 33). Aggregates are usually described according to several criteria (size, reversibility, conformation, and shape), although the size has been adopted as the most convenient to suggest a classification (34), summarized in Table 1. Interestingly, the type of generated aggregates depends on the nature of the applied stress as well as the chosen monoclonal antibody. In particular, aggregated antibody preparations under accelerated experimental stresses induce a wide variety of aggregates. A classification scheme was proposed for antibody aggregates, based on several biophysical characterizations and the visible or subvisible criteria (35). Nevertheless, a few reports dealt with the occurrence of antibody aggregation that could take place during or just before the administration of the BP (36). For example, dilution of antibody preparations in PBS or in the manufacturer's

TABLE 1 | Classification of protein aggregates (34).

Size	Preferred terminology	Characteristics
<100 nm	Nanometer aggregated	Oligomers, soluble
100–1000 nm	Submicron aggregates	Soluble
1–100 μm	Micron aggregates	Subvisible particles, insoluble
>100 μm	Aggregates greater than 100 μm	Visible particles, insoluble

formulation led to a reduction in the monomer concentration (37). Moreover, nanometer, submicron, and micron protein particles have been evidenced in intravenous saline bags (38, 39). Furthermore, micron aggregates were detected in ejected solutions from prefillable syringes due to increased antibody adsorption to the syringe surface (40). Interestingly, it was shown that bedside filtration could significantly reduce the quantity of submicron- and micron-sized aggregates before BP injection (41). Finally, it is suggested that subcutaneous administration of antibody solutions could also favor aggregation due to the forced interaction between monomers in highly concentrated solutions (42, 43).

ANTIBODY AGGREGATES ACT AS DANGER SIGNALS FOR INNATE IMMUNE CELLS

In vitro cell-based models are valuable tools in an attempt to describe the interaction of exogenous molecules with the immune system. Monocytes and DCs are professional APCs acting as sensors while continuously capturing exogenous molecules that could represent a potential danger. The so-called “danger signal” concept (44) includes exogenous pathogen-associated molecular patterns (PAMPs), endogenous damage-associated molecular patterns (DAMPs), and the more recently described nanoparticle-associated molecular patterns (NAMPs) (45). Soluble submicron-sized protein aggregates can fall into the latter category (46). These molecular patterns can bind to pattern recognition receptors (PRR) expressed on innate immune cells and induce cell activation, through the activation of signaling pathways that lead to the activation of transcription factors such as nuclear factor kappa B (NF- κ B) and activator protein 1 (AP-1), resulting in the secretion of pro-inflammatory cytokines and chemokines.

Interaction of Antibody Aggregates With Peripheral Blood Mononuclear Cells

As a first approach, most cellular models that evidenced the danger signal role of antibody aggregates prepared under accelerated conditions used PBMCs from healthy donors. Joubert et al. first described a cytokine/chemokine signature resulting from PBMC activation in response to stir-stressed antibody preparations, compared to monomeric antibodies (47). Furthermore, using the same cellular model, a comparison of size-fractionated aggregates showed that aggregates having a size between 5 and 10 μ m were the most efficient to induce cytokine secretion (48). PBMC activation was also induced by aggregated polyvalent immunoglobulin preparations (IVIG) in terms of cytokine and chemokine secretion (49, 50), but also in terms of intracellular proteins involved in signaling pathways: the activation of mitogen-activated protein kinases (MAPKs) p38, Erk1/2, and Jnk, was observed within 30 min of PBMC stimulation with IVIG aggregates. Screening of the expression of over 100 genes in PBMCs in response to aggregated IVIG showed an increased expression of specific genes implicated in

cell signaling and/or linked to the activation and recruitment of innate immune cells (50).

Attempts to identify cellular receptors implicated in PBMC activation gave different outcomes, depending on the used cell-based model. Using specific blocking antibodies, the involvement of the toll-like receptors (TLRs), TLR2 and TLR4, and to a lesser extent the Fc-fragment receptors Fc γ RI and Fc γ RIII was evidenced via the decrease of aggregate-induced PBMC cytokine and chemokine secretion (47, 49). However, studies using reporter cell models allowing the evaluation of the individual implication of TLRs and/or Fc γ Rs showed opposing results. Indeed, Polumuri et al. showed no implication of TLRs, including TLR2 and TLR4, in the activation of HEK293 cells expressing TLRs in response to IVIG aggregates (50). More recently, the use of other reporter cell models showed that antibody aggregates induced Fc γ Rs activation, mainly Fc γ RIIIa and Fc γ RIIIb; however, they did not activate TLRs (51). Taken together, all these results indicate that the activation of PBMCs in response to aggregated antibodies is multifactorial, through the potential engagement of multiple receptors. The implication of multiple cell types in the observed response of PBMCs clearly shows the complexity of this cellular model to evaluate the specific pathways (mainly receptors and intracellular proteins) enabled by antibody aggregates. It would therefore be of interest to study the role of each cell type separately to deepen our understanding of the innate immune response to aggregates.

Interaction of Antibody Aggregates With Antigen-Presenting Cells

Aggregated antibody behavior as a danger signal was also demonstrated using APC models. The monocytic THP-1 cell line was compared to primary purified monocyte preparations to evaluate the impact of aggregated IVIG. Both pro-inflammatory cytokine secretion profiles were comparable, not in magnitude, but in terms of a dose-dependent response to increasing aggregates concentrations (49). Moreover, receptors involved in THP-1 or monocyte activation in response to aggregates were identified as TLR2, TLR4, and, to a lesser extent, Fc γ RII. Other studies focused on the impact of aggregated antibodies on DC maturation, using purified monocyte preparations from healthy donors that were differentiated into dendritic cells (moDCs). This primary cell *in vitro* model has been described and used for DC maturation studies under the action of coagulation Factor VIII preparations (52) and recombinant human growth hormone aggregates (53). The effect of monoclonal antibody aggregates on moDC has also been studied with rituximab (53–55), trastuzumab (54), monoclonal IgG1s (56), and adalimumab, natalizumab, and infliximab (55). These studies showed that antibodies in their native state did not induce maturation of moDCs whereas their aggregated counterparts increased the expression of surface markers, mainly CD83 and CD86, as well as the secretion of cytokines such as IL-6, IL-8, IL-10, IL-12p40, and chemokines such as CCL2, CCL3, and CCL4. Although moDC phenotypic alterations could be observed in response to different antibody aggregates, the degree of maturation varied depending on the used therapeutic antibody as well as

the size, structure, quantity, and type of generated aggregates. For instance, Morgan et al. compared the moDC response to infliximab and natalizumab aggregates. This study showed that infliximab had the highest propensity to aggregate when submitted to heat stress contrary to natalizumab. Evaluating moDC maturation then showed that heat-stressed infliximab induced phenotypic alterations of moDCs whereas stressed natalizumab did not have any effect on moDC activation (55). Moreover, it was shown that infliximab aggregates increased the phosphorylation of the kinases Syk, Erk, and Akt (55).

In order to correlate the innate immune response observed with aggregated antibodies with the adaptive immune response, T-cell proliferation and cytokine secretion have been evaluated in multiple studies through PBMC culture models or allogeneic DC–T-cell co-culture models. On one hand, cultures of healthy donors' PBMCs showed an increased proliferation of T cells and IL-2 production (57, 58) or IFN- γ production (56) in response to aggregated antibodies, whereas native antibodies induced little to no T-cell response. On the other hand, using an allogeneic moDC–T-cell co-culture model, we showed an increase in CD4 T-cell activation (proliferation and cytokine secretion) when moDC were treated with various types of protein aggregates [stir-stressed rituximab and stir-stressed recombinant human growth hormone (53)]. Interestingly, these aggregates did not induce similar cytokine profiles: rituximab aggregates induced IFN- γ , IL-5, IL-13, and IL-17 production, whereas growth hormone aggregates only induced IFN- γ secretion (53). These results strongly suggest that different mechanisms are implicated in the activation of moDCs depending on the origin and type of the generated protein aggregates. However, the results converge to show full DC activation, sufficient to trigger a T-cell response evaluated using orthogonal readouts such as proliferation and cytokine secretion (Figure 1). This suggests that antibody aggregates have the ability to initiate an adaptive immune response that could lead to ADA production.

ANTIBODY AGGREGATES AND THE GENERATION OF *DE NOVO* ANTIGENS

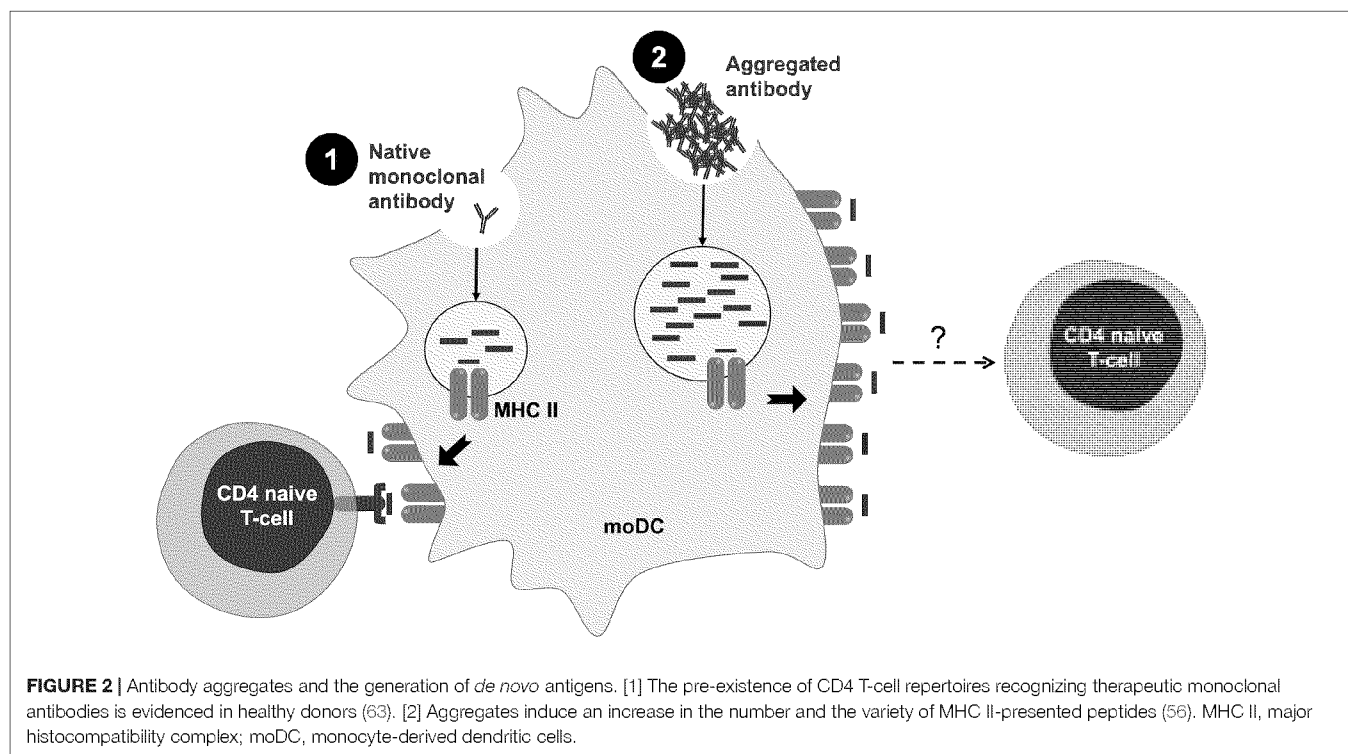
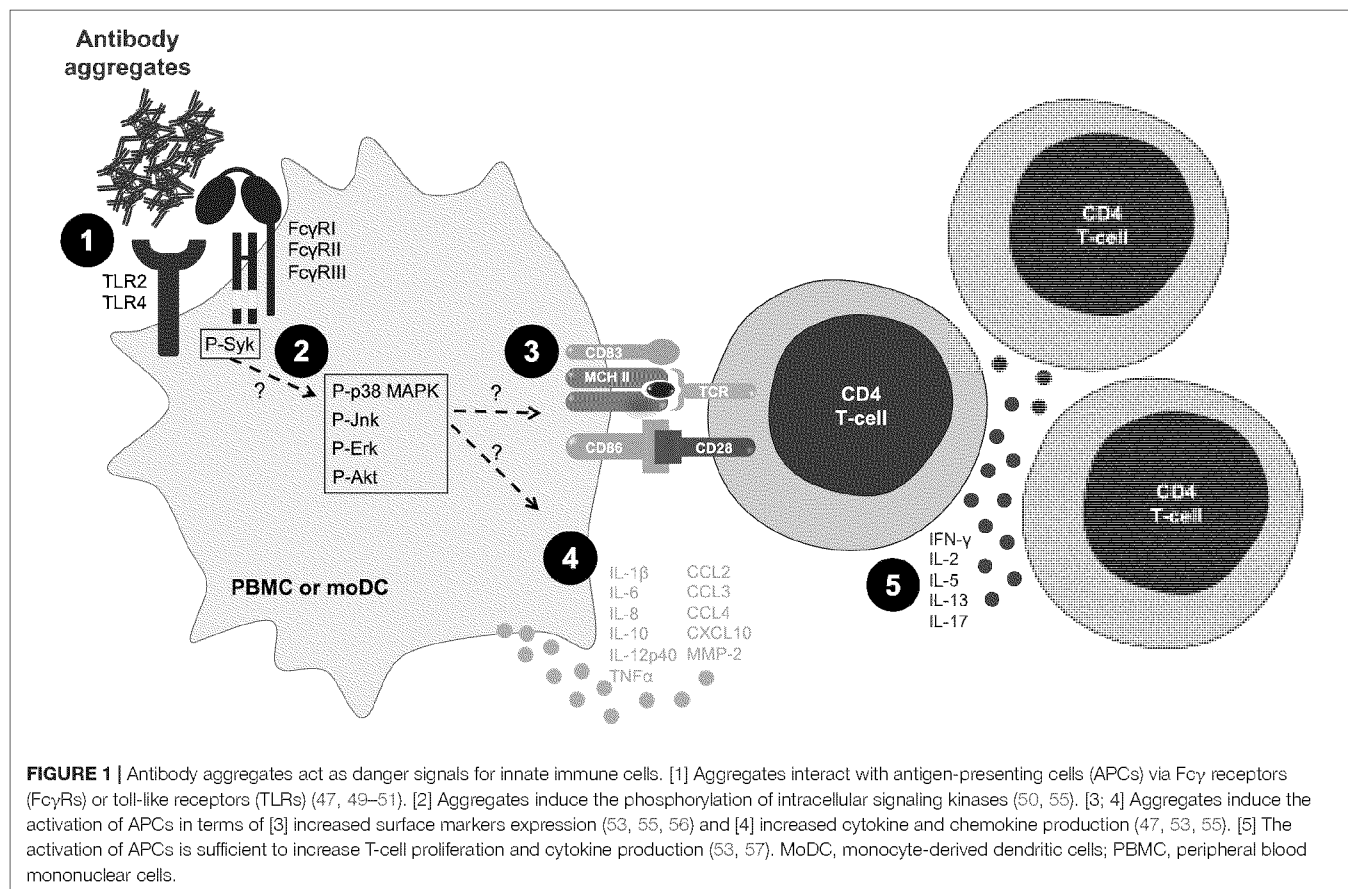
Producing high-affinity ADAs against therapeutic antibodies requires that patients develop a specific adaptive immune response through antigen recognition by T cells and B cells. In fact, specific T-cell activation requires efficient antigen presentation by fully mature DCs, through the establishment of the immune synapse (59, 60) that allows the transmission of three activation signals. The interaction between DCs and T cells is established via the HLA–peptide complex recognized by a specific T-cell receptor (TCR), the membrane co-stimulation proteins as well as the DC-secreted pro-inflammatory cytokines that allow T-cell proliferation and polarization (59, 61). Cell-based models presented in the previous section highlighted that aggregated antibodies could fulfill the two latter activation signals. However, the role of aggregates in the initiation of a specific T-cell response has been less explored (62), even though the specificity of the immune response to native monoclonal antibodies has been well documented over the past years, as described hereafter.

The CD4 T-Cell Repertoire Targeting Monoclonal Antibodies

In order to establish a link between the development of the adaptive immune response and the clinically observed immunogenicity of monoclonal antibodies, autologous DC–T-cell co-culture models have been developed. Briefly, CD4 T cells isolated from human healthy donors' blood are seeded in plates and stimulated by autologous mature moDCs loaded with monoclonal antibodies. After weekly rounds of stimulations with antibody-treated moDCs allowing specific T-cell expansion, an IFN- γ ELISpot assay is used to detect antibody-specific T-cell lines (CD4 T cells present in a single well) and determine a frequency of CD4 T cells recognizing monoclonal antibodies following the Poisson distribution for rare events. This cellular model first allowed Delluc et al. to identify the existence of T-cell repertoires recognizing monoclonal antibodies, notably rituximab, infliximab, and adalimumab. Interestingly, these results correlated with the clinical immunogenicity of BPs (63). More recently, studies helped identify the T-cell epitopes incriminated in monoclonal antibody immunogenicity, which is essential in the process of deimmunizing antibodies. Monoclonal antibodies can be uptaken and processed by APCs such as DCs and the derived-linear peptides will be presented to T cells on major histocompatibility complex (MHC) molecules. These peptides could then be identified through an MHC-Associated Peptide Proteomics (MAPPs) assay. Identified peptides are then tested in the co-culture model to determine the ones that can induce a T-cell response, which depends on the peptide's affinity to bind to MHC molecules and the recognition of the MHC–peptide complex by a specific TCR. This type of experiment has evaluated T-cell epitopes of different monoclonal antibodies such as infliximab, rituximab (64), natalizumab, adalimumab (65), secukinumab, and ixekizumab (66). Results showed that peptides inducing a CD4 T-cell response to a monoclonal antibody were mainly sequences deriving from CDRs and framework regions (FR) of the antibody variable domains. These studies clearly showed the pre-existence of CD4 T cells specific of peptides deriving from therapeutic antibodies which could favor their immunogenicity (Figure 2).

The Role of Antibody Aggregates in the Initiation of a Specific Immune Response

While studying the role of aggregates in the activation of DCs, it has been shown that aggregated antibodies can be internalized by these APCs (54). Different mechanisms can be involved in the uptake of particles: phagocytosis, macropinocytosis, or clathrin-mediated endocytosis. In particular, aggregated particles of size larger than 0.5 μm are internalized by phagocytosis or macropinocytosis, whereas smaller particles are internalized by clathrin-mediated endocytosis (67). Studies exploring the role of antibody aggregates mainly generate these aggregates by submitting antibody solutions to extreme and accelerated stress conditions that often induced the formation of submicron- and micron-sized aggregates. This strongly suggests that these aggregates would be internalized by either phagocytosis or macropinocytosis; however, no data has yet confirmed this



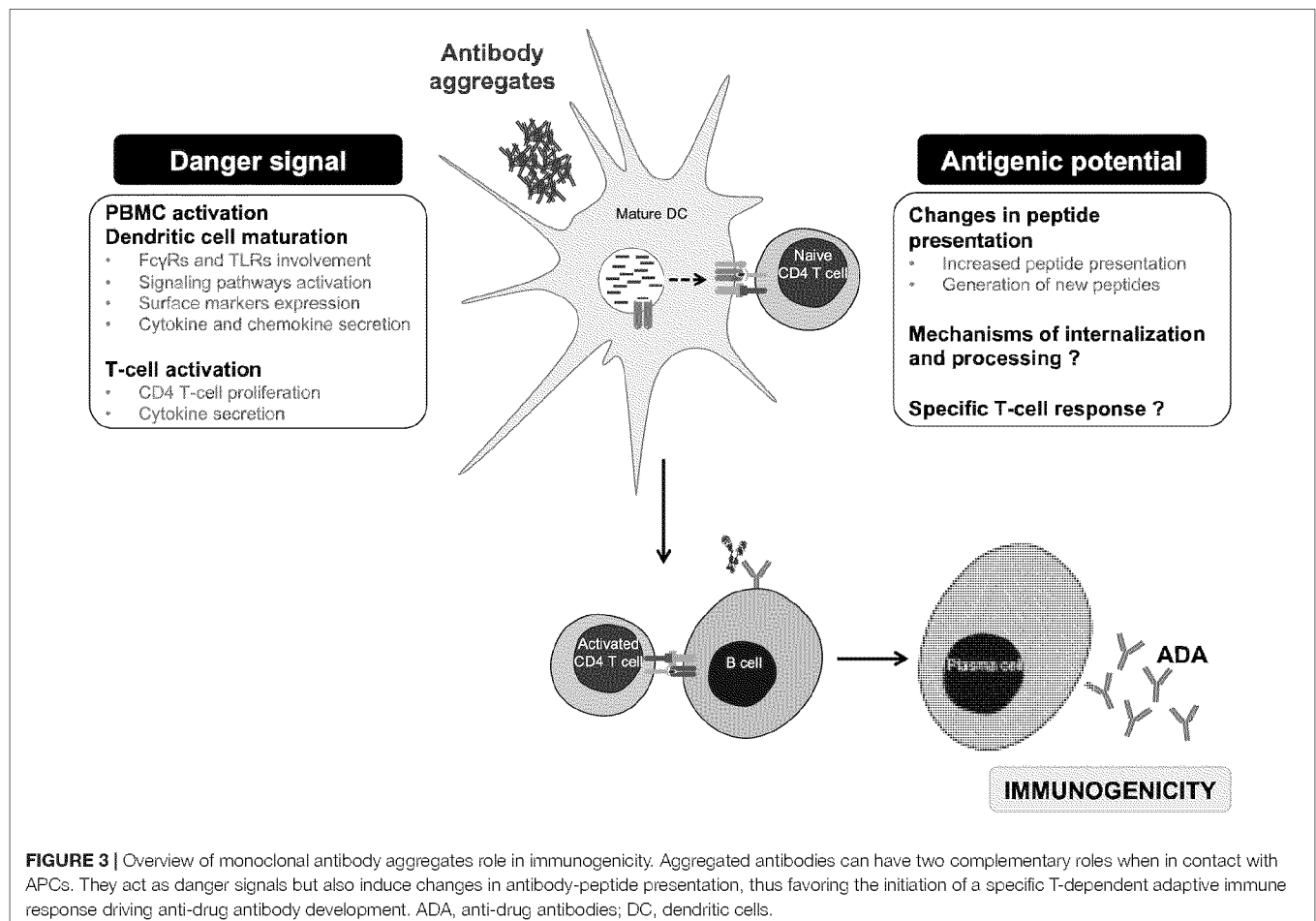
hypothesis. On the other hand, *in vivo* studies are currently focusing on the oligomeric antibodies and their immunogenicity (68, 69). Indeed, nanometric-sized aggregates could be found in preparations and are hardly ever detected and eliminated before the administration of the therapeutic antibody (38, 41). Contrary to subvisible aggregates, oligomers would probably be uptaken by endocytosis, which is yet to be confirmed (67). Further investigations are necessary to gain insight into the mechanisms of aggregate binding and trafficking into APCs.

Once internalized, particles are trafficked to the endocytic compartment to be processed and generated peptides are then loaded on MHC molecules. One study by Ahmadi et al. showed that rituximab aggregates were uptaken by moDCs and co-localized with HLA-DR molecules after 30 min of incubation (54). Moreover, Rombach-Riegraf et al. used the MAPPs assay to evaluate the peptides presented on MHC-II molecules from moDCs loaded with native or aggregated antibodies. This study showed that the aggregation of IgG monoclonal antibodies can induce an increase in the number as well as in the diversity of the peptides presented by MHC-II molecules compared to native monoclonal antibodies (56). This observation suggests that aggregation could induce alterations in the uptake and processing mechanisms of the antibody leading to changes in the peptides presented to CD4 T cells (Figure 2).

How could aggregation modulate the specific T-cell response detected for native antibodies? One study exploring the T-cell response to erythropoietin and heat- or tungsten-induced erythropoietin aggregates in an autologous moDC-T-cell co-culture model that aggregates induced an increase in T-cell proliferation compared to native erythropoietin (62). This is the only study to date evaluating the specific T-cell activation in response to BP aggregates. For antibodies, what is currently known about aggregation and T-cell epitopes is that the majority of hydrophobic APRs are found in the T-cell epitope sequences of therapeutic antibodies (70). More studies could help better understand if antibody aggregates could initiate a specific immune response. In particular, it remains to be clarified if aggregation favors the presentation of *de novo* T-cell epitopes that could be generated by possible alterations in protein cleavage, occurring at different sites compared with the monomeric antibody.

SUMMARY AND CONCLUSION

Immunogenicity of therapeutic monoclonal antibody aggregates has been widely explored in the past few years. In this review, we focused on the *in vitro* cellular models that have been used



to better understand the role of aggregates in the initiation of a T-dependent immune response leading to the production of high-affinity ADAs (Figure 3). Many experimental studies have explored the danger signal role of antibody aggregates by showing their potential to induce efficient activation of innate immune cells. However, current data show the implication of diverse receptors, signaling pathways, surface markers, as well as cytokines and chemokines without a clear signature for all antibody aggregates. Exploring the differences between existing results show that the detected immune response can vary depending on the cellular model, the nature of the monoclonal antibody, the types of generated aggregates, as well as the level of the selected stress. It is important to notice that most studies have used extreme stress conditions that would often lead to the formation of aggregates of various size ranges, quantities, and structures that do not resemble aggregates found in administered preparations. Thus, it is currently essential to focus on the role of antibody oligomers in the initiation of the immune response; oligomers being sometimes detected but not efficiently eliminated from preparations. Using homogeneous well-characterized oligomer preparations may allow one to evaluate the sensitivity of these cellular models and to also determine a threshold of particle number sufficient for cell activation. Finally, the specificity of the immune response induced by antibody aggregates has yet to be explored to gain insight into the antigenicity of aggregates.

The use of cell-based assays has clearly some benefits in assessing the impact of aggregated antibodies on the

establishment of the immune response. They allow one to directly work with a relevant mixture of human immune cells, and therefore to take into account the HLA diversity of donors. They also allow one to test a variety of samples and to compare different aggregate preparations. For these reasons, beyond their use for the assessment of induced cellular mechanisms, they can also find applications in the evaluation of BPs under development, to assess the potential risk of immunogenicity due to aggregation during the manufacturing process.

AUTHOR CONTRIBUTIONS

MN and IT wrote the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Immunogenicity of Protein Therapeutics: A Lymph Node Perspective

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The continuous development of molecular biology and protein engineering technologies enables the expansion of the breadth and complexity of protein therapeutics for *in vivo* administration. However, the immunogenicity and associated *in vivo* development of antibodies against therapeutics are a major restriction factor for their usage. The B cell follicular and particularly germinal center areas in secondary lymphoid organs are the anatomical sites where the development of antibody responses against pathogens and immunogens takes place. A growing body of data has revealed the importance of the orchestrated function of highly differentiated adaptive immunity cells, including follicular helper CD4T cells and germinal center B cells, for the optimal generation of these antibody responses. Understanding the cellular and molecular mechanisms mediating the antibody responses against therapeutics could lead to novel strategies to reduce their immunogenicity and increase their efficacy.

Keywords: therapeutics, follicle, germinal center, B cells, ADA, Tfh cell

INTRODUCTION

Protein therapeutics is a new class of drugs that, unlike small molecule drugs, are not chemically synthesized; instead, they are produced within living cells or organisms. Remarkable developments in molecular biology and protein engineering methodologies in the last few decades have enabled the generation of several new biotherapeutics for a wide range of diseases. Despite the potential of protein therapeutics, a drawback, often associated with them, is the generation of antidrug antibodies (ADAs), which diminishes the bioactivity and effectiveness of the therapeutic (1). The anatomical sites where the development of ADA occurs are the secondary lymphoid organs, including lymph nodes and spleen, which are central for humoral responses to immunogens and pathogens (2–4). The organogenesis (5, 6), architecture (7, 8), and cellular composition (9, 10) of lymph nodes as well as the immune reactions to pathogens mediating the development of humoral responses (11, 12) are well-studied and understood. Here, we review these principles with a focus on ADA development and potential strategies aiming to minimize the immunogenicity of biotherapeutics.

HISTORICAL BACKGROUND

The history of protein therapeutics starts probably with diphtheria antitoxin derived from horse serum. The extraction of insulin from bovine pancreas, a few decades later, was a milestone in the treatment of diabetes. The development and approval of recombinant insulin, a human

insulin expressed in *Escherichia coli*, in 1982 (13) resulted in the increased accessibility of insulin. Around the same time, murine monoclonal antibodies were being considered as therapeutic agents, with Orthoclone OKT3 (muromonab-CD3) being the first licensed monoclonal antibody, in 1986 (14). Many of the first-generation monoclonal antibodies were significantly immunogenic, because of their murine origin. Orthoclone OKT3 was eventually discontinued from the market in 2010 owing to the development of ADA (14–17). New generations of monoclonal antibodies are primarily being developed as humanized or human antibodies and are far less immunogenic. The use of a fully humanized IgG1 mAb against TNF α (adalimumab) triggered the development of ADA in patients with plaque psoriasis (18) and rheumatoid arthritis (19–22), indicating that ADA development to therapeutics can vary depending on factors like preexisting activation of the immune system and chronic inflammation.

Monoclonal antibodies constitute a large fraction of biotherapeutics and target a specific protein, usually to inhibit or modulate its function; in some cases, they may have a diagnostic role, or they may deliver a drug. The rest of protein therapeutics are primarily replacement therapies for proteins that are deficient owing to genetic or other reasons. Such protein therapeutics include coagulation factors, hormones, growth factors, and enzymes. Recently, fusion proteins are becoming an important class of biotherapeutics (Table 1). There are several examples of albumin fusion, Fc fusion, and antibody drug conjugates available in the market (33). The breadth and the complexity of protein therapeutics increase the potential for immune response generation. Protein therapeutic immunogenicity poses a great challenge in the field; and a better understanding of the risks, development, and mechanisms of ADA is needed to allow for strategies to reduce immunogenicity.

Generation and Mechanisms of Action of Antidrug Antibodies

Several factors contributing to the immunogenicity of biotherapeutics have been identified and in many cases eliminated to generate better drugs. For example, animal proteins have been for the most part phased out of the market, as these were very often associated with strong immune responses. Initially, many recombinant proteins were generated through bacterial systems (especially *E. coli*), which are effective and simple to use but lack the higher mechanisms for glycosylation and therefore are often immunogenic in humans. Mammalian expression systems are now commonly used for biotherapeutics to allow for glycosylation. However, even within mammalian species, glycosylation may differ contributing to the development of ADA. For example, cetuximab, a monoclonal antibody against epidermal growth factor receptor (EGFR) inhibitor (24, 34, 35) generated in a mouse myeloma cell line SP2/0 was associated with development of ADA owing to its glycosylation profile (24, 34–36). Introduction of alternative mammalian cell lines [Chinese hamster ovary (CHO)] has greatly contributed to overcoming immunogenicity due to glycosylation patterns (34, 35). L-Asparaginase, a highly immunogenic enzyme, is effective in its own native, *E. coli* derived form (37, 38). However, allergic reactions due to multiple doses caused silent hypersensitivity that in turns generates ADA. Use of a pegylated form (26) or increasing the enzyme binding to erythrocytes (39) was able to reduce the development of ADA during multiple doses of *E. coli* asparaginase.

In patients receiving replacement therapy, a significant factor affecting their risk to ADA development is the levels of endogenous protein, with patients expressing no or very little protein being at a much higher risk, presumably owing to compromised central tolerance induction (40). Even a few amino

TABLE 1 | Reported ADA development for licensed therapeutics.

Drug name	Type	Clinical use	ADA prevalence	Citation
Monoclonal antibodies				
Muromonab-CD3	Murine against CD3	Immunosuppression for the prevention of allograft rejection in transplants	43–91%	(14–17)
Infliximab	Chimeric human/mouse IgG1 against TNF α	Rheumatoid arthritis, inflammatory bowel disease, plaque psoriasis	5.4–43.6%	(19, 21, 23)
Cetuximab	Chimeric human/mouse IgG1 against EGFR	Colorectal cancer, squamous cell carcinoma (head/neck)	0.6–20.8%	(24)
Adalimumab	Human IgG1 against TNF α	Plaque psoriasis, rheumatoid arthritis, Crohn's disease, spondyloarthritis, psoriatic arthritis	17–49%	(18, 20–22, 25)
Other drugs				
L-Asparaginase	Enzyme	Acute lymphoblastic leukemia in adult and children	2% (PEG-Asp–neutralizing ADA); 26% (<i>Escherichia coli</i> -Asp)	(26, 27)
FVIII	Anti-hemorrhagic protein	Hemophilia A/B	3.6–33% (25–30% in those with severe hemophilia)	(28, 29)
IFN- β	Mammalian cytokine	Multiple sclerosis	2–53%	(30–32)

ADA, antidrug antibody; EGFR, epidermal growth factor receptor.

acid sequence changes between the endogenous protein and the administered biotherapeutic may lead to an increased risk in immunogenicity. Substitution of just three amino acids in the recombinant activated factor VII (rFVIIa) (1, 41) was shown to significantly increase immunogenicity of the therapeutic protein. In addition, dosing (42), protein folding/aggregation, route of administration, storage conditions, and excipients may also affect the development of ADA (43, 44). It has been proposed that even codon usage of the recombinant protein may affect protein conformation and modulate immunogenicity (45). The inhibitory activity of ADA can be mediated by several mechanisms. Development of anti-idiotypic antibodies against the therapeutic could lead to *in vivo* formation of immune complexes (ICs), which can diminish therapeutic efficacy by reducing the half-life of the therapeutic or engaging the complement cascade (46, 47). Larger ICs are removed from circulation faster than smaller ICs owing to engagement of FcR on macrophages, reducing drug levels and requiring more frequent administration (47, 48). Complement cascade activation (as seen with administration of therapeutic IFN- β for multiple sclerosis) enhances inflammatory responses (46, 47). Alternatively, generation of neutralizing antibodies (i.e., adalimumab and infliximab, anti-TNF α , and monoclonal Abs) could directly block the action of the administered antibody or modulate its *in vivo* half-life (18, 25, 49, 50). In rare cases, ADA generation may lead to anaphylactic shock and death (51).

Lymph Nodes: Primary Sites for the Development of Immune Responses Against Pathogens Structure

Lymph node positioning along lymphatic vessels enables the efficient draining and detection of pathogens and immunogens (Figure 1). The number of human LNs varies depending on age and disease status (52–56). The LN architecture is characterized by well-organized, distinct anatomical areas: cortex, paracortex, follicles, germinal centers (GCs), high endothelial venules (HEVs), medulla, and fibroblastic reticular cells (FRCs) (57, 58) (Figure 1). The formation of distinct LN areas contributes to the compartmentalization of cellular and molecular mechanisms involved in the generation of antigen-specific humoral responses. This compartmentalization further contributes to the control of relevant immune interactions and reduction of unwanted B cell responses. The cortex consists of many lymphocytes, mainly naive B cells (sIgD+IgM+) packed into primary follicles (absence of GC) or secondary follicles that are characterized by the formation of GC (58, 59). GCs are the areas where B cells proliferate in response to T cell-dependent antigen and create memory cells and plasma cells (57). Two major GC areas have been characterized, dark zone (DZ) and light zone (LZ), with different cellularities and roles for the development of B cell responses (60, 61). The deeper cortex, also known as the paracortex, contains HEVs, which are specialized blood vessels that allow circulating lymphocytes, such as T cells, and innate immunity cells to directly enter the LN (58). The local interaction between T and dendritic cell (DC) subsets initiates a cascade

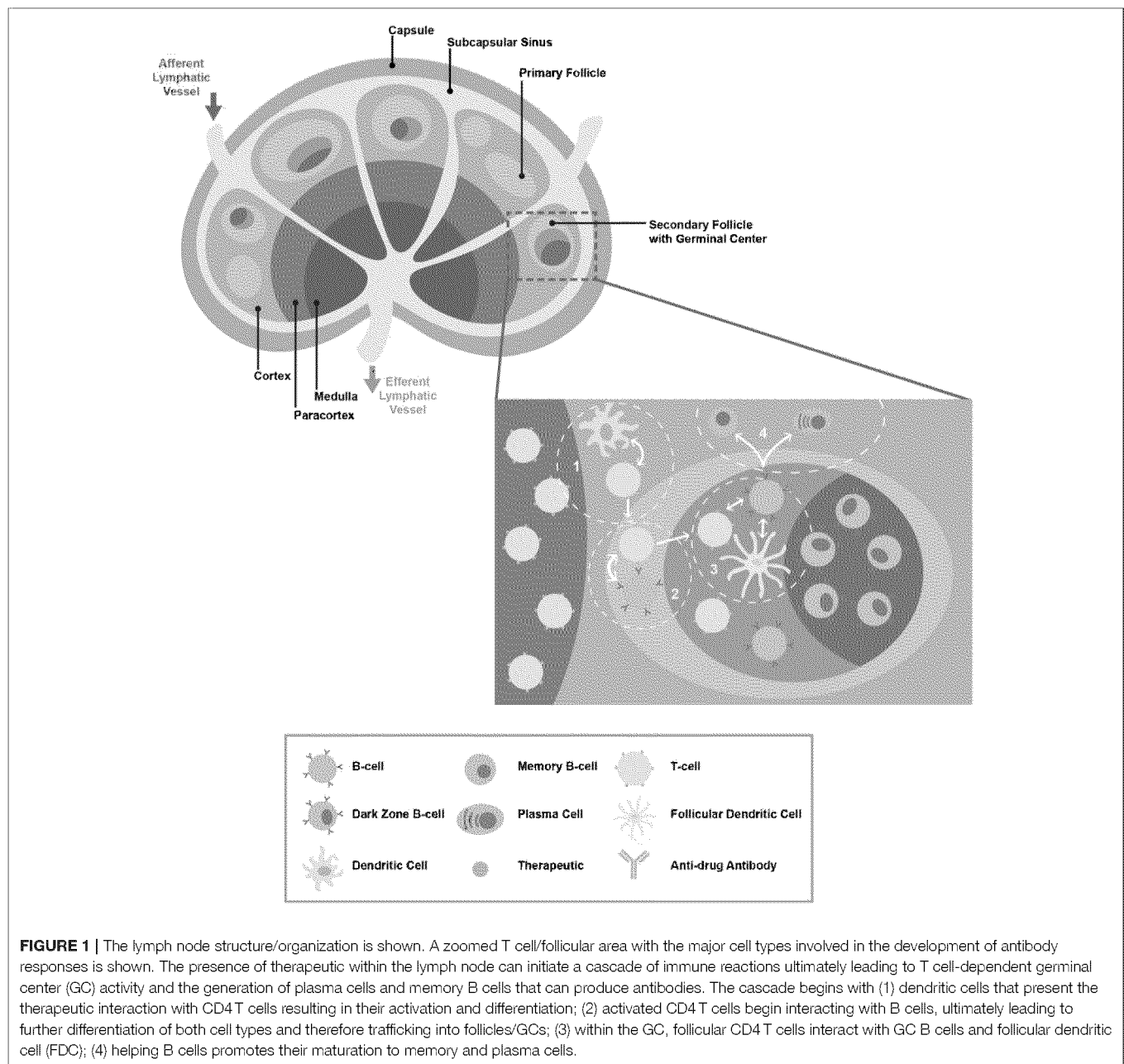
of immune reactions critical to the formation of mature GCs (57). The medulla, located on the efferent side where the lymph drains out of the LN, contains blood vessels and medullary cords enriched in B cells, macrophages, and plasma cells (Figure 1). Finally, the backbone of the LN architecture is the FRCs. The FRCs form a network that allow DCs and T cells to travel throughout the LN (62).

Major Cell Populations

T cell zone (paracortex) is populated with innate immunity cells (DC, monocytes, macrophages, and granulocytes), adaptive immunity cells (CD4 and CD8), and stromal cells (FRCs). Subcapsular sinus macrophages is the first lymph node population encountering pathogens from the lymph (63) that controls the pathogen dissemination and inflammation and affects B cell responses to subsequent infections (64). These cells can trigger responses to lipid antigens, a mechanism mediated by activation of LN invariant natural killer cells (iNKTs) (65). Recirculating monocytes can traffic to LNs and either keep their classical status (66) or further differentiate to macrophages or DCs and initiate adaptive responses (67, 68). T cell zone macrophages can also function as scavengers for apoptotic cells (69). DC and monocyte in LNs are main producers of IL-6, an important cytokine for the differentiation development of Tfh cells (70, 71). FRCs provide a vital network for (i) recruitment of naive T cells and DCs through CCL21 and CCL19, the CCR7 ligands (72), a major chemokine receptor mediating tissue trafficking of several cell types (73, 74); (ii) T cell survival through IL-7, a survival factor particularly for naive T cells (75, 76); and (iii) trafficking of CD4 T cells toward the GC (62). When an antigen is present, major rearrangements take place within this area. Studies using mouse models have shown that the presence of antigen triggers the activation and repositioning of DC cells (77, 78), which have an important effect on CD4 T cell activation as well as the initiation of CD4 T and B cell interaction. B cells will follow a CCR7 gradient toward the follicle/T-zone (T-B) boundary where they could bind to multiple helper CD4 T cells at once, whereas T cells would only bind one at a time (79). The interaction of CD4 T and B cells in the T cell zone will activate a cascade of immune dynamics associated with major changes in differentiation status (phenotypes, transcriptome profile, and trafficking) (80) of both CD4 and B cells, ultimately enabling their trafficking into the GC area. In fact, the interaction between CD4, B, and antigen-presenting cells (APCs) in the T cell zone and T-B borders is required for optimal differentiation of CD4 T cells to Tfh cells (81, 82). These early interactions are also critical to the further development of B cell responses (83, 84).

GERMINAL CENTER: THE LABORATORY FOR THE DEVELOPMENT OF B CELL RESPONSES

The role of the Tfh cells is to help create high-affinity memory B and plasma cells (85); thus, this subset of CD4 T cells is crucial in the immune response. GCs, the structures found in



mature, secondary follicles (59), are populated with activated B cells, follicular DCs (FDCs), Tfh cells, and macrophages (tingible body macrophages) (59). Upon antigen stimulation, naive B cells traffic to the T–B border following a CCR7 gradient (79). Further interaction with CD4 T cells and receipt of co-stimulatory signals (86) trigger a rigorous proliferation of B cells and the formation of a tight cluster within the follicle, which becomes the GC. Within the GC, the B cells undergo somatic hypermutation, affinity maturation, class switch recombination, and plasma/memory B cell production (87–89). Formation of GC is mediated by help from FDCs and the function of G-protein-coupled receptors (GPCR) like S1PR2 and P2RY8 (59, 90, 91). In

primary follicles, FDCs help B cells form a follicle (92), whereas in secondary follicles, FDCs support GC B cell survival (93–97). B cell survival was impaired when FDCs were exposed to HIV-1 (98), smaller GCs formed and lower antibody titers were obtained when FDC activation was blocked through TLR4 (99). FDCs modulate antigen availability by cycling the antigens between the FDC surface and other endosomal compartments (100) or accumulating ICs bound to Fc receptors on their cell surface (100), a process critical to the affinity maturation of B cells (101, 102). Tfh cells are a subset of CD4 T cells that are specialized to help B cells. They are located inside B cell follicles of secondary lymphoid organs and are responsible for activation,

isotype switching, affinity maturation, and differentiation of B cells (103–105). Tfh cells express a unique phenotype and transcriptome signature (106–109). A mutual regulation through modulation of Bcl-6 between Tfh and GC B cells has been proposed (110–112). Tfh cells produce cytokines like IL-21 and IL-4, which are important for the GC B cell dynamics (105, 113–115) and maintenance of Tfh cells. Two distinct GC areas have been identified; the DZ, where B cell proliferation and somatic hypermutation occurs, and the LZ, where B cells interact with Tfh cells and FDCs (60, 116). Expression of the chemokine receptor CXCR4, which is highly expressed on Tfh and GC B cells (117, 118), and local production of its ligand CXCL12 (SDF-1) in DZ by CXCL12-expressing reticular cells (CRC) (119, 120) play a critical role for the organization of DZ and LZ (116, 121, 122). LZ is less compact and more diverse than is DZ. B cells continuously move between the DZ and LZ, helping in the further differentiation and affinity maturation of GC B cells (123–126). Within the LZ, B cells continuously interact with Tfh, FDC, and antigen, interactions that dictate their survival and clonal selection (84, 127–129).

Germinal Center Reactivity Against Therapeutics

Several studies have investigated GC dynamics after drug administration. Understanding how the LN and GC react to therapeutics (antibodies, recombinant proteins, cytokines, vaccines, enzymes, etc.) is important to reduce/eliminate ADA development. ADA can be generated by T cell-dependent (Td) and T cell-independent (Ti) pathways (130–132). The Td pathway involves an antigen-activated T cell that then stimulates B cell activation and differentiation to plasma cells. Neutralizing IgG4 ADA against FVIII (6, 133) was triggered by a Th2 CD4 T cell response (134), whereas initiation of Th1 responses was found to induce IgG1 and IgG2 ADA against FVIII when patients were on immunosuppressive therapy (134), which may sometimes be non-neutralizing (133, 134). Administration of IFN- β was found to induce either non-neutralizing and transient neutralizing ADA, mainly of low titers and affinity IgG1 and IgG3 subclasses, or persistent neutralizing ADA, which had mostly IgG2 and IgG4 antibodies (135). ADA binding affinity was positively correlated with IgG4 production and neutralizing ADA titers but negatively correlated with IgG3 production. Similarly, generation of high-affinity antibodies to biopharmaceuticals is CD4 T cell dependent (136, 137). In fact several studies have shown the development of neutralizing antibodies ADA (138–142) mainly of IgG4 subtype (143). Similar polyclonal IgG1 responses consisting of neutralizing and non-neutralizing specificities have also been detected in response to natalizumab (NZM) administration in multiple sclerosis patients. Neutralizing antibodies in these patients carry a higher load of somatic mutations in the complementary determining regions (CDRs) and have a higher affinity than have non-neutralizing, binding antibody specificities, which is consistent with LN-specific antigen-driven selection (144).

These considerations indicate that alternative cytokine milieu and initial programming of CD4 T cells in the LN can affect the outcome of the GC B cell responses to a given therapeutic in

a way that parallels the LN-associated changes seen in vaccine-specific or pathogen-associated antibody production. The Ti pathway is triggered when the B cell is activated directly by the antigen. In general, polyvalent antigens, such as an aggregated biologic (145, 146), are more likely to induce Ti-B cell responses (147, 148). Ti responses lead to IgM or low affinity IgG ADA owing to lack of T cell help (130). Because most ADAs are IgG, the possible role of complement activation by ADA needs further investigation (130). Neutralizing antibodies, particularly the broad neutralizing antibodies (bNABs), are characterized by high levels of somatic mutations (149) and are indicative of GC maturation. The mechanisms leading to such maturation process in the GC are not well-understood. Presumably, antigen concentration within LN/GC and the co-evolution of Tfh and GC B cells (selection of TCR and BCR clones) are major biological factors affecting this process. Studies using mouse models have shown that the quality of Tfh help to GC B cell is an important biological factor for the development of high-affinity antibodies (127, 129, 150). Specifically, Tfh helps regulate the metabolic programming of LZ B cells that support their proliferation in DZ (129). Furthermore, this helps prolong the duration of B cell cycle in the DZ, a process associated with the generation of high-affinity GC B cells (151). Shuttling/binding of antigen to FDC (152) as well as the amount of antigen presented to Tfh by GC B cells (150) can have a significant impact on the development of high-affinity B cell responses. Dynamics and factors in the follicular, non-GC area can also affect the maturation of B cell responses. High-affinity B cell clones can be selected during early interaction between CD4 T and B cells at the T–B cell border (84), whereas class switch recombination can be started outside GC (153). Furthermore, recently identified populations like the CD25+FoxP3+ T follicular IL-10-producing cells (154) and Tbet+ B cells, mainly localized around the GC (155), could be important regulators for the development of neutralizing antibodies. Host genetics are also relevant. The presentation of MHC class II-restricted drug-specific peptides on CD4+ T cells can further contribute to the emergence and maintenance of polyclonal drug-specific B cell responses in lymphoid localities (144). Conversely, elimination of specific drug-associated T cell epitopes in mice treated with recombinant immunotoxins curtails the development of high-affinity antidrug IgG responses in primary as well as anamnestic responses (156). Taken together, these findings suggest that at least for some types of biologic pharmaceuticals, T-dependent pathways in LNs are central in the induction of neutralizing ADAs. Therefore, understanding in more detail the nature, trafficking/distribution of each biopharmaceutical into LN and its availability/sustainability on FDCs is warranted, as these are factors could direct the cellular and molecular mechanisms immobilized in the LN, which lead to the development of specific types of antibody responses, especially in the absence of adjuvants, which trigger innate immunity.

CONCLUSIONS

The cellular and molecular mechanisms governing the development of ADA responses in humans are not well-understood. This is a highly coordinated process taking

place in secondary lymphoid organs where the nature of the “antigen,” tissue structure, and spatial positioning of relevant cell populations, particularly in the follicular/GC area, play a critical role for the host–therapeutic interplay leading to the differentiation of adaptive immune cells to enable the generation of antibody-secreting B cells. Today, the link between ADA and changes in LN function is still not well-studied. Major aspects related to ADA development that need further investigation include the following:

1. The trafficking and sustainability of a particular therapeutic in the LN areas, particularly the GC. Formulation for the *in vivo* delivery of an immunogen could significantly change its dynamics in the GCs with major impact on the B cell response development (157, 158).
2. The activation of specific innate immunity cells and the concomitant changes in the local cytokine/chemokine milieu are factors regulating the degree of CD4 T cell help for the B cell responses (78).
3. The possible association between ADA titer and affinity maturation and particular GC dynamics (i.e., magnitude of Tfh cell responses and expansion of particular Tfh subsets).
4. The possible role of preexisting immune activation and inflammation within the lymphoid organs.

Of particular interest is the investigation of ADA development in aging where the GC dynamics are different compared with those in young individuals (159–161) as well as in chronic inflammatory diseases like HIV and autoimmunity. For example, altered antibody responses are expected in HIV-infected individuals where chronic infection is associated with

LN inflammation, architecture damage (fibrosis), and dramatic changes to GC dynamics (100, 155, 162, 163). Such LN changes could have a major impact in the ADA development to a specific therapeutic. Despite the limited predictive value for the drug immunogenicity in humans based on non-human primate (NHP) studies (164), NHP represents the only model for testing antibody development under such conditions. However, we need to keep in mind that compared with that in humans, ADA in NHP is mainly directed against the Fc region of the monoclonal antibody, causing loss of efficacy and adverse effects. Supplemental to human studies, investigation of therapeutic immunogenicity, when it occurs, in NHPs could lead to identification of particular cell types, molecules, and molecular pathways driving the responses to a particular therapeutic. The wide range of titers, subtypes, and function of ADA induced by different therapeutics argues for the need for identification of “LN molecular/cellular signatures” specific to certain therapeutic, which could lead to targets for “individualized” *in vivo* manipulation of ADA development.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Development of ImmTOR Tolerogenic Nanoparticles for the Mitigation of Anti-drug Antibodies

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The development of anti-drug antibodies (ADAs) is a common cause for treatment failure and hypersensitivity reactions for many biologics. The focus of this review is the development of ImmTOR, a platform technology designed to prevent the formation of ADAs that can be applied broadly across a wide variety of biologics by inducing immunological tolerance with ImmTOR nanoparticles encapsulating rapamycin. The induction of tolerance is antigen-specific and dependent on the incorporation of rapamycin in nanoparticles and the presence of the antigen at the time of administration of ImmTOR. Evidence for the induction of specific immune tolerance vs. general immune suppression is supported by the findings that: (1) ImmTOR induces regulatory T cells specific to the co-administered antigen; (2) tolerance can be transferred by adoptive transfer of splenocytes from treated animals to naïve recipients; (3) the tolerance is durable to subsequent challenge with antigen alone; and (4) animals tolerized to a specific antigen are capable of responding to an unrelated antigen. ImmTOR nanoparticles can be added to new or existing biologics without the need to modify or reformulate the biologic drug. The ability of ImmTOR to mitigate the formation of ADAs has been demonstrated for coagulation factor VIII in a mouse model of hemophilia A, an anti-TNF α monoclonal antibody in a mouse model of inflammatory arthritis, pegylated uricase in hyperuricemic mice and in non-human primates, acid alpha-glucosidase in a mouse model of Pompe disease, recombinant immunotoxin in a mouse model of mesothelioma, and adeno-associated vectors in a model of repeat dosing of gene therapy vectors in mice and in non-human primates. Human proof-of concept for the mitigation of ADAs has been demonstrated with SEL-212, a combination product consisting of ImmTOR + pegadricase, a highly immunogenic enzyme therapy for the treatment of gout. ImmTOR represents a promising approach to preventing the formation of ADAs to a broad range of biologic drugs.

Keywords: nanoparticles, immune tolerance, rapamycin, regulatory T cells, anti-drug antibodies

INTRODUCTION

The rise of biological therapies, first from natural sources and more recently from recombinant DNA technology, has heralded a revolution in medicine (1, 2). However, from early on, it was recognized that immune responses to biologic therapies could compromise the efficacy and safety of treatment (3–6). The formation of anti-drug antibodies (ADAs) can neutralize the activity or alter

the pharmacokinetics and biodistribution of biologic drugs (5–7), cause hypersensitivity reactions, including life-threatening anaphylaxis (3, 5, 6), or cross-react with endogenous proteins (5, 8). Protein engineering has aided in reducing the risk of immunogenicity, but even biologics derived from human sequences, such as growth factors (8) and therapeutic monoclonal antibodies (9) can elicit ADAs resulting in late stage clinical trial failure (10, 11). Moreover, the current trend in protein design is to create novel structures, such as bispecific antibodies or chimeric proteins, which are foreign to the immune system (12). Prevention of ADAs in an antigen-selective manner would be desirable to reduce late stage clinical failure of promising novel biologics in development and to improve the safety and efficacy of existing products (13, 14). Here we describe the development of tolerogenic ImmTOR nanoparticles incorporating rapamycin that can be applied broadly to mitigate the immunogenicity of biologic therapies. This review describes the development of ImmTOR (section Development of Tolerogenic ImmTOR Nanoparticles), its putative mechanism of action (section Tolerogenic Properties of ImmTOR), and its application to various biologic therapies in animal models of disease (section Application of ImmTOR to Mitigating Immune Responses Against Biologic Therapies). For an overview of other immune tolerance technologies and ADA mitigation strategies, the reader is referred to other recent reviews on THE subject (15–21).

DEVELOPMENT OF TOLEROGENIC IMMTOR NANOPARTICLES

Primary adaptive immune responses are initiated in lymphoid organs where antigen-presenting cells are poised to capture antigen and then process and present peptide fragments to T cells. Professional antigen-presenting cells, such as dendritic cells (DCs), sit at the crossroad of immune stimulation and immune tolerance (22, 23). The context in which DCs encounter antigen influences outcome of the immune response. ‘Danger signals’ comprised of pathogen-associated molecular patterns (PAMPs), such as microbial toll-like receptor (TLR) agonists, or damage-associated molecular patterns (DAMPs) associated with tissue injury can activate DCs to express co-stimulatory molecules and pro-inflammatory cytokines that promote immune stimulation (24, 25). For example, traditional vaccines rely on either exogenous adjuvants or TLR agonists that are integral to microbial components of a vaccine to promote antigen-specific effector T cell responses. The purpose of an adjuvant in vaccines is to provide the pro-stimulatory context to an antigen that ensures DC activation and maturation and a robust immune response (26). Conversely immature or quiescent DCs process and present antigen that results in the formation of regulatory T cells (Tregs) (22, 23). Antigen administered in the absence of PAMPs can be tolerogenic, but there is a potential risk that the same antigen could be immunogenic if administered in a setting of inflammation. Our goal was to identify a ‘tolerogenic adjuvant’ that could provide context to antigens, specifically biologic drugs, that

would promote immune tolerance programming even in the face of inflammatory signals (**Figure 1**).

ImmTOR (previously known as SVP-rapamycin) are synthetic, biodegradable nanoparticles comprised of PLA (poly(D,L-lactide) and PLA-PEG [poly(D,L-lactide)-block-poly(ethylene-glycol)] polymers encapsulating rapamycin. We were guided by the following design criteria in developing ImmTOR: (1) use of nano-sized particles to allow for efficient targeting of DCs in lymphoid organs; (2) use of biocompatible and biodegradable polymers that have been approved for human use in multiple products, (3) use of a small-molecule immunomodulatory agent that has been validated in humans and is capable of inducing tolerogenic DCs and antigen-specific Tregs, and (4) a universal approach that could be applied to a broad range of biologic drugs in a manner that allows for immediate therapeutic benefit without the need to alter the biologic drug product. Rapamycin, alone or in combination with other immunomodulators, has been shown to have tolerogenic properties, both *in vitro* (27, 28) and *in vivo* (29–31); however, *in vivo* applications require extended daily or 3X/week administration. Our goal was to develop a technology that allows for dosing only at the time of administration of the biologic therapy.

Why Nanoparticles?

Nanoparticles are an effective means to target DCs and other APCs in lymphoid tissues (32, 33). The immune system has evolved to filter out and interrogate nanoparticulates, which are virus size and represent a potential threat. In peripheral tissues, nanoparticulates can be endocytosed by resident DCs and myeloid cells which migrate to draining lymph nodes or can flow directly to regional lymph nodes through the draining lymphatics. Blood borne nanoparticulates are filtered out in the spleen and liver. Indeed, whole animal imaging of mice injected with fluorescent labeled ImmTOR showed accumulation of ImmTOR in the draining popliteal, iliac, and renal lymph nodes within 1 h after subcutaneous (s.c.) injection in the hind limb and similarly rapid accumulation in the spleen and liver following intravenous (i.v.) administration (34). Within the spleen, immunohistochemistry showed co-localization of ImmTOR particles with dendritic cells in the marginal zone as well as within macrophages (34). These findings were confirmed by flow cytometric analysis of splenocytes, showing a significant fraction of conventional DCs, plasmacytoid DCs, monocytes and macrophages had endocytosed fluorescent-labeled ImmTOR (34, 35). In contrast, 1% or less of CD4 T cells, CD8 T cells, B cells, and neutrophils were positive for fluorescent ImmTOR (35). These results indicate that ImmTOR leverages the natural disposition of nanoparticulates to target APCs in lymphoid organs.

Use of PLA Polymers

ImmTOR is primarily composed of the biodegradable polymers PLA and PLA-PEG. PLA is part of the broader PLGA [poly(lactide-co-glycolide)] family of biodegradable polymers that have more than 30 years of clinical use and are formulation components in a number of approved products, including Zoladex®, Risperdal® Consta®, Vivitrol® and Lupron Depot® (36). PLA- and PLGA-based nanoparticles are hydrolyzed in an

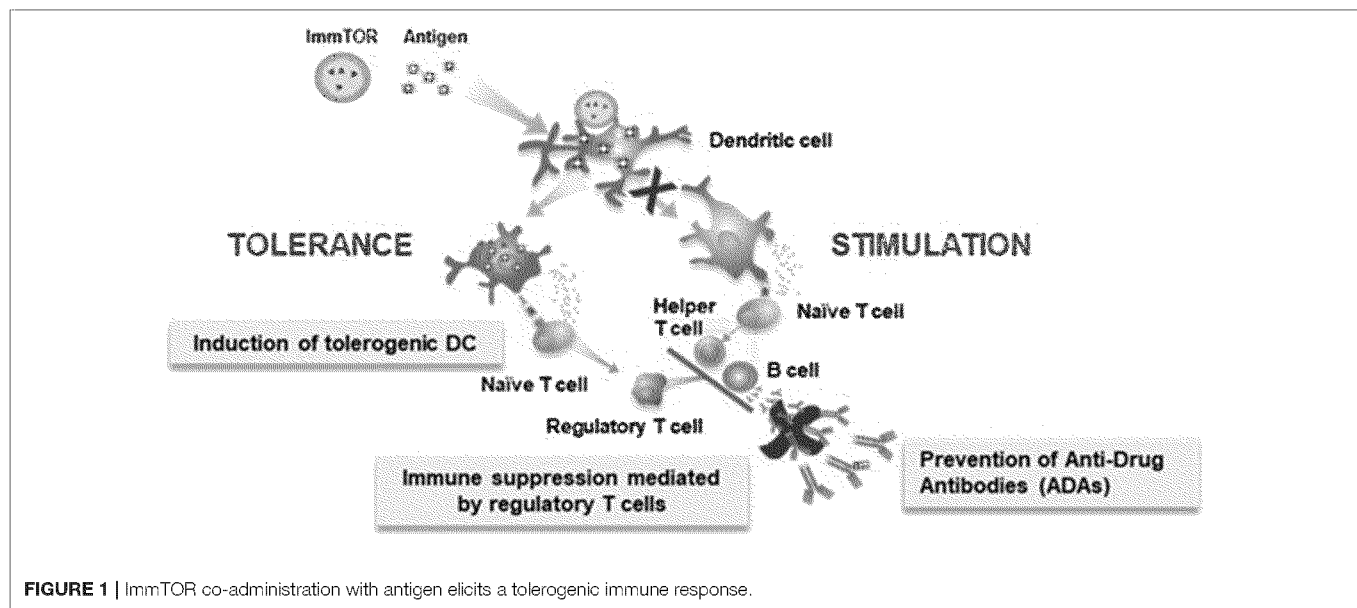


FIGURE 1 | ImmTOR co-administration with antigen elicits a tolerogenic immune response.

acidic environment, such as that of the endosome, and the release of the payload can be tuned for optimal activity *in vivo* (37). PLA is hydrolyzed to lactic acid, a natural metabolite that is rapidly cleared. PEG has also been widely studied in clinical trials and is also a formulation component in many approved biological products (38).

Selection of Rapamycin

Rapamycin, a natural macrolide compound that inhibits the mammalian target of rapamycin (mTOR) pathway, has been shown to have tolerogenic properties *in vitro* (27, 28) and *in vivo* (29–31).

Thomson and colleagues demonstrated that treatment of DCs *in vitro* with rapamycin induced a tolerogenic phenotype that promoted the induction of Tregs (27). Murine bone-marrow-derived DCs propagated *ex vivo* in the presence of rapamycin express low levels of MHC class II and significantly reduced levels of co-stimulatory molecules CD40, CD80, and CD86 (27).

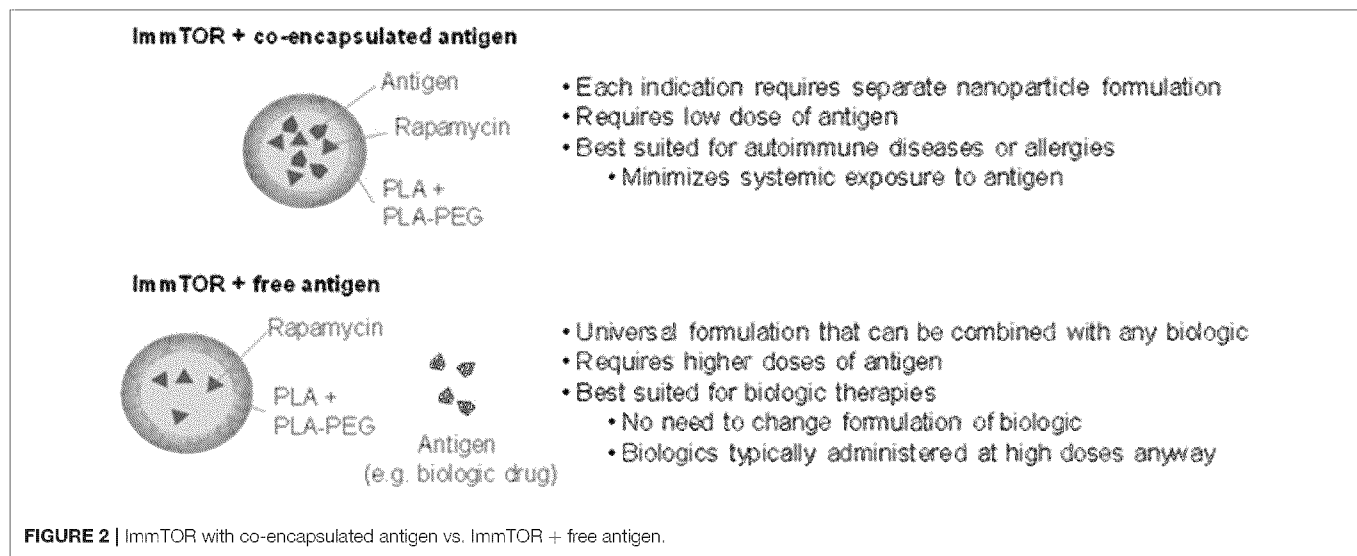
The mTOR pathway also differentially regulates effector T cell vs. Treg activation and differentiation (28, 39, 40). IL-2 promotes proliferation of effector T cells through activation of the JAK/STAT5 pathway and the phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR pathway downstream of the IL-2 receptor. While IL-2 is a critical survival factor for Treg, it does not promote robust proliferation due to expression of PTEN, a negative regulator of the PI3K/Akt/mTOR pathway (40). The mTOR pathway promotes effector T cell expansion by regulating the metabolic switch to glycolysis, which meets the energetic requirements of rapidly proliferating cells (39). In contrast, Tregs rely on mitochondrial oxidative metabolism rather than glycolysis. Rapamycin has been shown to selectively suppress the activation of effector T cells by inhibiting the PI3K/Akt/mTOR pathway, while permitting the differentiation and expansion of Tregs (41, 42). Rapamycin is approved for the prevention of renal transplant rejection (43), but does not induce tolerance in transplantation, perhaps in part due to its use in combination

with calcineurin inhibitors that inhibit both effector T cells and Tregs (44).

Universal Approach to ADA Mitigation

We initially demonstrated that nanoparticles that co-encapsulated both rapamycin and antigen were effective at inducing durable antigen-specific immunological tolerance *in vivo*, including against coagulation factor VIII in a mouse model of hemophilia A (Figure 2) (34). This approach utilizes encapsulation of the biologic in the nanoparticle to induce immune tolerance with either concomitant or subsequent treatment with the free biologic to provide therapeutic activity. The advantage of this approach is that it ensures efficient co-delivery of both the rapamycin and antigen to the same antigen presenting cells. However, the disadvantage for applications involving ADA mitigation is that this approach requires encapsulation of the biologic drug, which would alter its biodistribution and activity. It is possible that ImmTOR + co-encapsulated antigen particle could be used as an initial tolerizing therapy prior to or concurrent with administration of the free biologic drug (45). However, this would still require new formulation development for each biologic drug and GMP manufacturing of both free and nanoparticle-encapsulated drug. For these reasons, the ImmTOR + co-encapsulated antigen approach may be best suited for use as tolerogenic therapies for autoimmune disease or allergies, in which minimizing systemic exposure of autoantigens or allergens, respectively, would be desirable (34, 46).

For the purpose of inhibiting ADAs, it was desirable to have a universal approach that can be applied to any biologic drug therapy without the need to optimize the nanoparticle for each specific biologic and without having to alter the biologic itself or its intended dose route or regimen. We discovered that ImmTOR particles containing only rapamycin could simply be co-administered with a free antigen to induce immunological tolerance (Figure 2) (47). As with ImmTOR



particles encapsulating both rapamycin and antigen, ImmTOR particles containing only rapamycin and co-administered with free antigen were capable of inducing tolerogenic dendritic cells and Tregs that were specific to the co-administered antigen (35, 47, 48).

In the case of ImmTOR-rapamycin particles co-administered with free antigen, the ImmTOR nanoparticles show limited biodistribution to APCs in the liver and spleen, following i.v. administration (34), while the free antigen typically biodistributes broadly. However, as long as some portion of the antigen co-localizes with the APCs that take up ImmTOR particles (35), the resultant tolerogenic DCs can induce antigen-specific Tregs that, in turn, can circulate to other tissues to suppress the immune response against the target antigen. A key advantage of this approach is that the formulation of the biologic drug does not have to be altered—the biologic is simply administered as intended together with the ImmTOR-rapamycin particle. It is not necessary to physically admix the ImmTOR with the biologic prior to injection; the two components can be injected sequentially (35).

TOLEROGENIC PROPERTIES OF IMMATOR

ImmTOR vs. Free Rapamycin

Moghimi et al. (31) reported that free rapamycin administered daily mitigated the formation of antibodies to a sub-therapeutic dose of coagulation factor VIII but not to a therapeutic dose (see section Coagulation Factor VIII for additional detail). In our hands, free rapamycin, even administered daily, did not have the same tolerizing effect as ImmTOR against a highly immunogenic antigen, keyhole limpet hemocyanin (KLH) (47). Mice immunized with 3 weekly doses of KLH and treated concurrently with either daily doses of 50 µg free rapamycin for 3 weeks (5 days per week) or with weekly doses of ImmTOR particles containing 50 µg rapamycin were tested for the generation of immune tolerance to KLH. During the treatment period, both free rapamycin and ImmTOR were similarly

effective in suppressing the anti-KLH antibody response. The treated mice were then challenged with 3 weekly injections of KLH alone. The mice treated with free rapamycin + KLH developed a robust antibody response to KLH that was indistinguishable from naïve mice that had received only the three injections of KLH alone. In contrast, the animals that had been treated with ImmTOR + KLH were still seronegative after the three KLH challenge injections, even though the total rapamycin exposure was five times lower in the ImmTOR group than that of the mice treated with free rapamycin (47). These results highlight the difference between immune suppression vs. immune tolerance, where immune suppression is mediated by a drug with no lasting immunosuppressive effect after the drug is cleared. Whereas, immune tolerance is mediated by immune cells which maintain tolerance even after the drug is cleared. The basis for this difference between free rapamycin and ImmTOR is not entirely clear. *In vitro*, in a static tissue culture well, ImmTOR is not more effective than free rapamycin in inducing Tregs (unpublished observation); therefore, we hypothesize that the difference *in vivo* is related to the selective biodistribution of ImmTOR to lymphoid organs and its preferential uptake by antigen-presenting cells (34, 35). In contrast, rapamycin distributes broadly and extensively into organs and tissues (49). It is known that different doses of rapamycin are needed to inhibit phosphorylation of different mTOR substrates and in different cell types (50). It is possible that conventional dosing of free rapamycin cannot achieve the local concentration necessary to induce a robust tolerogenic phenotype in dendritic cells.

Tolerogenic Window

The ImmTOR particles opens a tolerogenic window that is defined both temporally and spatially. Temporally the free antigen must be concomitantly administered with ImmTOR (35, 47), indicating that ImmTOR is not simply acting as a slow release formulation of rapamycin that mediates chronic immune suppression. This is consistent with findings that adoptive transfer of tolerance requires treatment of donor animals with

both ImmTOR and antigen, as either alone were incapable of inducing regulatory cells capable of transferring tolerance to naïve recipients (35, 46, 48). Spatially, as noted above, fluorescent-labeled nanoparticles show restricted biodistribution to APCs in the spleen and liver following i.v. administration (34). While free antigen is expected to biodistribute broadly, the APCs that take up ImmTOR are also flooded with free antigen during the temporal window which enables peptide epitopes from the free antigen to effectively compete for presentation on MHC molecules expressed by the ImmTOR modified APCs (35). In contrast pathogen-derived antigens, which are likely to enter the body through the lung, gut or skin, will be concentrated in regional lymph nodes draining these tissues.

In vivo Induction of Tolerogenic Dendritic Cells

We evaluated the ability of ImmTOR to induce tolerogenic DCs *in vivo* by treating mice with ovalbumin 323–339 peptide (OVA peptide) alone or in combination with ImmTOR (47). The next day, splenic DCs were isolated and co-cultured with OVA peptide-specific OTII T cells. The DCs isolated from animals treated with OVA peptide + ImmTOR increased the percentage of Foxp3⁺, CD25^{hi} OTII T cells, while DCs isolated from mice treated with OVA peptide alone increased the percentage of interferon- γ producing effector OTII T cells (47). These results demonstrate the ability of ImmTOR co-administered with antigen to induce tolerogenic DCs *in vivo* that are capable of promoting antigen-specific Tregs.

In vivo Induction of Antigen-Specific Treg

The ability of ImmTOR to induce antigen-specific T cells *in vivo* was first demonstrated using nanoparticles encapsulating both rapamycin and OVA peptide (34). OVA peptide-specific OTII transgenic T cells were adoptively transferred into naïve mice and then treated the next day. Mice treated with ImmTOR particles containing OVA peptide and rapamycin show reduced numbers of total OTII T cells and an increased percentage of Foxp3⁺, CD25^{hi} OTII T cells compared to mice treated with nanoparticles containing OVA peptide alone. OVA peptide particles co-administered with free rapamycin actually showed the reverse trend, with a lower percentage of Foxp3⁺, CD25^{hi} OTII T cells compared to control animals treated with OVA peptide particles alone, suggesting that ImmTOR mediates fundamentally different biological outcomes than free rapamycin (34). Similar induction of OVA-specific Tregs was shown for ImmTOR particles containing rapamycin alone co-administered with free OVA peptide (47).

The use of adoptively transferred transgenic OTII cells specific for OVA may not reflect an endogenous T cell response. We assessed the ability of ImmTOR to induce endogenous antigen-specific Treg using the 2W1S peptide described by Nelson et al. (51). Mice treated with ImmTOR particles containing both 2W1S peptide and rapamycin substantially increased the number and percentage of endogenous 2W1S-specific, Foxp3⁺, CD25^{hi} Treg as detected using a 2W1S-MHC class II tetramer (46). The increased number and percentage of 2W1S-specific, Foxp3⁺, CD25^{hi} Treg were maintained even following challenge with

2W1S peptide co-administered with a potent TLR7/8 agonist or emulsified in complete Freund's adjuvant. Meliani et al. (48) also showed that ImmTOR increased the percentage of lymph node T cells with a follicular regulatory (CXCR5⁺, PD1⁺, Foxp3⁺) phenotype, which may play a key role in inhibiting germinal center B cell responses.

Another hallmark of immune tolerance is the ability to transfer tolerance from treated animals to naïve animals by adoptive transfer of immune cells. Adoptive transfer of tolerance induced by ImmTOR was demonstrated by three separate laboratories (35, 46, 48). The transfer of tolerance required treatment of donor mice with both ImmTOR and antigen; donor mice treated with ImmTOR particles containing rapamycin without co-encapsulated or co-administered antigen were unable to confer tolerance to recipient mice (35, 46). Moreover, the tolerogenic activity of ImmTOR was partially negated by depletion of CD25⁺ T cells, which are enriched for Tregs (35, 48). The inability of anti-CD25 depleting antibodies to completely restore the immune response in ImmTOR-treated animals may reflect additional mechanisms of tolerance mediated by other (non-CD25⁺) regulatory cells or simply incomplete depletion of CD25⁺ Tregs.

Effect of ImmTOR on Effector T and B Cell Responses

Effector and Memory T Cell Responses

Animals treated with ImmTOR + antigen showed reduced antigen-specific T cell activation, proliferation, interferon- γ production, and *ex vivo* antigen-recall responses (47, 48). Adoptive transfer of antigen-experienced immune cells into tolerized donor mice that had previously been treated with ImmTOR + antigen were inhibited in responding to *in vivo* antigen challenge (34, 46). Similarly, recipient SJL mice treated with ImmTOR nanoparticles containing rapamycin and PLP peptide, but not with ImmTOR particles containing rapamycin without antigen, were protected from the development of experimental autoimmune encephalomyelitis following subsequent transfer of activated PLP-specific encephalitogenic T cells (46). These results provide further evidence for an induction of an antigen-specific regulatory cell population capable of inhibiting activated effector T cells.

The ability of ImmTOR to inhibit memory T cells was evaluated by immunizing donor mice with adeno-associated viral (AAV) vector and allowing memory T cells to form (48). Sixty-two days after immunization, antigen-experienced CD4 T cells were transferred into naïve recipient mice which were subsequently challenged with AAV alone or AAV + ImmTOR. The addition of ImmTOR enabled inhibition of the antibody response to AAV even in the presence of antigen-experienced memory T cells (48).

Effector B Cell Activation and Antibody Production

Inhibition of antigen-specific B cells was demonstrated with adoptively transferred hen egg lysozyme (HEL)-specific transgenic MD4 B cells. Treatment of mice with ImmTOR containing rapamycin and either co-encapsulated HEL or co-administered free HEL inhibited MD4 B cell activation

and proliferation compared to mice treated with HEL alone (34, 47). Treatment of mice with ImmTOR + AAV inhibited the expansion of endogenous antigen-specific B cells, as determined by ELISpot analysis for both IgG- and IgM-secreting splenic B cells, without affecting the total number of B cells (48). ImmTOR treatment also strongly reduced the presence of activated germinal center B cells (34, 47, 48). Moreover, the percentage of B cells expressing an anergic or regulatory phenotype was significantly higher in animals treated with ImmTOR + antigen vs. antigen alone (47, 48).

These results are consistent with the ability of ImmTOR to inhibit antibody responses to a variety of antigens. ImmTOR was capable of completely inhibiting the formation of antigen-specific IgG1, IgG2a, IgG2b, and IgG3 antibodies in mice (35, 48). Importantly, ImmTOR also inhibited the formation of antigen-specific IgE antibodies that could potentially cause hypersensitivity responses (34). ImmTOR has shown varying activity in inhibiting an IgM response (35, 47, 48).

ImmTOR treatment had no apparent effect on pre-existing bone marrow plasma cells (35), as expected, as long-lived plasma cells do not require T cell help (52). From a safety perspective, it would be undesirable to deplete long-lived plasma cells as these cells produce protective immunity against previously encountered pathogens and vaccines.

APPLICATION OF IMMTOR TO MITIGATING IMMUNE RESPONSES AGAINST BIOLOGIC THERAPIES

We have tested the ability of ImmTOR to mitigate the formation of ADAs against a variety of highly immunogenic biologic therapies with different physicochemical properties, dose routes and dose regimens (Table 1).

Coagulation Factor VIII

Replacement coagulation factors has been a mainstay therapy for the treatment of hemophilia patients, such as Factor VIII (FVIII) therapy for the treatment of hemophilia A and Factor IX therapy for the treatment of hemophilia B (54). The formation of neutralizing antibodies (inhibitors) against FVIII occurs in 20–30% of patients with severe hemophilia A treated with replacement factor, exposing these patients to increased risk of bleeding episodes. While bypass therapies exist, such as Factor VIIa (55) and the bi-specific emicizumab antibody (56), the development of ADAs is still a major complication for patients with hemophilia A (57).

Initially nanoparticles containing both rapamycin and FVIII were used to demonstrate the induction of durable antigen-specific immunological tolerance in a mouse model of hemophilia A (34). Zhang et al. (45) studied ImmTOR particles containing co-encapsulated FVIII and ImmTOR co-administered with free FVIII. Both strategies were effective in mitigating ADAs against FVIII, even after multiple challenge injections of FVIII alone. However, the two types of ImmTOR particles (Figure 2) were studied using different treatment regimens and different challenge injections, so the results cannot be directly compared. In the former case, hemophilia A mice

received two tolerizing doses of ImmTOR + co-encapsulated FVIII followed by three additional weekly injections of the nanoparticle concurrently with 3 weekly injections of a therapeutic dose of free FVIII. The mice were then challenged with 4 injections of FVIII alone. The anti-FVIII response was effectively inhibited, with the exception of one mouse that showed high titers. Mice treated with ImmTOR + co-encapsulated FVIII showed normalized bleeding responses to repetitive treatment with FVIII (45). While effective in inhibiting the formation of total anti-FVIII IgG and anti-FVIII neutralizing antibodies, this approach utilizes encapsulation of the FVIII in the nanoparticle with either concomitant or subsequent treatment with the free FVIII (34, 45), which is not ideal for drug development (see section Universal Approach to ADA Mitigation). Thus, subsequent studies investigated ImmTOR co-administered with free factor VIII.

ImmTOR particles containing rapamycin alone co-administered with free FVIII was similarly effective and specific in mitigating the formation of ADAs (45). The advantage of this approach from a drug-development perspective is that the biologic drug is not physically altered. Five weekly co-administrations of ImmTOR particles containing rapamycin with therapeutic doses of free FVIII induced durable mitigation of ADAs that was maintained for at least 5 months despite repeated challenges of FVIII alone but did comprise the immune response to other antigens. Moghimi et al. (31) previously reported that free rapamycin administered daily (6x/week) for 1 month with factor VIII mitigated the ADA response to subsequent dosing of factor VIII alone (31). Both Zhang et al. (45) and Moghimi et al. (31) administered the same amount of rapamycin (in ImmTOR or as free drug, respectively) per dose, but due to the daily dosing of free rapamycin vs. weekly dosing of ImmTOR, the cumulative rapamycin dose was 6X higher for free rapamycin than that required with ImmTOR over the same 1 month tolerizing period (31, 45). In addition, the free rapamycin had to be administered with a sub-therapeutic dose of factor VIII during the tolerizing period. A key advantage of the ImmTOR approach is being able to administer the tolerizing therapy with therapeutic doses of the biologic such that the patient can receive therapeutic benefit immediately from the beginning of therapy, without the need for a lead-in tolerization period.

Zhang et al. (45) also showed that ImmTOR + free FVIII was therapeutically efficacious in controlling the ADA response in hemophilia A mice that were pre-sensitized to factor VIII. Initially, mice that had low levels of anti-FVIII antibodies prior to the start of treatment showed an initial increase in anti-FVIII antibody levels, but the levels steadily decreased after a second course of therapy; whereas, control mice treated with empty nanoparticles + FVIII showed increasing titers over time.

Pegylated Uricase

Humans lack endogenous uricase, an enzyme that metabolizes uric acid, and consequently can develop gout, a disease caused by deposition of urate crystals in joints and soft tissues leading to painful gout flares, bone remodeling, and disability (58). Recombinant pegylated uricase has been developed as a promising therapy for the treatment of chronic

TABLE 1 | Mitigation of antibodies against biologic therapies by ImmTOR in preclinical animal models.

Biologic (disease)	Preclinical model	Key results	Reference
Pegadricase (chronic refractory gout)	Hyperuricemia in uricase-deficient mice	- Mitigated ADA formation and enabled sustained reduction of serum uric acid in uricase deficient mice - Mitigated ADA formation and prolonged serum uricase activity in non-human primates	(47)
Adalimumab (autoimmune diseases)	Inflammatory arthritis in transgenic mice expressing human TNF α	- Sustained mitigation of ADAs even after 9 challenge injections of adalimumab alone - Improved clinical outcome as measured by arthritis score, histopathology of joints, and radiographic imaging	(47)
Coagulation factor VIII (hemophilia A)	Factor VII-deficient mice	- Sustained mitigation of ADAs even after multiple challenge injections of factor VIII alone administered over 5.5 months - Sustained blood coagulation with repeated dosing - Did not affect immune responses to other unrelated antigens administered during challenge period - Mitigated ADA response in animals previously exposed to Factor VIII	(45)
LMB-100 immunotoxin (mesothelioma)	Mesothelioma tumor in mice	- Sustained mitigation of ADAs even after 11 challenge injections of LMB-100 alone - Did not affect immune responses to other unrelated antigens administered during challenge period - Mitigated ADA response in animals previously exposed to LMB-100 - Improved survival in tumor bearing animals - Adoptive transfer of splenocytes from treated animals to naïve animals mitigates subsequent ADA formation	(35)
Acid alpha-glucosidase (Pompe disease)	Acid alpha-glucosidase-deficient mice	- More durable inhibition of ADA responses compared to animals treated with methotrexate - Higher glycogen clearance in skeletal muscles and improved motor function - No decrease in body weight compared to animals treated with methotrexate	(53)
Adeno-associated vectors (gene therapy of inherited diseases)	Human factor IX in mice, mouse models of methylmalonic acidemia and ornithine transcarbamylase deficiency	- Mitigation of anti-AAV antibodies, enabling redosing of AAV vector in mice and non-human primates - Antigen-selective to specific serotype of AAV - Inhibition of antigen-specific effector T and B cell responses - Adoptive transfer of splenocytes from treated animals to naïve animals mitigates subsequent ADA formation - Depletion of CD25+ cells partially restores immune response in ImmTOR-treated animals	(48)

gout refractory to oral therapies and has been shown to rapidly and efficiently reduce tissue urate crystal deposits (59). However, pegylated uricases are highly immunogenic tetrameric enzymes that are foreign to the human immune system. The marketed product, pegloticase, induces anti-drug antibodies (ADAs) in ~90% of patients (59, 60). The formation of high titer ADAs correlates with the loss of efficacy and increased risk of infusion reactions (59).

The addition of ImmTOR to pegadricase (formerly known as pegsiticase), a pegylated recombinant uricase derived from *Candida utilis*, prevented the formation of ADAs in uricase-deficient mice and enabled sustained control of serum uric acid in these hyperuricemic mice (47). Similarly, ImmTOR mitigated the immunogenicity of pegadricase and prolonged the pharmacodynamic activity of the enzyme in non-human primates. In addition to inhibiting the anti-uricase IgG response, ImmTOR also inhibited the IgM response to the pegylated enzyme (47). SEL-212, a combination product consisting of ImmTOR + pegadricase, is currently being evaluated in a Phase 2 study in patients with chronic gout refractory to oral therapies (61) (see section Clinical Translation).

Adalimumab

Adalimumab is a monoclonal antibody directed against tumor necrosis factor- α (TNF- α) and approved for the treatment of rheumatoid arthritis, ankylosing spondylitis, Crohn's disease, ulcerative colitis, plaque psoriasis and other autoimmune diseases (62). It has been the best-selling drug for many years with annual sales approaching \$20 billion. Despite being the first fully human monoclonal antibody approved by the FDA, adalimumab is highly immunogenic (9). Greater than 70% of healthy volunteers develop ADAs after a single injection (63–65). The formation of ADAs in rheumatoid arthritis patients was associated with accelerated drug clearance and poor outcomes. Only 3.9% of patients that developed ADAs experienced sustained remission, compared to 34% for patients that did not develop ADAs (9).

Adalimumab, unlike FVIII or pegylated uricase, is administered by subcutaneous (s.c.) administration. ImmTOR administered s.c. localizes to the draining regional lymph nodes (34). We evaluated the ability of ImmTOR to mitigate the immunogenicity of adalimumab administered s.c. in a transgenic mice expressing human TNF α which spontaneously develop

inflammatory arthritis. Co-administration of ImmTOR with adalimumab for 7 weekly injections mitigated the formation of ADAs that was sustained even after 9 additional weekly injections of adalimumab alone (47). Although low titers of antibodies (<1:100) developed by the end of the study, these did not appear to affect clinical outcome. The combination treatment normalized adalimumab blood levels throughout the 16 week treatment period and prevented the development of arthritis as assessed by clinical scores, histopathology, and microCT imaging.

Immunotoxin

Recombinant immunotoxins are chimeric proteins containing a tumor-targeting antibody fragment linked to a protein toxin, such as pseudomonas exoprotein A (66). Recombinant immunotoxins have shown promising clinical activity, highlighted by the recent approval of moxetumomab dasudotox for the treatment of relapsed or refractory hairy cell leukemia (67); however, the bacterial toxin moiety is highly immunogenic, which limits the efficacy of immunotoxins in patients that do not have comprised immune systems. Pastan et al. (68) undertook the herculean task to deimmunize the pseudomonas exoprotein A toxin by painstakingly mapping antibody and T-cell epitopes through mutagenesis and functional analysis, rather than by *in silico* prediction which is prone to artifacts. While immunogenicity could be substantially reduced, it could not be fully eliminated without compromising the activity of the immunotoxin.

The Pastan group showed in preclinical studies that ImmTOR was capable of inducing immune tolerance to LMB-100, a partially de-immunized mesothelin-targeted immunotoxin being developed for the treatment of mesothelioma and other solid tumors (35). LMB-100 is administered in cycles, in which a cycle consists of three infusions of LMB-100 administered every other day at the beginning of each cycle. ImmTOR administered at the first dose of each cycle was sufficient to mitigate the ADA response, and two such cycles of treatment was sufficient to enable immune tolerance that allowed for at least three additional cycles (nine injections) of LMB-100 alone without compromising the immune response to other antigens (35). Interestingly, administering ImmTOR on the second dose of LMB-100 in each cycle (2 days after the first dose of LMB-100) was ineffective, underscoring the need to administer ImmTOR within a narrow time window of the first dose of antigen (see also section Tolerogenic Window). ImmTOR mitigated the formation of all IgG subtypes specific for LMB-100 but had no apparent effect on the IgM response. The mitigation of immunogenicity enabled repeated administration and allowed for control of tumor growth and improved survival in a mouse model of mesothelioma, even in mice that were pre-sensitized to LMB-100 prior to treatment (35). This is significant, because some patients have pre-existing antibodies that cross-react with LMB-100, presumably from prior exposure to pseudomonas bacteria (69).

The activity of ImmTOR in pre-sensitized mice was further investigated in two studies (35). In the first study, mice were sensitized with 6 doses of LMB-100 and then rested for 6 weeks prior to treatment. The sensitized animals showed low

ADA titers at the time of treatment. Titers remained low following treatment with LMB-100 + ImmTOR and subsequent re-challenge with LMB-100 alone. In contrast, sensitized mice re-challenged with LMB-100 alone showed a large increase in ADA titer, characteristic of an anamnestic response. In the second study, mice were pre-sensitized with 12 injections of LMB-100 to induce high ADA titers (~10,000–30,000). Subsequent treatment with LMB-100 + ImmTOR was able to reduce titers about 5–10-fold. However, this level of reduction may not be sufficient to allow for therapeutic activity of the immunotoxin. These results suggest that ImmTOR, which targets the dendritic cell-T cell axis, may not be sufficient to mitigate high levels of pre-existing antibodies.

Mazor et al. (35) also studied the combination of LMB-100 with anti-CTLA4 or anti-OX40 checkpoint inhibitors. The checkpoint inhibition enhanced the ADA response to LMB-100, particularly in the case of CTLA4 blockade which increased anti-LMB-100 titers ~8-fold. Interestingly ImmTOR was able to inhibit the formation of ADAs in the presence of checkpoint blockade. However, the effect of ImmTOR on the anti-tumor activity of the checkpoint inhibitors was not investigated. It is possible that different regimens would have to be explored in order to successfully combine ImmTOR with LMB-100 and checkpoint inhibitors (e.g., dosing checkpoint inhibitors after LMB-100 + ImmTOR therapy).

Alglucosidase Alfa

Pompe disease is a rare metabolic disease caused by a deficiency of lysosomal enzyme acid- α -glucosidase and characterized by accumulation of glycogen in lysosomes leading to progressive muscle weakening which can result in death due to cardiorespiratory failure (70). Recombinant alglucosidase alfa (GAA) is a life-saving replacement enzyme therapy (71). However, severely deficient patients are prone to develop neutralizing ADAs that comprises activity (72). There is currently no approved rescue therapy for patients that develop ADAs. Kishnani et al. (73) have pioneered the use of methotrexate, rituximab, and IVIG to mitigate the immunogenicity of alglucosidase alfa, which has saved patients' lives.

Joseph et al. (74) showed that transient dosing of methotrexate on days 1, 3, and 5 after each of the first three treatments of GAA also mitigated the ADA response to subsequent challenge injections of GAA alone in a mouse model of Pompe. Recently this finding was translated in a small human clinical trial (75) in Pompe patients that were positive for GAA cross-reactive immunological material (so called CRIM⁺ patients). Because CRIM⁺ patients usually have some level of natural immune tolerance to GAA due to endogenous expression of low levels of GAA or mutant GAA, these patients tend to have less pronounced ADA responses compared to CRIM⁻ patients, and consequently have better clinical responses to GAA therapy (76). Twelve of 14 treatment-naïve CRIM⁺ Pompe patients treated with transient dosing of methotrexate on days 1, 3, and 5 after each of the first three treatments of GAA developed only low titers (<12,800) of anti-GAA antibodies. There was no concurrent control in this pilot clinical study, but the results compare favorably with a retrospective analysis showing the

development of high ADA titers in 9 of 23 (39%) CRIM+ patients (77).

The Kishnani group conducted a small pilot study comparing the ability of ImmTOR vs. transient dosing with methotrexate, as described by Joseph et al. (74), for the ability to mitigate the immunogenicity of GAA in a mouse model of Pompe disease (53). ImmTOR treated animals showed more durable inhibition of ADA formation, higher glycogen clearance in skeletal muscles, and improved motor function compared with animals treated with GAA + methotrexate. Moreover, the animals treated with GAA + methotrexate showed a ~5% loss in body weight during the treatment phase, while mice treated with GAA + ImmTOR showed a ~4% gain in body weight over the same period. The body weights of the GAA + methotrexate-treated mice lagged behind those of the GAA + ImmTOR treated mice throughout the duration of the study (10 weeks after the treatment phase). Antibody titers against GAA developed by week 6, after 3 weekly challenges of GAA alone in the GAA + methotrexate-treated animals. In contrast, anti-GAA antibody titers remained low through 10 weeks, after 7 GAA challenge injections, in the GAA + ImmTOR-treated group. However, antibody titers developed by 12 weeks, the last time point measured in the study. These results indicate that tolerance was broken after repeated challenge injections of GAA. It is possible that the durability of tolerance could be extended by additional co-injections of ImmTOR, either at the beginning of therapy or intermittently to reinforce tolerance, analogous to a booster injection used in vaccines (see also section Durability of Tolerance).

Adeno-Associated Virus

Gene therapy is one of the most promising approaches for the treatment of thousands of rare genetic diseases. The field has experienced a renaissance since the development of AAV as a vector for *in vivo* gene delivery (78). AAV is a non-pathogenic and largely non-integrating virus capable of transducing multiple cell types, including non-dividing cells, but does not induce a strong immune response. However, AAV does elicit the formation of neutralizing antibodies (79, 80). Due to the non-integrating nature of AAV, transgene expression can wane over time due to cell turnover. For many inherited metabolic and degenerative diseases, correction of the defective gene is often needed in infancy or early childhood to limit irreversible progression of disease. However, as the child grows, the target organ, such as the liver, may also increase in mass by several fold. In addition, liver injury, caused by infection or chemicals, may cause further turnover of hepatocytes resulting in further dilution of the transgene. These patients may require retreatment to restore therapeutic benefit. However, currently retreatment is not possible due to the formation of neutralizing antibodies that occur after the initial treatment with AAV vectors. Mitigating the immunogenicity to AAV is particularly challenging because of its size, the repetitive display of antigenic epitopes on the capsid, and the high degree of antibody suppression required to prevent vector neutralization (79, 80).

Mingozzi et al. (48) investigated the ability of ImmTOR to mitigate the formation of anti-AAV antibodies and enable vector

re-dosing. In these experiments, animals were transduced with an AAV8 vector expressing an irrelevant transgene on day 0 and then treated with a second AAV8 vector expressing human factor IX on day 21. The rationale behind this design was that expression of the human factor IX transgene should only be observed if the immune response to the initial dose of AAV was sufficiently inhibited to allow efficient transduction on day 21. These investigators demonstrated that co-administration of ImmTOR with AAV vector prevented the formation of anti-AAV antibodies in both mice and non-human primates and enabled productive expression of the factor IX transgene upon repeat dosing (48). ImmTOR combined with the AAV8 serotype vector did not compromise the immune response to AAV5 serotype, demonstrating antigen selectivity to the co-administered capsid. AAV transduction of hepatocytes in the liver appears to be a stochastic process. Using two different transgenes for the first and second administrations, Meliani et al. (48) showed that a second dose of AAV, enabled by the use of ImmTOR, was capable of transducing hepatocytes that were not transduced after the first dose. This may be particularly important for the correction of metabolic diseases of the liver, where the total percentage of transduced cells may be critical for efficacy. Redosing for gene therapy is different from redosing of most biologic therapies, which are typically administered on a regular schedule. In the case of gene therapy, the interval of redosing would likely be a minimum of several months if not years after the initial dose. Meliani et al. reported that optimal mitigation of anti-AAV antibodies required administration of ImmTOR at both the initial and repeat dose of AAV (48). Due to the particulate nature of AAV capsid, which contributes to its immunogenicity, and the fact that even low titers of antibodies can neutralize AAV transduction, the therapeutic dose of SEL-110 for AAV gene therapy applications was typically higher than that required for protein therapies (100–200 µg vs. 50–100 µg). In addition to mitigating the formation of ADAs, ImmTOR treatment inhibited the appearance of CD8 T cells in the liver (48), an event which may be associated with liver inflammation following systemic AAV administration in human patients (81).

Mitigation of Hypersensitivity Responses

Immune-mediated hypersensitivity reactions are a common cause of adverse events associated with biologic therapies (5). ImmTOR has been shown to inhibit antigen-specific T cell mediated delayed type hypersensitivity reactions, even when administered in the presence of a potent TLR agonist (47). Similarly, ImmTOR was shown to inhibit injection site reactions associated with repeated s.c. injections of adalimumab (47).

Systemic hypersensitivity reactions are more serious and can result in anaphylaxis. Anaphylaxis can be mediated by IgE antibodies that provoke mast cell activation or by IgG immune complexes that can result in complement activation and myeloid cell activation. ImmTOR co-administered with ovalbumin has been shown to inhibit the formation of antigen-specific IgE antibodies and IgE-mediated allergic reactions (34). Repeated high doses of KLH administered i.v. induced

the formation of high titer IgG antibodies that led to anaphylaxis in animals. Co-administration of ImmTOR with KLH inhibited both antibody formation and the anaphylactic response (47).

Pre-existing Immunity

Mitigating or reversing pre-existing immunity is challenging, particularly for pre-existing antibodies. One of the salient features of the adaptive immune system is the formation of memory T and B cells that enable rapid and robust anamnestic responses and the formation of long-lived plasma cells (LLPCs) that continue to produce antibodies even in the absence of further antigen stimulation (52).

For emerging antibody responses against coagulation FVIII, low titer antibodies induced by 3–6 injections of FVIII alone could be reduced in the majority of animals by repeated therapeutic treatment with FVIII + ImmTOR (45). Mazor et al. (35) induced low levels of ADAs against recombinant immunotoxin and rested animals for 6 weeks to allow memory cells to form. Challenging the mice with immunotoxin alone induced a massive anamnestic response resulting in titers that were approximately ten times higher. However, therapeutically treating the animals with ImmTOR + immunotoxin not only prevented the boost in antibody titer, but actually further reduced titers close to baseline levels. In the presence of high titer antibodies (>10,000), induced with 12 injections of recombinant immunotoxin, ImmTOR + immunotoxin could reduce titers 5–10-fold (35). However, even a 5–10-fold reduction in high antibody titers may still affect the activity or pharmacokinetics of a biologic therapy. Thus, the ability of ImmTOR to mitigate pre-existing antibody titers may vary with the antigen and the level of pre-existing antibodies. For T cell-mediated disease, such as EAE, a single dose of ImmTOR containing both rapamycin and PLP antigen administered at the peak of disease was sufficient to resolve disease symptoms and prevent disease relapse (34, 48).

Durability of Tolerance

There are two types of Foxp3+ Tregs (82). Natural Tregs (nTregs) are selected in the thymus based on their reactivity to self-antigens and are critical to maintain tolerance to self. However, naïve T cells that are weakly reactive to self-antigen can escape the thymus and have the potential to become self-reactive. Adaptive Tregs (aTreg) can be induced in the periphery to limit autoimmune responses. The aTreg are also critical for the induction of tolerance to beneficial commensal bacteria, food antigens and harmless environmental antigens. Immune tolerance to biologic agents can leverage nTreg in the case of replacement enzyme or protein therapies, such as FVIII or aglucosidase alpha. However, patients that are completely deficient in the expression of the endogenous protein may lack nTreg specific to the protein and thus are more likely form ADAs (72). Immune tolerance induction in such patients may require induction of aTregs. Similarly, induction of aTreg are critical for biologics that are foreign to the human immune system, like uricase or AAV. The aTreg are more plastic than nTreg and may become unstable

in certain inflammatory conditions (82). This plasticity is important in the event that a “harmless” microbe becomes pathogenic. Thus, induction of immune tolerance is not an irreversible on-off switch. Rather maintenance of tolerance is a dynamic process between pro-tolerogenic and pro-stimulatory signals. The ratio of Treg to effector T cells can determine the outcome of immune tolerance vs. immune stimulation (29). One of the key outstanding questions in the translation of immune tolerance technologies is the durability of aTreg-mediated tolerance.

The durability of tolerance to a biologic therapy may be impacted by a number of factors, including drug-related properties and patient or disease-related factors (83–85). The inherent immunogenicity of the biologic drug can impact the durability of tolerance, as repeated challenge with highly immunogenic antigens could provide an overwhelming immunostimulatory bias (5, 6). Key factors that promote immunogenicity are repetitive display of antigenic-epitopes (e.g., multimeric proteins), the propensity to form micro-aggregates, dose route and regimen, antigens that cause tissue damage or inflammation, and the absence of natural tolerance (e.g., proteins that are foreign to the immune system). Patient and disease-specific factors may include an inflammatory milieu, pre-existing immunity, immune status, co-medications, and genetics. The age of the patient may also be a factor, as the production of naïve T cells wanes with the involution of the thymus, and the T cell repertoire becomes comprised primarily of antigen-experienced memory T cells (86, 87).

Preclinical studies have shown the ability of ImmTOR to induce durable tolerance to a variety of highly immunogenic proteins that withstands multiple challenges with antigen alone. For KLH, five s.c. co-administrations of KLH + ImmTOR maintained tolerance for at least 5 months during which animals were challenged 11 times with KLH alone (47). Similarly, for adalimumab, 7 co-treatments enabled tolerance that was maintained after 9 challenge injections (47). For coagulation FVIII, 5 combination treatment provided sustained mitigation of ADAs for at least 5 months after treatment (45), and for recombinant immunotoxin, two cycles of treatment induced tolerance that allowed for three additional cycles (9 injections) of immunotoxin alone (35). Finally, for recombinant aglucosidase alpha, 3 weekly combination treatments mitigated the formation of ADAs for 7 challenge injections; however, ADA developed by the time of the 10th challenge injection (53). Immune tolerance is a dynamic process balancing pro-stimulatory and pro-tolerogenic signals, and can be broken by repeated injections of a highly immunogenic antigen. It is possible that additional co-treatments with ImmTOR may be required for more durable tolerance or that periodic retreatment with ImmTOR might be needed to reinforce immune tolerance. However, the number and timing of such additional treatments may need to be determined empirically for each biologic and disease setting. This may be a key challenge for successful clinical translation of applications in which patients require life-long therapy, such as the case for GAA in Pompe disease or FVIII in hemophilia A.

Clinical Translation

Most of the preclinical studies with ImmTOR have been performed in inbred strains of laboratory mice, which have their obvious limitations with respect to their anatomy, immune system, genetic diversity, lifespan, microbiome, and environmental factors for translating findings to humans. Oral tolerance therapies for autoimmune disease that looked promising in animal studies have not translated to humans (88). While there are many promising immune tolerance technologies and strategies on the horizon (15–21), translation to humans remains a formidable challenge. Applying immune tolerance strategies to autoimmune diseases adds a layer of risk due to heterogeneous disease presentation and progression, antigen uncertainty, generally poor animal models, and the requirement to reverse a well-established immune response. We have tried to mitigate some of this risk by focusing initially on mitigation of ADAs to biologic therapies, which has the advantage of a well-defined antigen, a robust biomarker readout (ADA levels), and the ability to treat prophylactically.

The ability of ImmTOR to mitigate the formation of ADAs in human has been evaluated in combination with pegadricase, a highly immunogenic, pegylated uricase enzyme of fungal origin, in patients with hyperuricemia. A Phase 1b single ascending dose, open-label, multi-center clinical trial (NCT02648269) conducted in the United States showed a dose-dependent inhibition of uricase-specific ADAs (89). The activity of pegadricase was monitored through the measurement of serum uric acid (SUA). In gout, the therapeutic goal is to lower SUA levels below 6 mg/dL, as higher levels can result in the deposition of urate crystals in joints and soft tissues. Patients were selected for baseline SUA >6 mg/dL. All patients treated with enzyme alone showed an initial drop in serum uric acid (SUA) levels that was maintained for the first week after treatment. However, by day 14, SUA levels started to rebound and by day 30, 4 of 5 patients were back to baseline levels of SUA. All patients treated with a single dose of pegadricase alone developed high titers of ADAs by day 14, which correlated with rapid clearance of serum uricase activity. The addition of ImmTOR showed a dose-dependent inhibition of ADA formation. Mitigation of ADAs correlated with prolonged pharmacodynamic activity of pegadricase and sustained reduction in SUA levels for at least 30 days after a single dose (89). These results suggest that combination of ImmTOR + pegadricase would support monthly dosing. SEL-110 was generally well-tolerated at doses up to 0.3 mg/kg. No deaths or life-threatening treatment emergent adverse events (TEAEs) were reported during the study, and overall, there were no notable trends in the nature or frequency of TEAEs. There were no clinically significant changes in clinical laboratory values,

vital signs or ECGs during the course of the study. Interim data from a Phase 2 multidose, open-label, multi-center clinical trial (NCT02959918) indicate that multiple monthly doses of SEL-212, the combination of ImmTOR + pegadricase, is able to maintain SUA < 6 mg/dL in the majority of patients (61).

CONCLUSION

The full impact of ADAs on healthcare is largely unknown, as ADAs are not routinely measured after drug approval due to the lack of effective ADA mitigation strategies. However, patients that develop ADAs may experience disease progression due to ADAs that compromise efficacy and may be exposed to an increased risk of adverse events (5, 6). ADAs also place a burden on healthcare costs (5). In addition, there are opportunity costs related to the late-stage abandonment of promising but immunogenic biologic drugs in the pipeline (10, 11). While companies strive to minimize immunogenicity during development on a product-specific basis, there is a need for an approach to ADA mitigation that can be applied broadly across many types of biologic therapies. The use of ImmTOR nanoparticles is a promising approach to mitigate the immunogenicity of a diverse array of biologics without the need to reformulate or alter the biologic therapy. Treatment with ImmTOR induces dendritic cells with a tolerogenic phenotype and regulatory T cells specific to the co-administered biologic therapy resulting in inhibition of T and B cell activation and ADA formation. Early clinical studies of SEL-212, a combination product of ImmTOR + a pegylated uricase enzyme, provide proof-of-concept for ADA mitigation against a highly immunogenic enzyme in humans. ImmTOR has the potential to improve the efficacy and safety of biologic therapies for patients and warrants further study.

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TK wrote the manuscript.

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Conflict of Interest: TK is an employee and shareholder of Selecta Biosciences, and ImmTOR is being developed by Selecta Biosciences.

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A Telomerase-Derived Peptide Exerts an Anti-Hepatitis B Virus Effect *via* Mitochondrial DNA Stress-Dependent Type I Interferon Production

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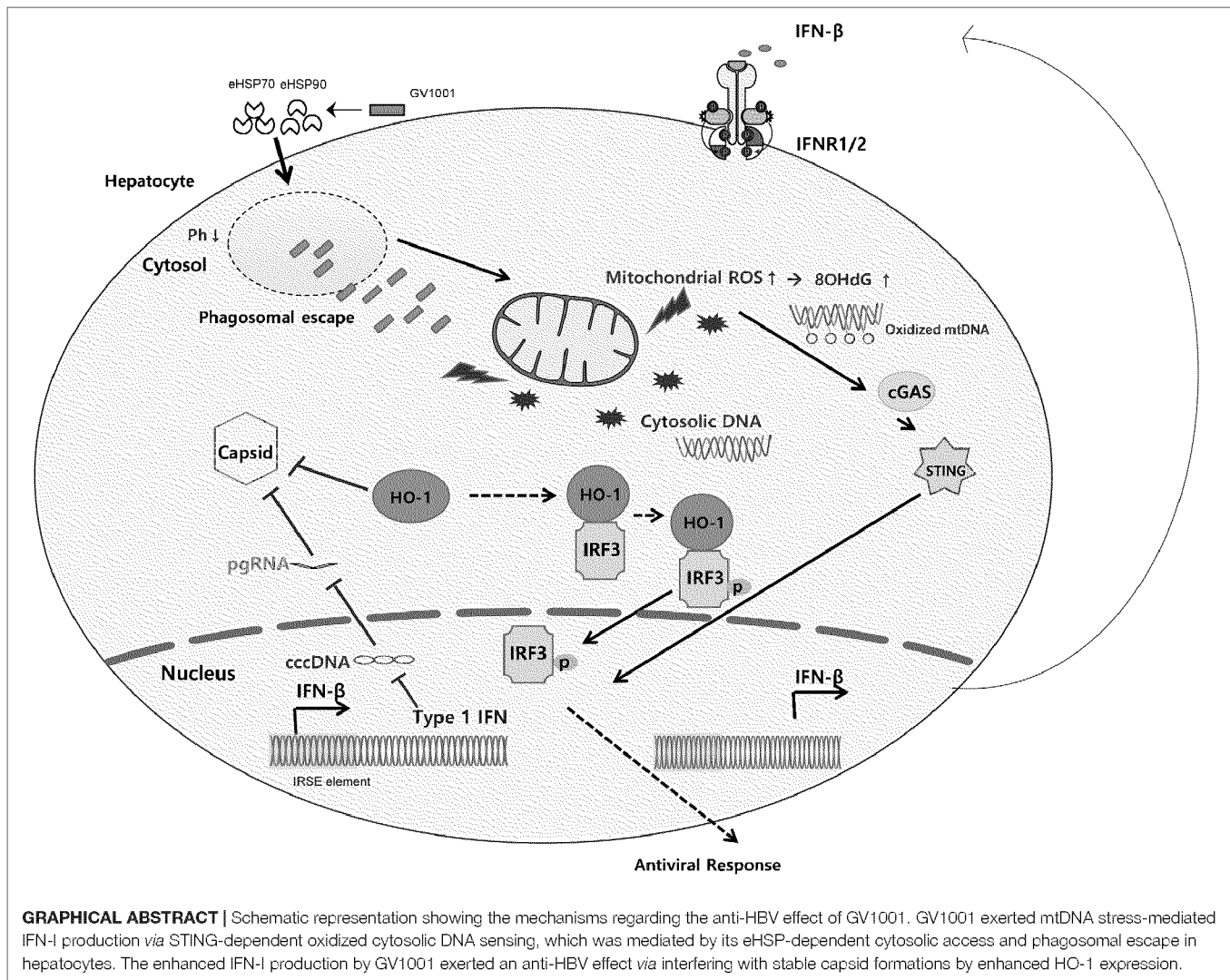
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Previously, a telomerase-derived 16-mer peptide, GV1001, developed as an anticancer vaccine, was reported to exert antiviral effects on human immunodeficiency virus or hepatitis C virus in a heat shock protein-dependent manner. Here we investigated whether GV1001 exerts antiviral effects on hepatitis B virus (HBV) and elucidated its underlying mechanisms. GV1001 inhibited HBV replication and hepatitis B surface antigen (HBsAg) secretion in a dose-dependent manner, showing synergistic antiviral effects with nucleos(t)ide analogs (NAs) including entecavir and lamivudine. This peptide also inhibited viral cccDNA and pgRNA. The intravenous GV1001 treatment of transgenic mice had anti-HBV effects. Our mechanistic studies revealed that GV1001 suppresses HBV replication by inhibiting capsid formation *via* type I interferon-mediated induction of heme oxygenase-1 (HO-1). GV1001 promoted the mitochondrial DNA stress-mediated release of oxidized DNA into the cytosol, resulting in IFN-I-dependent anti-HBV effects *via* the STING-IRF3 axis. We found that the anti-HBV effect of GV1001 was due to its ability to penetrate into the cytosol *via* extracellular heat shock protein, leading to phagosomal escape-mediated mtDNA stress. We demonstrated that the cell-penetrating and cytosolic localization capacity of GV1001 results in antiviral effects on HBV infections *via* mtDNA stress-mediated IFN-I production. Thus, GV1001, a peptide proven to be safe for human use, may be an anti-HBV drug that can be synergistically used with nucleot(s)ide analog.

Keywords: covalently closed circular DNA, heme oxygenase 1, mitochondrial ROS, phagosomal escape, type I interferons

INTRODUCTION

Hepatitis B virus infection is associated with adverse outcomes of liver diseases, including cirrhosis, hepatic decompensation, and hepatocellular carcinoma (HCC). The annual number of deaths caused by HBV-related diseases is approximately 887,000 worldwide (1). Although there is variation according to geography, endemicity, and viral genotypes or the prevalence of vertical



transmission, approximately 12–20% of the infected patients will have a 5-year progression from CHB to liver cirrhosis (LC), and the 5-year cumulative risk of HCC progression is estimated to be between 10 and 17% in LC patients (2).

Unfortunately, despite their high efficacies, all currently approved HBV life cycle inhibitors, including two exogenous interferon (IFN)-based therapies—IFN and pegylated IFN—and five oral nucleos(t)ide analogs (NAs)—lamivudine (LMV), adefovir dipivoxil, entecavir (ETV), telbivudine, and tenofovir disoproxil fumarate, have their own limitations. Long-term NA treatment results in NA-resistant viral strains and cannot completely eradicate HBV cccDNAs in infected hepatocytes (3).

Abbreviations: cccDNA, covalently closed DNA; CHB, chronic hepatitis B; HBeAg, hepatitis B e-antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HO-1, heme oxygenase-1; LAM, lamivudine; mtROS, mitochondrial ROS; NAs, nucleos(t)ide analogs; pgRNA, pregenomic RNA; qPCR, quantitative polymerase chain reaction; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; type 1 IFN, type 1 interferon.

Exogenous IFN-related treatments can eliminate HBV cccDNA in infected hepatocytes *via* epigenetic regulation, which could lead to HBsAg seroconversion in chronic patients, a signature of complete remission. However, these treatments are associated with a high incidence of adverse effects (4). Therefore, novel anti-HBV agents with improved efficacy and safety are urgently needed.

Mitochondria are central eukaryotic organelles of energy production, which maintain mitochondrial DNA (mtDNA) encoding essential protein subunits involved in driving mitochondrial respiration and ATP production (5). In addition to energy production, mitochondria are involved in other cellular functions, including anabolic and catabolic pathways, apoptosis regulation, calcium homeostasis, and reactive oxygen stress (ROS) signaling (6, 7). Moreover, mitochondria were shown to trigger innate immune responses *via* the release of damage-associated molecular patterns (DAMPs), such as oxidized mtDNA during cellular stress, infections, or injury (8). Cytosolic mtDNA has antiviral activity against various viral infections, including HBV, hepatitis C virus (HCV),

and HSV, *via* production of innate cytokines such as type I interferon (IFN-I) or IL-1 β (9, 10). Therefore, agents that induce mtDNA stress have therapeutic potential as antiviral drugs for HBV infections.

GV1001, a human telomerase reverse transcriptase-derived 16-amino-acid peptide, was designed as an anticancer vaccine for several cancers, including advanced pancreatic cancer, non-small cell lung cancer, and melanoma (11–13). In addition to its anticancer effects, GV1001 has various biological activities including anti-inflammatory (14), anticancer (15), anti-apoptotic, and antioxidant roles (16). Furthermore, we recently reported that GV1001 has antiviral effects against HCV and human immunodeficiency virus type 1 (HIV-1) *via* extracellular heat shock protein (eHSP)-GV1001 binding-mediated cell signaling (17, 18). Therefore, we aimed to explore the possible antiviral role of GV1001, a safe drug in human, in HBV infections and to elucidate its underlying mechanism against HBV infections, mainly focusing on mtDNA stress-mediated IFN-I production.

MATERIALS AND METHODS

Cells and Reagents

HepG2 cells were maintained in Eagle's minimum essential medium (MEM) containing 10% fetal bovine serum (FBS), penicillin/streptomycin (PS) (100 U/ml), and N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (25 mM). HepG2-2.15 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, and PS (100 U/ml). Huh-7 cells were maintained in RPMI 1640 medium containing 10% FBS and PS (100 U/ml). Antibodies against HSP90 (sc-101494), HSP70 (sc-32239), heme oxygenase 1 (sc-10789), GAPDH (sc-25778 and sc-293335), HBsAg (sc-52410), pSTAT1 (sc-7988), LAMP-1 (sc-20011 and sc-17768), heme oxygenase 1 siRNA (sc-35554), and control siRNA (sc-30007) were purchased from Santa Cruz Biotechnology (Santa Cruz, Dallas, TX, United States). Antibodies against IRF3 (#4962), pIRF3 (#4947S), STAT1 (#9172), and LC3B (#2775S) were purchased from Cell Signaling Technology, Inc. (CST). Antibodies against HBV core protein were purchased from Dako (B0586) and Abcam (ab18686). Bafilomycin-A1 (B 1793) was obtained from Sigma-Aldrich (St. Louis, MO, United States). A luciferase assay kit (E1501) was purchased from Promega, and the MitoSOX Red mitochondrial superoxide indicator (M36008) was purchased from Invitrogen.

In vivo Assay and Hydrodynamic Injection

Transgenic (TG) mice were generated by transferring the pHY92-1.1x-HBV-full genome plasmid (genotype A2) into C57B1/6N mice, and the TG mice used in this study constitutively express the HBV genome with the W4P mutation in the preS1 region (19). The age-matched TG mice were injected with GV1001 (50 μ g/kg) and LMV (500 μ g/kg) twice per week. Serum was collected from the orbital sinus of the mice at 4 and at 8 weeks. All animal experiments were approved by the Institutional Animal

Care and Use Committee of the Seoul National University College of Medicine (SNU-170308).

Anti-viral Effect Assay

For the analysis of the anti-HBV effect of GV1001, HepG2, Huh-7, or HepG2-2.15 cells were treated with PBS, entecavir, lamivudine, or GV1001 and incubated for 24 or 48 h. The supernatants were collected, and HBsAg and HBeAg ELISAs were conducted using a commercial Bioelisa HBsAg color ELISA Kit (BIOKIT, Barcelona, Spain) and a HBeAg ELISA kit (AccuDiagTM, DIAGNOSTIC AUTOMATION, INC., Woodland Hills, CA, United States), respectively, according to the manufacturer's procedures. Cell pellets were harvested and subjected to RNA extraction for RT-qPCR to determine the viral amount.

HBV Viral Quantification and mRNA

For evaluating the HBV viral titers, HBV genomes from cell culture supernatants and pellets were purified using the QIAamp Blood DNA extraction kit (QIAGEN, Hilden, Germany) and quantitated by qPCR using a primer pair specific to the small S gene (SF: 5'-TTG ACA AGA ATC CTC ACA ATA CC-3') and antisense primer SR (positions 309–328, 5'-GGA GGT TGG GGA CTG CGA AT-3'). The HBV DNA plasma standard containing 1×10^6 HBV genomic DNA copies/ml (HBV DNA Quantiplex, Chiron) was used to standardize the viral titers. The following primer sets were used to investigate the mRNA expression levels with RT-qPCR: 18S-F: 5'-AGTCCCTGCCCTTTGTACACA-3' and 18S-R: 5'-CGATCCGAGGGCCTCACTA-3', HO-1-F: 5'-TTG CCAGTGCCACCAAGTTC-3', and HO-1-R: 5'-TCAGCAG CTCCTGCAACTCC-3', IFN β -F: 5'-TTGTGCTTCTCCACTA CAGC-3' and hIFN β -R: 5'-CTGTAAGTCTGTAAATGAAG-3', mtDNA1-F: 5'-CATGCCCCATCGTCCTAGAAT-3' and mtDNA1-R: 5'-ACGGGCCCTATTTCAAAGAT-3', mtDNA2-F: 5'-CCCTAACACCAGCCTAACCA-3' and mtDNA2-R: 5'-AA AGTGCATACCGCC7AAAAG-3', mtDNA3-F: 5'-TCCAACT CATGAGACCCACA-3' and mtDNA3-R: 5'-TGAGGCT TGGATTAGCGTTT-3').

HBV pgRNA Assay

Total RNA from cell pellets was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, United States) according to the provided protocol. The RNA samples were incubated with RQ1 DNase (Promega, United Kingdom) for 60 min at 37°C, mixed with 1 μ l of stop solution, incubated at 65°C for 10 min to inactivate the DNase, and stored at –80°C until use. For detection of viral pgRNA, 2 μ g of RNA was reverse-transcribed and amplified by the Reverse Transcription System (Promega, United Kingdom). Next, 2 μ l of each cDNA was quantified by RT-qPCR analysis, and the 18S rRNA gene was used to normalize the RNA samples.

HBV cccDNA Assay

HepG2 cells transiently transfected with a linearized 1.2x genotype C2 HBV plasmid were collected and incubated in lysis

buffer A (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 50 mM NaCl, and 1% NP-40) for 10 min at 4°C. The lysates were centrifuged for 5 min at 14,000 rpm, and the nuclear pellet in lysis buffer B (10 mM Tris-HCl, 10 mM EDTA, 150 mM NaCl, 0.5% SDS) was sonicated at three to four pulses of 60% power and incubated overnight at 37°C after treatment with 0.5 mg/ml proteinase K. After the lysates were extracted with phenol-chloroform (1:1) and precipitated with ethanol, 1 µg DNA was treated with 10 U of PSAD (Plasmid safe DNase I, Epicenter, PA, United States) for 45 min at 37°C. The reaction was stopped by incubating for 30 min at 70°C. RT-qPCR was performed, and the 18S rRNA was used for normalization.

siRNA Transfection of HepG2 and HepG2-2.15 Cells

HepG2 cells and HepG2-2.15 cells were grown in six-well plates to 70–80% confluency. siRNA transfection was carried out using Lipofectamine 3000 following the manufacturers' procedures (Santa Cruz and Thermo Fisher Scientific). The cells were incubated with 1 ml of OPTI-MEM containing a mixture of siRNA (75 pmol) and Lipofectamine 3000 reagent (7.5 µl) for 6 h. The transfection solutions were replaced with MEM or DMEM containing 2% FBS in the presence of GV1001 or PBS. Then, the cells were incubated for 24 h, and the supernatants and pellets were collected for ELISAs and RT-qPCR assays.

Cellular Uptake Mechanism

HepG2-2.15 cells were treated with anti-HSP70 (1 µg/ml), anti-HSP90 (1 µg/ml), or anti-GAPDH (1 µg/ml) antibodies for 1 h. After neutralization with HSP70, HSP90, or GAPDH antibodies, the cells were inoculated for 24 h in the presence of GV1001 or PBS. The role of the corresponding proteins in the uptake process was confirmed, and the effect on the virion level in the presence of GV1001 was measured by qPCR.

Cell Cytotoxicity Assay

HepG2 and Huh-7 cells were seeded (1×10^4 cells) in 96-well microplates and incubated with increasing concentrations of GV1001 for 3 days. Cell viability was determined using the MTT assay kit (Promega, Fitchburg, WI, United States). For analysis of the cytotoxicity of GV1001 and entecavir, CytoTox 96 Non-Radioactive Cytotoxicity Assays (G1780, Promega, Fitchburg, WI, United States) of the collected supernatant were performed according to the manufacturer's protocol.

Western Blot Analysis

The harvested cells were lysed using RIPA buffer (CST, #9806) containing protease inhibitor and phosphate inhibitor (Hoffmann-La Roche Inc.) and incubated for 20 min on ice. The lysed cells were centrifuged for 30 min at 13,000 rpm, and the lysates were collected for Western blotting. A Bradford assay was performed for protein quantification. Then, 5X loading buffer and PBS were added for protein quantification, and the samples were boiled for 5 min and chilled on ice. Protein samples were separated by electrophoresis, transferred to NC membranes, and blocked for 1 h with 5% skim milk or bovine

serum albumin. The membranes were incubated overnight at 4°C with primary antibodies (1:1,000). On the next day, the membranes were washed with 0.1% Tween-20 in Tris-buffered saline and incubated in HRP secondary antibodies (1:2,000) for 2 h. After ECL solution was applied to the membrane, proteins were detected on an imager (LAS 2000).

Confocal Microscopy

Confocal Microscopy With Fluorescein

Isothiocyanate-Labeled Peptides and Bafilomycin A1

Cells were seeded and cultivated in two-chamber glass slides (Nunc, Roskilde, Denmark) for 12 h. After the cells were washed with PBS, they were incubated in serum-free OPTI-MEM for an hour. Fluorescein isothiocyanate (FITC)-labeled peptides were added to the cells for 2 h in the presence of Bafilomycin A1 or PBS. The cells were fixed with 4% paraformaldehyde solution for 10 min at room temperature (RT). After fixation, the cells were permeabilized with 0.1% Triton-X 100 for 10 min. Nuclear staining was performed with DAPI, and the cells were mounted in a mounting medium (VECTASHIELD Antifade Mounting Medium, H-1000).

Confocal Microscopy With MitoSOX

The cells were seeded and cultivated in two-chamber glass slides (Nunc, Roskilde, Denmark) for 2 h. After the cells were washed with PBS, they were incubated in DMEM containing 2% FBS in the presence of GV1001 or PBS for 12 h. The cells were washed with PBS and stained with MitoSOX (1 µM) for 10 min. The cells were fixed with 4% paraformaldehyde solution for 10 min at room temperature. After fixation, the cells were permeabilized with 0.1% Triton-X 100 for 10 min. Nuclear staining was performed with DAPI, and the cells were mounted in a mounting medium (VECTASHIELD Antifade Mounting Medium, H-1000). Images were captured using a 100× oil immersion objective lens.

Flow Cytometry With MitoSOX

To determine the mitochondrial superoxide level by flow cytometry, we treated the cells with GV1001 or PBS for either 6 or 12 h and stained them with 5 µM MitoSOX Red according to the manufacturer's protocol. The measurements were carried out using a FACS Calibur system (BD Bioscience, San Jose, CA, United States).

ELISAs for 8-OHdG

After the HepG2-2.15 cells were seeded into six-well plates for 12 h, they were treated with PBS (0.5%) or GV1001 (5 or 10 µM) for 12 h. From the pellets, genomic DNA was extracted using a QIAamp Blood DNA extraction kit (QIAGEN, Hilden, Germany). For the detection of 8-hydroxy-2'-deoxyguanosine (8-OHdG) activity, competitive ELISAs from an 8-OHdG analysis kit (OxiSelect Oxidative DNA Damage ELISA kit, Cell Biolabs, San Diego, CA, United States) was used according to the manufacturer's protocol.

Type I IFN Bioassay and Neutralization Assay

For the indirect measurement of IFN levels using luciferase reporter genes, hMH55-293-ISRE cells integrating IFN-sensitive response elements (ISREs) associated with the luciferase reporter gene at the 3' end were established using 5 µg of puromycin (Sigma-Aldrich, St. Louis, MO, United States). After the supernatant was collected from the cells treated with each reagent for 24 or 48 h, it was added to the hMH55-293-ISRE cells for 6 h. After incubation, the cells were washed with PBS and lysed by Reporter Lysis Buffer (E1500, Promega, Fitchburg, WI, United States) for 30 min at RT. Then, the luciferase assay reagent (E1500, Promega, Fitchburg, WI, United States) was added, and the luminescence was measured using a TECAN m200 reader (TECAN, Switzerland). For the neutralization assay, the HepG2-2.15 cells were seeded into six-well plates for 12 h. On the next day, the cells were pre-incubated with anti-IFNAR2 and anti-GAPDH antibodies for 2 h at RT on the rotator. After the cells were washed with PBS, they were treated with PBS (0.5%) or GV1001 (5 or 10 µM) for 12 h.

Statistical Analysis

Statistical comparisons between the control and tested groups were analyzed using one-way ANOVA. The *p*-value of statistical significance was set at *p* < 0.05 (*), 0.01 (**), or 0.001 (***). All the experiments were independently repeated three times.

RESULTS

Anti-HBV Effect of GV1001 on *in vitro* Hepatocyte Cultures

To determine the antiviral effect of GV1001 on HBV, we first examined its effect on hepatocyte, HepG2, and Huh-7 cells transiently transfected with a 1.2x genotype C2 HBV genome plasmid *via* the analysis of secreted virion and HBsAg levels. We observed an anti-HBV effect of GV1001 on both transfected cells in a dose-dependent manner 48 h after transfection with no cell cytotoxicity. After the treatment with PBS, LMV, or GV1001 for 2 days, the HBsAg and the extracellular virion DNA levels were measured. The GV1001-treated cells in both groups showed decreased HBsAg and extracellular HBV virion level compared with the PBS groups, and the LMV-treated group showed a similar effect to the GV1001-treated group (Figure 1A). No cytotoxicity was observed in either cell lines after the GV1001 treatment, which was confirmed by MTS assay (Figure 1B). Furthermore, extracellular viral DNA was significantly decreased by GV1001 in a dose-dependent manner in HepG2 cells. The mean IC₅₀ of GV1001 on HepG2 cells, transfected with the 1.2x genotype C2 HBV genome plasmid, was approximately 0.67 µM (Figure 1C). Next, we evaluated the anti-HBV effect of GV1001 in stable HepG2-2.15 cell lines that constitutively expressed HBV virions. GV1001 reduced the extracellular HBV virion and HBeAg levels in HepG2-2.15 cells in a dose-dependent manner (Figures 1D,E), while the reduction in HBeAg level was not definite compared with the reduction level in extracellular HBV

virion and in IC₅₀ calculation. GV1001-induced cytotoxicity was not observed on both HepG2 and HepG2-2.15 cells (Figure 1F). Together our data indicated that GV1001 had an antiviral effect on HBV infection in a dose-dependent manner in hepatocyte cultures *in vitro*.

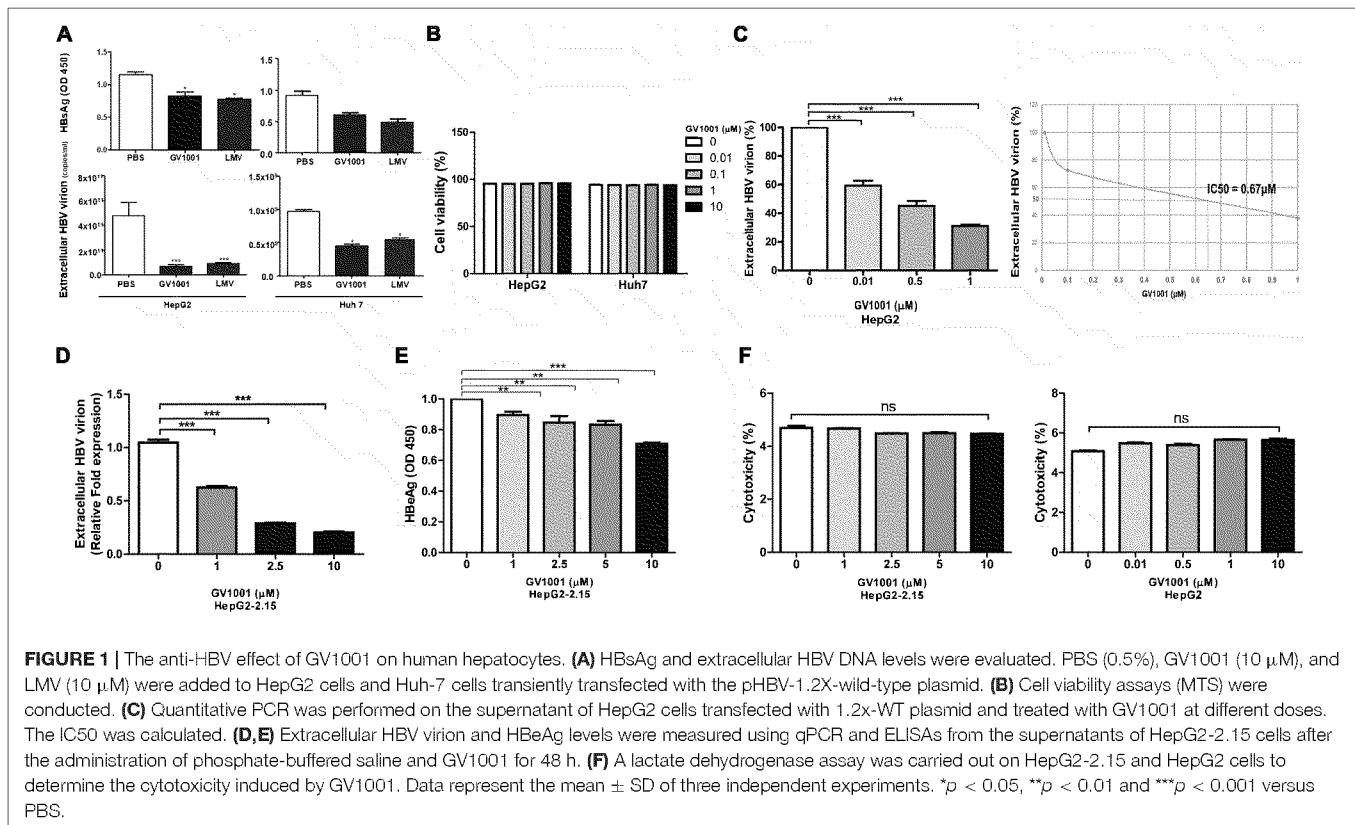
Suppressive Effect on HBV cccDNA, pgRNA, and Nucleocapsid Formation by GV1001 in HepG2 Cells

The cccDNA, which can serve as a template for viral RNAs as an episome in the nucleus, is a major challenge in HBV therapy. Current NA-based treatment has rarely been reported to eliminate cccDNA or pgRNA in the nuclei of infected hepatocytes (20). Therefore, we examined whether GV1001 could inhibit HBV cccDNA or pgRNA in infected hepatocytes. To this end, we analyzed HepG2 cells transiently transfected by a linearized 1.2x genotype C2 HBV plasmid and assessed those replication capacity depending on the treatment of GV1001. The cccDNA and pgRNA transcript levels were significantly reduced by GV1001, but not ETV, compared with PBS (Figures 2A,B). Stable nucleocapsid formation could contribute to persistent HBV infections by promoting cccDNA production *via* its nuclear transport (21). To determine the inhibitory effect of GV1001 on HBV nucleocapsid formation, we conducted Western blotting for capsid detection on non-denatured gels using a cell pellet of HepG2 cells transiently transfected with the linearized 1.2x genotype C2 HBV plasmid. GV1001 treatment reduced the virion nucleocapsid levels in a dose-dependent manner (Figure 2C).

Next, we examined whether co-treatment with GV1001 and NAs had a synergistic antiviral effect. Co-treatment of GV1001 (10 µM) with LMV (10 µM) or ETV (10 µM) into HepG2.2.15 cells significantly reduced the pgRNA, cccDNA, extracellular virion DNA, HBsAg, and nucleocapsid levels compared with single-drug treatments (Figures 2C–G). Together, these results suggest that the anti-HBV mechanism induced by GV1001 may be attributed to the inhibition of cccDNA, pgRNA, and nucleocapsid formation *via* modulation of the host cell signaling involved in epigenetic modification, unlike NAs, which directly act on HBV polymerase.

Anti-HBV Effect of GV1001 *in vivo*

To examine the anti-viral effect of GV1001 in an *in vivo* mouse model, we intravenously injected GV1001 (50 µg/kg) or LMV (500 µg/kg) into a transgenic mouse model expressing HBV virions containing W4P mutation in the preS1 region (22) twice per week. The HBV DNA and HBsAg levels were measured from the mouse serum obtained *via* orbital sinus blood collection at 4 and 8 weeks. GV1001 did not significantly reduce the serum HBsAg or extracellular virion levels in mice after 4 weeks of infection compared with PBS, but a significant reduction in serum virions was found in GV1001-treated mice after 8 weeks of infection. The treatment of TG mice with GV1001 led to a mean HBsAg reduction of 20% compared to PBS, which had a limited effect on HBsAg

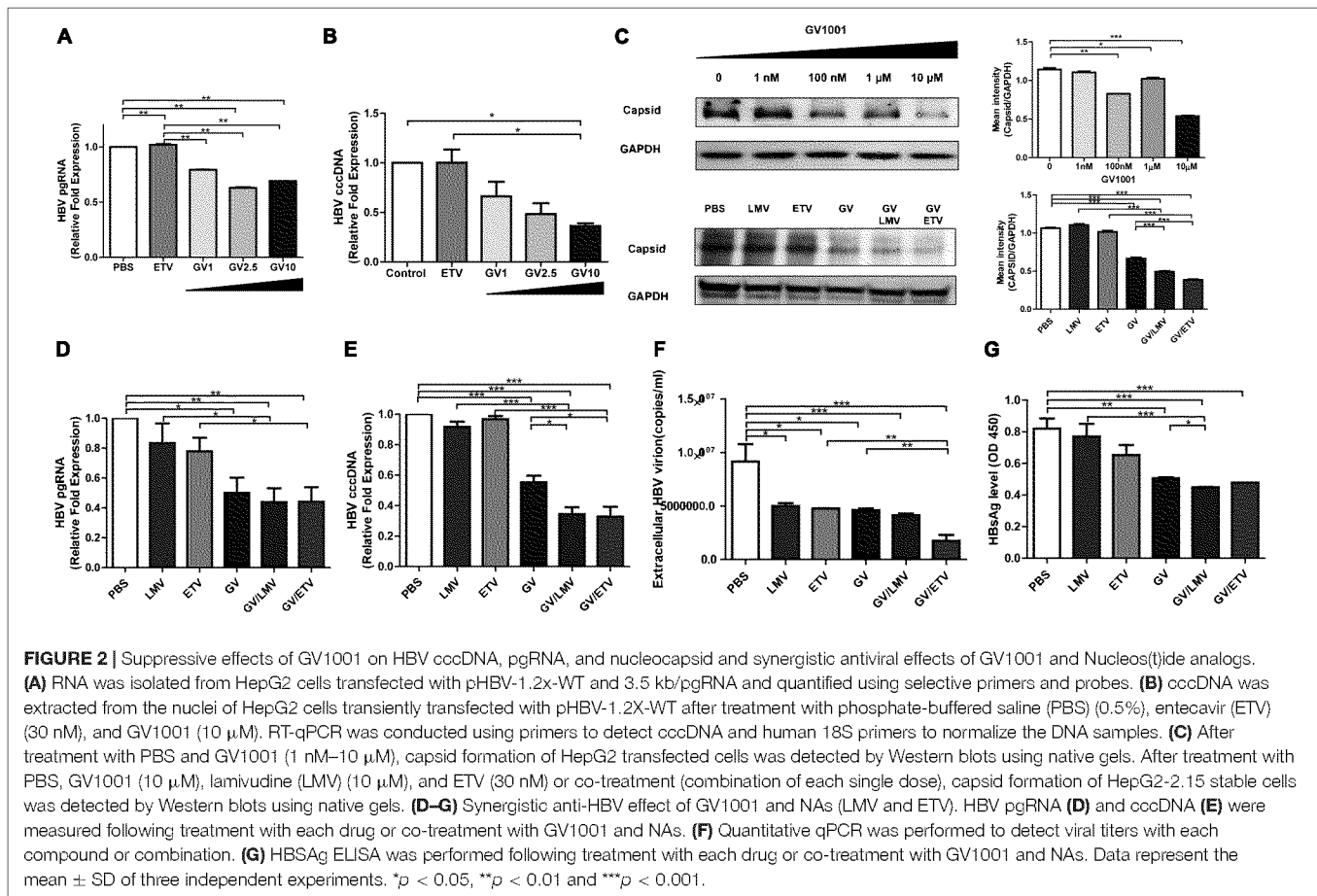


level (Figures 3A,B). The LMV treatment had similar anti-HBV effects as GV1001 (Figure 3B). These results suggest that GV1001 also exerted antiviral effects on HBV infection in HBV transgenic mice.

The Anti-HBV Effect of GV1001 Depends on IFN-I Production via a STING-IRF3 Axis

The above results showing the anti-HBV effect of GV1001 were due to the inhibition of cccDNA and HBV pgRNA, suggesting that the antiviral activity may be mediated *via* IFN-I production. To address this issue, we first examined whether GV1001 induced the transcription of IFN-I, which is responsible for the reduction of HBV cccDNA and nucleocapsid formation, using RT-qPCR. Our data showed that the gene transcriptions of IFN- β and TNF- α were significantly increased in GV1001-treated HepG2 cells transiently transfected with the HBV genome compared with PBS- or ETV-treated cells (Figure 4A), suggesting a positive role of IFN-I signaling in the anti-HBV effect of GV1001, which was distinct from the ETV-mediated anti-HBV effect. Then, we measured the secreted IFN-I level using HEK293-ISRE-Luc cells, which express luciferase luminescence according to the IFN level (23). The luminescence induced by GV1001 was significantly increased in a dose-dependent manner, confirming that GV1001 induces IFN-I production (Figure 4B). GV1001 also increased the phosphorylated IRF3 and phosphorylated STAT-1 levels, which are both considered as key regulatory factors inducing

IFN-I (24), as confirmed by Western blot (Figure 4C), suggesting that the anti-HBV effect of GV1001 may be exerted *via* the IRF-3–IFN-I axis. There are two general upstream signaling pathways for IFN-I production related to HBV infections: the RIG-dependent pathway (25) and the cGAS–STING-dependent pathway (26). Because there was no difference in the antiviral effect of GV1001 between Huh-7 and Huh-7.5 cell lines defective in the RIG-I pathway (data not shown), we hypothesized that IFN-I production by GV1001 may be due to the cGAS–STING dependent pathway. First, we knocked down STING with siRNA to demonstrate the involvement of cGAS–STING signaling in the IFN-I production of GV1001. We found that the reduced HBsAg, HBeAg, and extracellular HBV virion levels mediated by GV1001 in scramble (sc) siRNA-transfected HepG2 cells were not observed or even increased in STING siRNA-transfected cells, suggesting that the anti-HBV effect depends on the STING-1-mediated signal pathway (Figures 4D,E). In addition, to further assess the IFN-I pathway dependence of the anti-HBV effect of GV1001, we assayed the anti-HBV effect of GV1001 after treatment with an IFN receptor-neutralizing antibody (IFNAR2). The anti-HBV effect of GV1001 observed in the GAPDH group was not found in the IFNAR2 group (Figure 4F). There was no difference in the IFN-I levels between the cells with and without GV1001 in the IFNAR2 group, suggesting that the anti-HBV effect of GV1001 depends on the IFN-I pathway. Taken together, these results demonstrated that GV1001 exerts anti-HBV effects *via* the stimulation of IFN-I production by the cGAS–STING–IRF3 pathway.



The Induction of IFN-I by GV1001 Is Dependent on the Release of Oxidized DNAs Into the Cytosol by Mitochondrial DNA Stress

Previously, it was reported that mitochondrial stress-mediated oxidized DNA release into the cytosol contributed to IFN-I production *via* cGAS–STING axis (27, 28). Therefore, we investigated whether GV1001 exerts an anti-HBV effect *via* mitochondrial stress-mediated signaling. To this end, we first evaluated the mtROS and mtDNA levels in the cytoplasm. After the isolation of cytosolic mitochondrial DNA from cells, three regions of mtDNA—mtDNA 1, mtDNA 2, and mtDNA3, which detect ND5, ND1/ND2, and COII/ATPase6/8—were amplified. The GV1001 treatment significantly enhanced the cytosolic mtDNA levels in a dose-dependent manner (Figure 5A), suggesting that GV1001 enhances mtDNA release into the cytosol. To validate mtROS induction by GV1001, we evaluated mitochondrial superoxide *via* confocal microscopy and flow cytometry using MitoSOX. Our confocal images showed a dramatic increase in the mitochondrial fluorescence intensity of MitoSOX in HepG2-2.15 cells treated with 10 μ M GV1001 compared with PBS-treated cells (Figure 5B and Supplementary Figure S1). The flow cytometry analysis also showed a significant histogram shift in HepG2-2.15 cells treated with 10 μ M

GV1001 compared with those treated with PBS at 6 and 12 h (Figure 5C and Supplementary Figure S2), suggesting that GV1001 enhanced mtROS production together with mtDNA.

Exposure to intracellular ROS in the mitochondria can cause oxidative mtDNA damage, indicated by increased mitochondrial 8-OHdG, which may thus be a useful biomarker to detect mtROS (29). We found that 8-OHdG, a ubiquitous marker of oxidative stress, was significantly elevated in a dose-dependent manner after 12 h of GV1001 treatment (Figure 5D).

Finally, we investigated the relationship between mitochondrial stress and the IFN-I-mediated anti-HBV effect by GV1001 using MitoTEMPO, a mitochondria-targeted antioxidant that protects mitochondria from oxidative damage. MitoTEMPO abrogated the anti-HBV effect and IFN-I production of GV1001 (Figure 5E). Taken together, our data suggest that GV1001 promotes mitochondrial stress-mediated oxidized mtDNA release into the cytosol, resulting in an IFN-I-mediated anti-HBV effect *via* the cGAS–STING axis.

The Anti-HBV Effect of GV1001 Is Mediated in an Extracellular HSP90

Previously, it was reported that GV1001 could translocate into the cell cytosol *via* extracellular HSP90 and HSP70 binding and exerted its antiviral effect on HCV or HIV-1 infections in

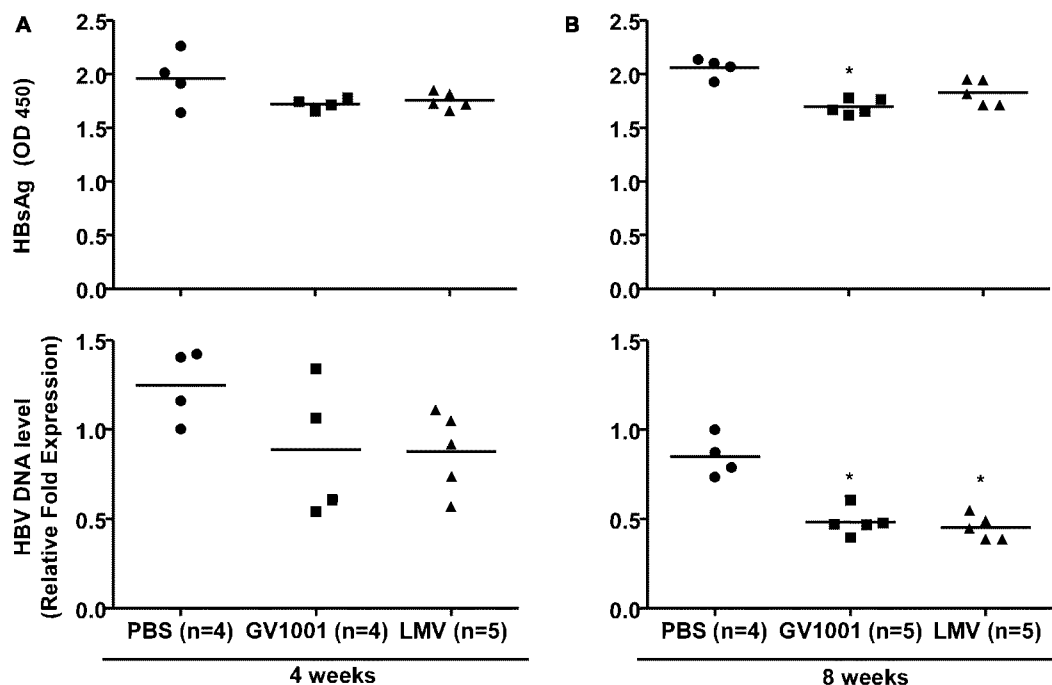


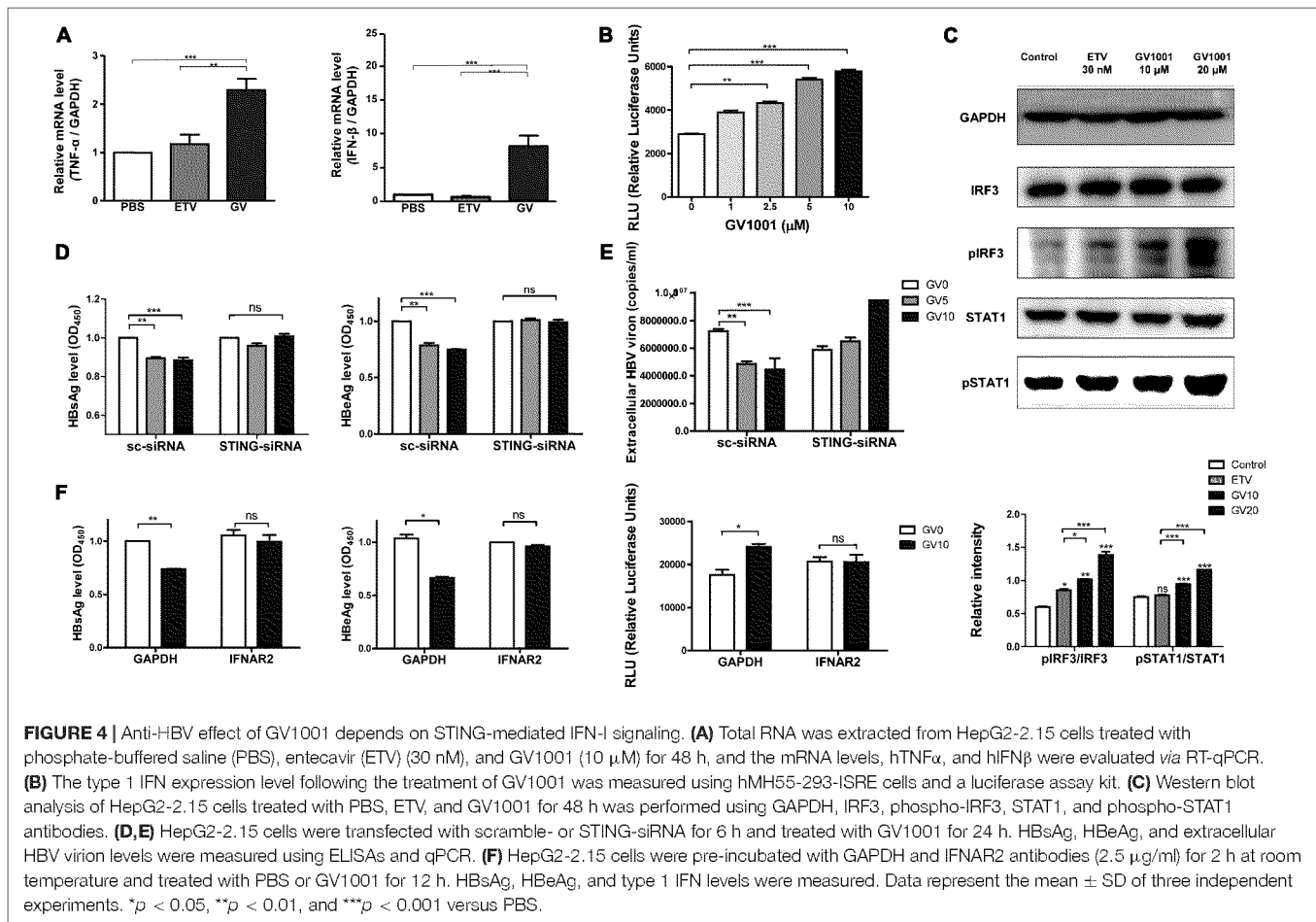
FIGURE 3 | Anti-HBV effect of GV1001 on a transgenic mouse model. **(A)** Female transgenic mice were injected with phosphate-buffered saline, lamivudine, (0.5 mg/kg) or GV1001 (0.05 mg/kg), twice weekly. At 4 and at 8 weeks later, HBsAg from mouse serum was measured by ELISAs. **(B)** After the extraction of viral DNA from serum, HBV DNA was quantified by qPCR. This study was conducted in accordance with the guidelines established by the Seoul National University Institutional Animal Care and Use Committee (Approval No. SNU-111025-6-3). Data represent the mean $\pm$ SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus PBS.

eHSP90- and eHSP70-dependent manner (18). Therefore, we assessed the involvement of HSP90 and HSP70 in the anti-HBV effect of GV1001. Interestingly, the GV1001-mediated suppression of HBV replication in stable HepG2-2.15 cells was completely restored by anti-HSP90 or anti-HSP70 neutralizing antibody (Figures 6A,B). However, an isotype control GAPDH antibody showed no significant effect on GV1001 treatment. These results suggested that GV1001 regulates the anti-HBV effect through its interactions with eHSP90 and eHSP70.

Cytosolic Localization of GV1001 After Phagosomal Escape Is Essential for the Antiviral Effect of GV1001 *via* IFN-I Signaling

In previous studies, GV1001, a cell-penetrating peptide (CPP), was localized in the cytoplasm rather than in the nucleus of MCF7, Huh-7, and HepG2 cells (30). In addition, it has been reported that the eHSP-CPP complex could be taken up by antigen-presenting cells and could escape into the cytosolic space from the phagosome (31). In this regard, drug delivery strategies for endolysosomal escape and cytosolic access mimicking the escape mechanism of pathogens (32) have emerged (33). Chitosan was reported to produce IFN-I in dendritic cells *via* its escape into the cytosol followed by endosome rupture, which can contribute to its strong adjuvant effect in vaccine application (34). Therefore, to investigate whether the anti-HBV effect of

GV1001 is due to its CPP nature with cytosolic preferential localization, which could contribute to IFN-I production *via* vacuole escape, as shown in chitosan (35), we used Bafilomycin A, which inhibits the acidification of phagosomes. In the presence of Bafilomycin, treatment with GV1001 failed to reduce the extracellular HBV virion and HBeAg levels compared with those of the control group (Figures 6C,D). In addition, cells treated with GV1001 induced IFN-I expression, but no significant difference was shown in the Bafilomycin-GV1001 co-treated group (Figure 6E). We also observed differences between the cells treated with and without Bafilomycin in confocal images using GV1001-FITC. In a case without treatment, GV1001 was diffused or released throughout the cytosolic space, but when Bafilomycin was added, GV1001 was not diffused to the cytosolic space due to the inhibition of phagosome acidification (Figure 6F and Supplementary Figures S3A,B). In addition, to investigate the phagosomal escape and cytosolic access of GV1001, we observed the colocalization of GV1001 with the late endosomal/lysosomal marker LAMP-1 in the presence or absence of Bafilomycin. In the presence of Bafilomycin, GV1001-FITC failed to escape into the cytosol and colocalized with LAMP-1 in both HepG2-2.15 and HepG2 cells, but without interruption of Bafilomycin A, the GV1001-FITC was diffused throughout the cytosolic space and did not colocalize with LAMP-1 (Figure 6G). Together, our results suggest that phagosome acidification or escape is required to induce IFN-I and the associated antiviral effect of GV1001.

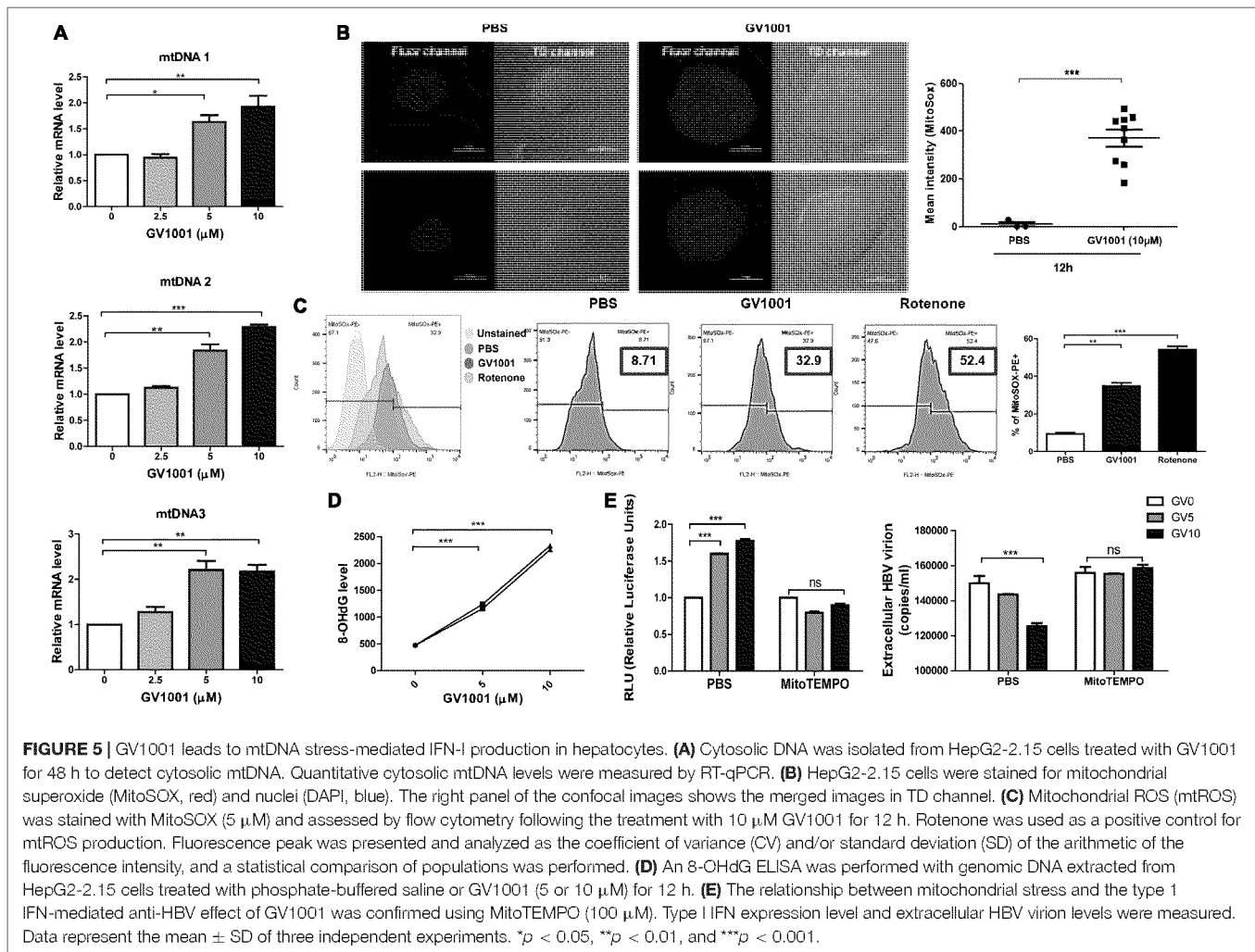


The Antiviral Effect of GV1001 Is Dependent on Enhanced HO-1 Expression

Heme-oxygenase-1 (HO-1) was shown to exert an anti-HBV effect *via* inhibition of the viral core/capsid formation (36). Next, we verified that GV1001 treatment increased HO-1 protein expression by Western blot analysis. We found that GV1001 enhanced HO-1 expression in a dose-dependent manner. Furthermore, GV1001 led to a reciprocal reduction of viral capsids in a dose-dependent manner, suggesting an inverse association between HO-1 expression and viral capsid formation (Figure 2C and Figure 7A). In addition, HO-1 gene transcription was significantly increased in GV1001-treated HepG2 cells (Figure 7B). Therefore, to determine the HO-1 involvement in viral capsid formation, we compared the anti-HBV effect of GV1001 between HBV genome-transfected HepG2 cells cotransfected with HO-1 siRNA or scramble siRNA. Notably, the reduced HBsAg, extracellular HBV virion levels, and increased IFN production level mediated by GV1001 in scramble siRNA-transfected HepG2 cells were not shown in HO-1 siRNA-transfected cells (Figures 7C-E). These results suggested that the anti-HBV effect of GV1001 is dependent on enhanced HO-1 expression.

DISCUSSION

Current NA-based antiviral agents can control, but not completely cure, HBV infection in chronic patients due to the persistence of HBV cccDNA in infected hepatocytes (37). Exogenous IFN- α treatment for CHB patients can lead to complete viral clearance in a proportion of patients. However, this treatment was not efficacious in genotype C-infected patients, and high doses are not tolerated (38). Therefore, the development of new drugs for the efficient elimination of HBV cccDNA is urgently needed. A novel candidate anti-HBV drug, GV1001, a telomerase-derived peptide whose antiviral effects against HCV and HIV-1 have already been described, was introduced in this study (17, 18). Here we demonstrated that the anti-HBV effect of GV1001 is due to its capacity to produce endogenous IFN-I *via* mitochondrial DNA stress. We also demonstrated that mtDNA stress elicited by GV1001 is attributed to cell cytosol access *via* phagosomal escape after eHSP-mediated cell penetration, which leads to the release of oxidized mtDNA into the cytosol. The resulting cytosolic mtDNA following GV1001 treatment induced antiviral innate immune responses *via* IFN-I production in a STING-dependent manner (Graphical Abstract).



Hepatitis B virus is a stealth virus capable of escaping IFN-I-dependent antiviral responses (39). HBV has developed various strategies to escape IFN-I production through RNA sensing *via* RIG-I or TLR-3 or -7 pathogen recognition receptors, which could be mediated by virally encoded proteins, including HBsAg, HBeAg, HBV Pol, or HBxAg (40–43). Recently, an HBV strategy to modulate distinct STING pathways for IFN-I production has been introduced (44).

However, our *in vitro* and *in vivo* studies proved that mtDNA stress-mediated IFN-I production *via* the STING-IRF3 axis by GV1001 could override the IFN-I escape mechanism (44), identifying GV1001 as an HBV treatment. Furthermore, cytosolic mtDNA following GV1001 treatment can induce the production of other antiviral cytokines, such as IL-1 β , *via* NLRP inflammasome activation as well as IFN-I production in a cGAS-STING-dependent manner (45), possibly from liver Kupffer cells, providing an additional benefit of GV1001 over exogenous IFN treatment.

Previously, we identified GV1001 as a CPP, which preferentially localized into the cell cytosol in an eHSP-dependent manner, suggesting potential uses for cell delivery of various pharmaceutical agents, such as proteins, DNA, or siRNA (30). Here, our data clearly demonstrated that blocking the eHSP-mediated cell entry of GV1001 led to complete inhibition of antiviral activity (Figures 6A,B). Furthermore, we found that treatment with Bafilomycin, an inhibitor of phagosome acidification, inhibited the antiviral and IFN-I effects of GV1001 (Figures 6C–E), suggesting that the antiviral activity of GV1001 depends on phagosomal escape after eHSP-mediated cell entry. In fact, some pathogens, such as *Mycobacterium tuberculosis* (46), *Mycobacterium abscessus* (32), or *Listeria monocytogenes* (47), were shown to exploit host innate immune systems *via* enhanced IFN-I production by mitochondrial stress after active phagosome rupture, promoting their virulence. This finding suggests that the antiviral mechanism of GV1001 may mimic the strategy of some pathogens to modulate the host innate immune response, as shown in the mechanism of chitosan as a vaccine adjuvant (48).

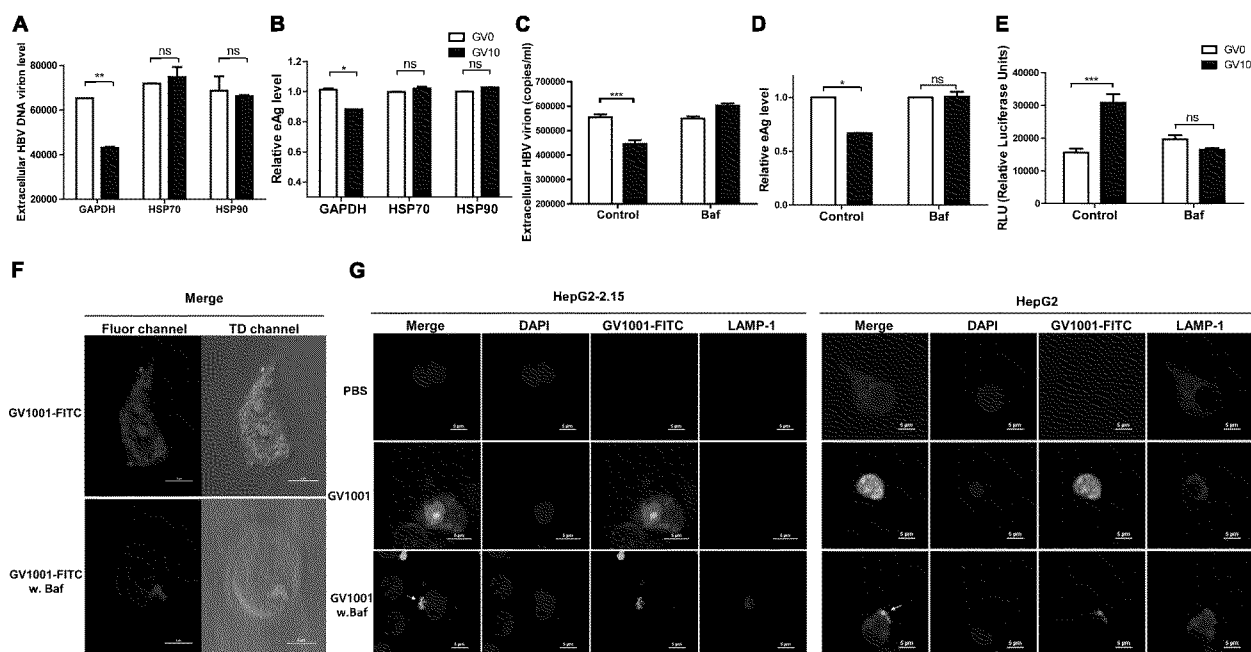


FIGURE 6 | GV1001 exerts an anti-HBV effect via its eHSP-mediated cytosolic access and phagosomal escape. For confirmation of eHSP70- or eHSP90-dependent translocation of GV1001, HepG2-2.15 cells were pre-incubated with HSP70, HSP90, and GAPDH antibodies for 2 h. After treatment with GV1001 for 24 h, (A,B) extracellular HBV DNA and HBeAg were evaluated via qPCR and ELISA. (C–E) Extracellular HBV DNA, HBeAg, and type 1 IFN-dependent luciferase levels in HepG2-2.15 cells treated with GV1001 in the presence of Bafilomycin A1 (white, GV0; dark blue, GV10). (F) Confocal microscopic analysis showed the intracellular location of GV1001 with or without Bafilomycin A1 (100 nM) at 12 h. HepG2-2.15 cells stained with DAPI (blue) for nuclei and with FITC-labeled GV1001 (GV1001-FITC, green) for the cytoplasmic display of GV1001. (G) The phagosome-associated protein LAMP-1 was detected in HepG2-2.15 and HepG2 cells with mouse anti-LAMP-1 and anti-mouse immunoglobulin-Alexa 594 (red). The white arrows represent the co-localization of GV1001-FITC with LAMP-1 (yellow). Data represent the mean $\pm$ SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

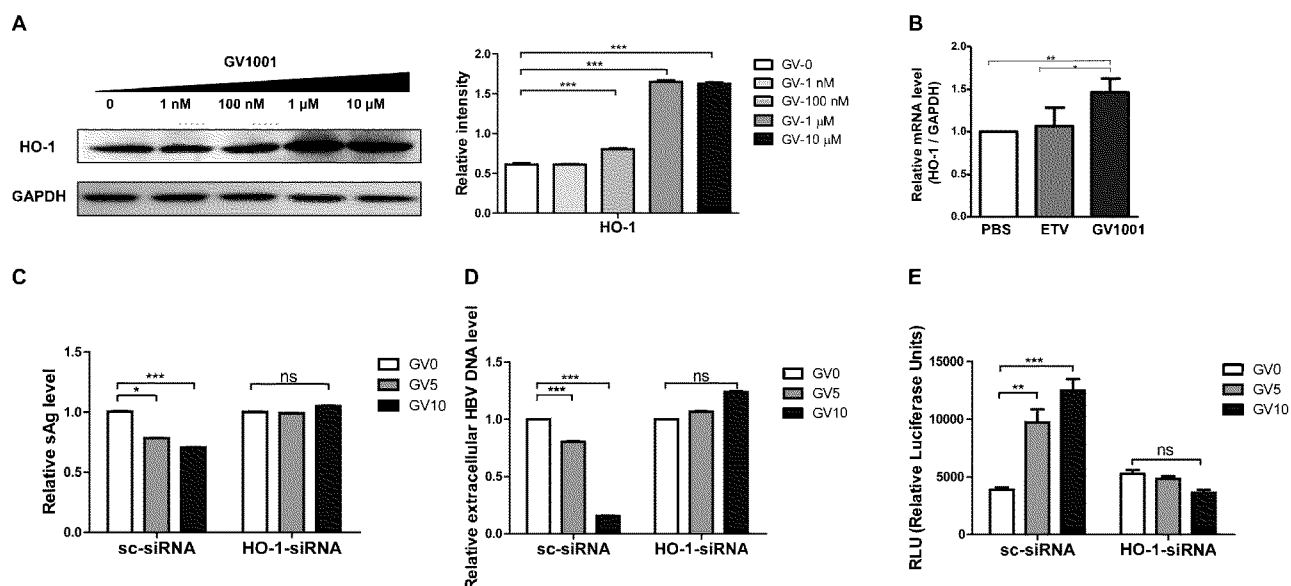


FIGURE 7 | The anti-HBV effect of GV1001 is dependent on the enhanced HO-1 expression. (A) Western blots of 1.2x-WT plasmid-transfected HepG2 cells treated with GV1001 showing the HO-1 expression level. (B) Total RNA was extracted from HepG2-2.15 cells treated with phosphate-buffered saline, entecavir (30 nM), and GV1001 (10 μM) for 48 h, and RT-qPCR was conducted to determine the HO-1 mRNA levels. (C–E) HepG2 cells transfected with both 1.2x-WT plasmid and HO-1 or scramble siRNA were treated with GV1001. HBeAg (C), extracellular HBV DNA (D), and type 1 IFN luciferase levels (E) were measured. Data represent the mean $\pm$ SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Previously, HO-1 was shown to exert an anti-HBV effect at a post-transcriptional stage by decreasing the stability of HBV capsid formation and blocking the refilling of nuclear HBV cccDNA (36). Furthermore, a byproduct of HO-1, biliverdin, could reduce HCV replication by increasing IFN-I signaling (49). Our data also indicated that GV1001 inhibited viral capsid formation in a dose-dependent manner *via* enhanced HO-1 expression (Figure 7A). Furthermore, the HO-1 knockdown experiment demonstrated that siRNA-mediated HO-1 inhibition abrogated the IFN-I production and the anti-HBV effects induced by GV1001, suggesting that GV1001 could exert an anti-HBV effect *via* inhibiting the virion capsid formation through the HO-1-IFN-I axis (Figures 7C–E).

In addition, our data, showing that GV1001 enhances IFN-I production, suggest that GV1001 could play dual roles in an anticancer vaccine: as a cancer-associated telomerase antigen and as an adjuvant *via* IFN-I-mediated anticancer cell-mediated immune responses. This finding could explain why GV1001 shows the strongest anticancer vaccine effect of various telomerase-derived peptides.

In summary, our data indicated that the cell-penetrating and cytosolic localization capacity of GV1001 exerts antiviral effects in HBV infections *via* mitochondrial stress-mediated IFN-I production (Graphical Abstract). These results suggest the potential use of GV1001, a peptide proven to be safe for human use, as an anti-HBV drug, which can be synergistically used with NA drugs.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/Supplementary Material.

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ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Seoul National University College of Medicine.

AUTHOR CONTRIBUTIONS

Y-MC, HK, and B-JK contributed to the conception and the design of the study and performed the statistical analysis. Y-MC, HK, S-AL, and S-YL organized the database. Y-MC, HK, and S-AL performed the lab work. Y-MC and B-JK wrote the draft of the manuscript and revised the manuscript. All the authors read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.00652/full#supplementary-material>

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Facing Real-World Challenges of Immunogenicity in Pediatric Inflammatory Bowel Disease

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INTRODUCTION

The advent of biological therapies drastically altered the landscape of inflammatory bowel disease (IBD) treatment, making long-term steroid-free remission possible for thousands of patients living with this chronic inflammatory condition that compromises the integrity of the gastrointestinal mucosa. Unfortunately, up to 65% of patients with IBD develop anti-drug antibodies to biologics (1). This is especially problematic for pediatrics, where treatment options are substantially more limited than for adult patients. Currently, only two biologics have approval from the United States (U.S.) Food and Drug Administration (FDA) for pediatric indications in IBD, anti-TNF- α agents infliximab (IFX), and adalimumab (ADM). The fear of losing these two agents to immunogenicity is very real for the providers and the families of the ~70,000 children affected by IBD in the U.S. (2).

GENERAL FACTORS CONTRIBUTING TO IMMUNOGENICITY

Immunogenicity, or the development of anti-drug antibodies (ADAs), is a major contributor to loss of treatment response to anti-TNF- α agents. Multiple factors play a role in ADA development and are frequently divided into drug properties, drug pharmacokinetics, and individual patient characteristics.

Drug properties, including compound structure and derivation, formulation and route of administration, play a significant role in immunogenicity. Briefly, compounds that are non-glycosylated, non-pegylated and/or non-human derived (i.e., chimeric) are more likely to elicit an immune response and be recognized as “non-self” by a patient’s immune system, triggering ADA formation (3). Similarly, ADA formation is more likely to occur when drug concentrations are low (e.g., trough before the next dose) and the addition of new drug may challenge the host immune system to recognize the drug as “foreign.” Known factors associated with low trough concentrations are low drug dose, infrequent dosing, and accelerated drug clearance, observed when inflammatory burden is high and serum albumin (a marker of reduced Fc Receptor-mediated protein recycling) is low (4, 5). Lastly, compared to less concentrated intravenous formulations administered directly into the intravascular space, biologics administered subcutaneously are prone to protein aggregation and more likely to predispose to ADA development due to prolonged contact time with cutaneous and subcutaneous immune cells (3, 6).

Interestingly, when comparing the subcutaneously administered humanized biologic, ADM, to the intravenously administered chimeric biologic, IFX, data from multiple clinical trials, early on, demonstrated similar degree of immunogenicity for these two anti-TNF- α agents in patients

with IBD (5). However, a more recent review of the IBD literature suggests that immunogenicity is up to two-fold greater for IFX than ADM (1), mirroring our clinical experience with these agents. Importantly, compared to all other autoimmune, inflammatory conditions treated with anti-TNF- α agents (e.g., rheumatoid arthritis, psoriasis, etc.), immunogenicity to IFX is highest in IBD (7).

IMMUNOGENICITY FACTORS UNIQUE TO IBD

Mucosal erosion of the gastrointestinal epithelium, characteristic of IBD, predisposes patients with IBD to protein losing enteropathy, a condition that results in significant, abnormal protein losses in the stool, including the loss of protein-based therapies (8). In patients with IBD, increased stool losses of IFX have been linked to lower circulating IFX drug concentrations and increased propensity for IFX ADA development, with subsequent therapeutic failure and the need for total parenteral nutrition dependence, surgical intervention, and permanent bowel resection (9). Thus, ADA development in IBD goes beyond clinical manifestations of infusion reaction, serum sickness, and decreased drug efficacy (10), and poses a serious threat to patient morbidity and mortality.

With loss of treatment response estimated as 13% per patient-year of IFX therapy (11), children, who inherently have longer treatment duration than patients with adult-onset disease, are at greatest risk for losing biological treatment options, especially when those options are already limited to anti-TNF- α agents.

IMMUNOGENICITY IN CHILDREN

Although, generally, the pharmacokinetics of anti-TNF- α agents are believed to be similar between adults and children (12–14), data specifically comparing immunogenicity in adult vs. pediatric patients are lacking, and are confounded by the use of different ADA assays across studies. Nevertheless, it is well-established that therapeutic immunogenicity susceptibility varies with age, with highest susceptibility observed in the elderly and the young (3). Anecdotally, younger children also appear to clear anti-TNF- α agents faster, requiring higher, more frequent drug dosing in order to avoid immunogenicity and maintain treatment response (15). One proposed mechanism for this increased drug clearance is age-related differences in metabolic rate (16, 17), which, on a kilocalorie-per-kilogram basis, is highest during childhood.

Unlike conventional low-molecular weight drugs (i.e., ≤ 1 kDa), systemic clearance of protein-based therapies depends on proteolytic degradation (i.e., catabolism), determined in large part by metabolic rate, which depends on age, size and body mass composition (18). Highest proteolytic catabolism is expected in young, small, thin children—the typical clinical phenotype of pediatric patients with IBD, whose growth is frequently stunted by disease (19). Indeed, it has been suggested that close therapeutic drug monitoring and ADA surveillance for biologics may be most important for those pediatric patients who weigh less (4).

THERAPEUTIC DRUG MONITORING

In our opinion, aside from medication adherence, therapeutic drug monitoring (TDM) is the single, most critical step for both preventing and overcoming immunogenicity in clinical practice. Clinical trial results from as early as 2014, demonstrate the cost-effectiveness of TDM for anti-TNF- α agents (20) and recent reports in pediatrics provide evidence that close TDM can help not only detect, but also reverse immunogenicity, with appropriate TDM-based dose adjustments (15).

At our center, between 2015 and 2018, TDM was performed 677 times for the ~ 350 children receiving anti-TNF- α therapy for IBD (21). Forty-five children (13%) were identified to have ADAs, and anti-TNF- α therapy was salvaged in 33% (14 IFX, 1 ADM) by increasing drug dose, shortening the dosing interval, and/or adding an immunomodulator to clear ADAs, as described by others (22). The other 30 children required prior authorization and appeals to third-party payers (e.g., letters of medical necessity, peer-to-peer communications) to secure off-label treatment with agents other than anti-TNF- α (e.g., ustekinumab, vedolizumab). To date, we have not detected immunogenicity with these newer agents.

ADA DETECTION PLATFORMS

In practice, the issue of testing for immunogenicity as part of proactive TDM is complicated by the availability of multiple ADA detection platforms. The intricacies of different ADA assay types are often unfamiliar to medical providers, with assay selection sometimes driven by third-party payer preference, or payment-support programs available to patients, especially if paying out of pocket. For example, based on financial considerations, providers at our institution alternate ordering ligand binding immunoassays, homogenous mobility shift and gene-reporter assays for therapeutic drug monitoring of biologics.

Of the currently available assays, providers are likely most familiar with ligand binding immunoassays (i.e., EIA, ELISA, ECLIA); however, there have been a number of novel ADA detection platforms developed, including homogenous mobility shift assays, gene-reporter assays, surface plasmon resonance, bio-layer interferometry, and mass spectrometry-based approaches (23). Although the overall correlation across these assays is acceptable (24), a major challenge in interpreting assay comparability is the use of different analytical standards and outcome measures that make interpretation of each assay highly dependent on the individual assay utilized (25). A major source for the observed variation amongst assays is the positive controls used in the assay, which commonly represent polyclonal ADAs developed through immunization of different animal species with the biological agent. The lack of uniform controls and reagents limits the comparability of results across assays and reveals the need for the development of ADA “standards” for the calibration and comparison of the various assays. This issue is perhaps best illustrated by comparing immunogenicity data from biosimilar development programs for IFX, which, overall, have failed to demonstrate a significant difference in the incidence of immunogenicity between the biosimilar and the innovator

product. However, if one reviews the actual reported ADA incidence from study to study, it varies from 26 to 60%, based on the immunogenicity assay used (26–29). One consequence of the deficiency in uniform assay standards is dissemination of assay-specific treatment recommendations (25), which are not always clinically useful or applicable.

An added challenge in immunogenicity interpretation is the issue of drug tolerance, or unreliable ADA detection when free drug is present in the blood sample being tested. Some ADA platforms have improved the drug tolerance of immunoassays by adding an acid dissociation step to liberate ADAs bound to drug, while others have not, making comparisons across assays difficult.

Another important consideration in evaluating the clinical implications of immunogenicity is the differentiation of neutralizing vs. non-neutralizing ADAs. The differentiation is based on the ability of an ADA to directly interfere with the binding site of the biological agent, preventing its intended function at the drug target and, effectively, neutralizing drug activity/efficacy. Although neutralizing ADAs are believed to have the most clinical relevance, as they affect drug pharmacodynamics, non-neutralizing ADAs may also have significant impact on pharmacodynamics through pharmacokinetic alterations that result in lower drug exposure, secondary to reduced drug bioavailability and/or enhanced drug clearance mediated by ADA binding (30). To our knowledge, differentiation of ADA types is not routinely communicated in clinical immunogenicity reports. Although this information may be of benefit for clinical decision making, it could potentially drive up assay costs as three separate, validated methods would need to be applied in a tiered fashion to provide meaningful drug concentration, neutralizing and non-neutralizing ADA data.

Lastly, although the turn-around time for immunogenicity reports has improved greatly, results may still take up to 5 business days and point-of-care platforms, though available (31), are not yet integrated into routine clinical care.

DISCUSSION

In summary, despite the outlined evidence that pediatric patients with IBD are at increased risk for immunogenicity, and the knowledge that approved biologic treatments for children are limited to anti-TNF- α , clinicians face many challenges in

implementing judicious, proactive therapeutic drug monitoring to detect immunogenicity in every-day IBD practice. A common barrier to implementing TDM is third-party payers denials to cover testing (21), despite the growing number of publications describing the clinical benefit and cost-effectiveness of TDM, specifically for anti-TNF- α therapy in IBD. (20, 32, 33) In practice, assay selection for TDM is often driven by financial considerations, and multiple ADA platforms may be used interchangeably for a given patient, confounding both the reliability and interpretability of test results.

In our opinion, uniformly validated ADA detection methods (e.g., standard reagents and positive controls), and provider education regarding limitations of different ADA assay types, could facilitate comparability of results across the different ADA platforms available. While, language regarding treat-to-target approaches and routine ADA assessment in the drug label, along with integration of point-of-care assays into clinical practice, could facilitate accessibility and affordability of TDM and ADA surveillance for patients and providers, preserving drug efficacy over time.

AUTHOR CONTRIBUTIONS

VS conceived the original idea for manuscript. JB, RF, RH, and VS provided expert opinion. KG, JB, RF, RM, RH, and VS conducted pertinent literature review, wrote and edited the manuscript, reviewed, and approved the final manuscript.

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Immunopeptidomic Data Integration to Artificial Neural Networks Enhances Protein-Drug Immunogenicity Prediction

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Recombinant DNA technology has, in the last decades, contributed to a vast expansion of the use of protein drugs as pharmaceutical agents. However, such biological drugs can lead to the formation of anti-drug antibodies (ADAs) that may result in adverse effects, including allergic reactions and compromised therapeutic efficacy. Production of ADAs is most often associated with activation of CD4 T cell responses resulting from proteolysis of the biotherapeutic and loading of drug-specific peptides into major histocompatibility complex (MHC) class II on professional antigen-presenting cells. Recently, readouts from MHC-associated peptide proteomics (MAPPs) assays have been shown to correlate with the presence of CD4 T cell epitopes. However, the limited sensitivity of MAPPs challenges its use as an immunogenicity biomarker. In this work, MAPPs data was used to construct an artificial neural network (ANN) model for MHC class II antigen presentation. Using Infliximab and Rituximab as showcase stories, the model demonstrated an unprecedented performance for predicting MAPPs and CD4 T cell epitopes in the context of protein-drug immunogenicity, complementing results from MAPPs assays and outperforming conventional prediction models trained on binding affinity data.

Keywords: MHC-II prediction, machine-learning, protein-drug immunogenicity, artificial neural-networks, immunopeptidomics, bioinformatics

INTRODUCTION

The advent of recombinant DNA technology in the last decades has boosted the use of protein drugs as pharmaceutical agents. However, a major potential problem of these—compared to lower molecular weight pharmaceutical counterparts—is adverse effects associated with protein immunogenicity. Immunogenicity is generated because the drug is recognized as non-self, involving an unwanted activation of CD4 T cells, and the formation of anti-drug antibodies (ADAs), potentially producing a hypersensitivity reaction in treated patients.

Protein drug activation of CD4 T cells depends on the internalization of the drug into endosomal compartments in antigen-presenting cells (APCs), where proteolytic enzymes digest the protein

into smaller peptides (1). According to specific rules, a small proportion of those peptides are loaded into major histocompatibility complex class II (MHC-II) molecules. Then, stable peptide-MHC-II complexes are exported to APCs' surface for presentation to CD4 T cells, which can initiate, maintain, and regulate immune responses, including the production of ADAs (2). As a consequence, finely characterizing the rules of MHC-II binding and antigen presentation is of high interest to promote a general understanding of T cell immunogenicity and for the development of biotherapeutics.

Each MHC-II complex has distinct peptide-binding preferences predominantly determined by residues in the MHC binding groove. The MHC binding groove interacts with a stretch of 9 amino acids termed the peptide core. For every MHC-II molecule, a few pockets accommodate specific positions of the peptide core with a narrow or broader specificity for different residues (3). These pockets and pocket specificities are dependent on the class II molecule of study. MHC-II in humans comprises three major gene pairs called HLA-DR, -DP, and -DQ, all having an α - and a β -chain. The MHC presentation of peptides is fundamentally determined by the amino acid sequence of the peptide and the MHC-II alleles expressed by the host. However, other factors, such as protein internalization or peptidase cleavage sites, influences which peptides are presented.

Historically, peptide-MHC binding affinity (BA) measurements have been used to characterize MHC binding preferences (4), and collections of BA data have been used to develop methods such as NetMHCII and NetMHCIIpan (5–8) with the ability to predict peptide binding to different MHC class II molecules. However, the predictive power of these methods for CD4 T cell epitopes remains limited. Recently, the introduction of ligandome data as obtained by mass spectrometry (MS) immunopeptidome assays (9) has improved MHC predictors' performance substantially (10–14).

Analyzing MS-data has allowed us to learn the rules of MHC-II peptide presentation beyond peptide-MHC binding, including peptide cleavage specificities. The incorporation of such data to MHC-II models has demonstrated to improve state-of-the-art prediction for “natural binders” (14). Currently, MHC-associated peptide proteomics (MAPPs) are used to assess the immunogenicity of protein drugs (15, 16). However, several factors entangle assay performance and interpretation. First, most of the peptides detected by MAPPs are of self-origin, and only a small fraction of the peptides come from the protein drug of interest. Thus, to increase the sensitivity toward the given protein of interest, the amount of sample required is very high, which can lead to aggregation of the protein drug *in vitro*, changing the immune response (17, 18). Second, although MS sensitivity has increased over the past years, still the comprehensive analysis of the peptide ligandome is highly challenging, making it necessary to perform several technical replicates to obtain the maximum amount of peptides identified (19–21). In addition, variations in MHC alleles dictate which peptides will be presented in a given MAPPs context, making necessary the study of several donors with different alleles, representing the population of interest, to accurately assess immunogenicity. Because of those reasons, learning the

specific rules of MHC-II presentation in the form of an *in-silico* predictor would constitute a definite step forward in the development of means to assess the immunogenicity of protein drugs effectively.

Recently, several publications have integrated MS data into MHC-II predictors applying different machine learning approaches (22–26). As regular cells can express up to 12 different HLA alleles including the HLA-DR, -DQ, and -DP genes, a large challenge of this integration lies in how to assign ligands to their HLA restriction element.

To tackle this question, different strategies have been proposed. Abelin et al. (24) used an experimental approach transfecting cells with modified HLA molecules able to be independently purified with a biotin-avidin system to perform “single allele” (SA) mass spectrometry. The peptides derived from each are then used to train allele-specific prediction models. The main disadvantage of this method is the limited set of predictable MHC-II alleles. Chen et al. (26) used a multimodal recurrent neural network to predict MHC class-II ligands, integrating binding affinity, mass-spectrometry data, and RNAseq expression levels. A recurrent neural network was trained on binding affinity data only to resolve the ligand HLA restriction. This method however did not show improved performance over netMHCIIpan, suggesting that Deep neural networks not necessarily outperform shallow neural networks in MHC-II prediction. This method was further suggested optimal for neoepitope discovery, where protein expression is relevant, a factor that is not applicable for prediction of protein drug immunogenicity. Finally, MixMHC2pred from Racle et al. (25) used a probabilistic framework to deconvolute MHC-II peptidomics to the specific allele, and after used a method based on scoring matrices for prediction, using a small set of relevant HLA-DR alleles. None of these recent methods, however, are pan-specific nor were conceived or previously used to predict protein drug immunogenicity.

We have recently developed a neural network framework, NNAlign_MA, that is able to deconvolute mass spectrometry data and at the same time train a predictor to learn the binding preferences of individual MHC molecules (22, 23, 27). In this work, we have trained an immunogenicity predictor based on this NNAlign_MA framework integrating ligand information obtained from in-house Infiximab MAPPs assays, and binding affinity measurements to build a prediction model for MHC-II antigen presentation. Using this model as a proxy for immunogenicity prediction, we showcase its performance on Infiximab and Rituximab, two well-known protein drug antibodies used to treat inflammatory diseases and known to generate an unwanted immune response (10–60% according to the analyzed disease, and how and when immunogenicity is screened) (28–30).

MATERIALS AND METHODS

Samples

Donors and Alleles

Peripheral blood mononuclear cells (PBMCs) were isolated from leukapheresis donated by seven healthy volunteers

(ethical protocol IXP-004 Belgium; Reg. Nr. B707201629385). Monocytes were isolated by positive magnetic separation and cultured for 5 days in DC medium supplemented with interleukin 4 (IL-4) and granulocyte-macrophage colony-stimulating factor (GM-CSF). Immature dendritic cells (iDCs) were pulsed with Infliximab at 50 µg/ml and further matured with Lipopolysaccharide (LPS) for ~20 h. Mature DCs (mDC) were collected, counted and washed with Dulbecco's Phosphate Buffered Saline (DPBS), and stored at -80°C as dry pellets without supernatant.

Allele genotypes of the donors were defined using Sequence-Based Typing (SBT) and are detailed in **Supplementary Table 1**.

Proteins and Peptides

Infliximab (Inflectra) was acquired from Hospira®. Peptides screened for T cell activation were purchased from Mimotopes and are listed in **Supplementary Table 2**.

MHC-Associated Peptide Proteomics (MAPPs) Assay

Cell Lysis

Dendritic cell pellets (1–6 million cells) were lysed in non-ionic detergents (4% CHAPS and 4% Triton X-100) in the presence of protease inhibitors (EDTA-free, Roche) and 590 units of nuclease (US Biologicals) for 45 min at 4°C with rotation. The cell lysate was clarified by centrifugation at 112,000 g for 30 min at 4°C.

Immuno-isolation of MHC II complexes. An isotype IgG (Southern Biotech) and the pan anti-MHC II class monoclonal antibody (L243) (BioXCell) were each coupled to individual HiTrap NHS-activated HP columns (GE Healthcare). The two columns were connected in series with the Isotype IgG column first for the immuno-isolation process. The cleared lysate was loaded on the immuno-isolation columns. The Isotype IgG column was removed, and the MHC II complexes were washed with a buffer and then eluted from the L243 column with 10% acetic acid. The MHC II peptides were desalted by solid-phase extraction using an MCX plate (Waters) into LoBind 96 well plates (Eppendorf) and then transferred to MS plates (Abgene), and vacuum evaporated.

Mass Spectrometry Analysis

Peptide samples were re-solubilized with 10 µL solubilization buffer [96/4 (v/v) water/acetonitrile (CAN) + 0.2% formic acid + 25 mM TCEP (Tris(2-carboxyethyl)phosphine)]. 7 µL were injected on a Waters nanoACQUITY UPLC system, and peptide separation was achieved with a Symmetry C18 trap column (100 Å, 180 µm x 20 mm, 5 µm particle size) and a BEHC18 column (300 Å, 150 µm x 100 mm, 1.7 µm particle size) coupled to a Q-Exactive Plus mass spectrometer (Thermo). Peptides were eluted with an ascending acetonitrile gradient over 105 min. MS spectra were acquired from 400 to 1,800 Da. The MS method consisted of a full MS scan followed by a dd-MS2 scan of the top 12 ions. The full MS scan was achieved with a resolution of 70,000 with an AGC value of 3×10^6 and a maximum IT level of 30 ms. The dd-MS2 scan was performed at a resolution of 17,500 with

an AGC value of 5×10^4 and a maximum IT level of 60 ms. Blank runs of resolubilization-buffer were injected between each sample.

MS Data Processing and Peptide Identification

A single custom database of protein sequences relevant to the experiment was created to include the Human proteome (Swissprot), common general and Caprion-specific laboratory contaminants, and Infliximab (Inflectra) sequence.

Peak alignment and extraction of intensity values of peptide ions and corresponding MS/MS spectra were performed using Rosetta Elucidator™ (Rosetta Biosoftware, version 3.3). MS/MS spectra were then exported for peptide identification in PEAKS Studio (Bioinformatics Solutions, version 7.5). Search parameters included the custom database described above, non-tryptic, oxidation of methionine and deamidation of asparagine as variable modifications, and error tolerance of 15 ppm for precursor mass and 0.025 Da for fragment ions. Data were filtered using a 2% FDR at the peptide level for database search results.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (<http://www.ebi.ac.uk/pride>) with the dataset identifier PXD018303.

Neural Network Architecture and Datasets Training Datasets

The NNAlign_MAC model was trained combining multi-allele (MA), and single-allele (SA) data including binding affinity (BA) peptide measurements and mass spectrometry (MS) data.

MA datasets included only self-protein MS eluted ligands obtained from in-house MAPPs assays. The alleles expressed by each donor are detailed in **Supplementary Table 1**. Infliximab and Rituximab are chimeric antibodies that bear the constant region from a human antibody. Therefore, it is expected that some naturally presented peptides share similarities to the protein-drug antibodies. To avoid a bias in the predictor when evaluating the protein-drug antibodies, we have excluded all peptides sharing a common motif of 9 amino acids (defined by the length of an MHC-II binding core) to both Infliximab and Rituximab proteins from the training dataset. This resulted in the removal of 262 peptide sequences from the mass spectrometry datasets. Additionally, the data were filtered to only include peptides with lengths 13 to 21 in the training datasets.

SA data included peptides derived from BA measurements or MS assays where cells were specifically-homozygous selected or were artificially and genetically engineered to only express a single HLA-DR allele. SA data was collected from previous NetMHCIIpan publications (5, 23), and updated with IEDB to date 01/28/2019.

Mass spectrometry data consists only of “positive” presented peptides. Therefore, a set of negative peptides was added to train artificial neural networks, randomly sampling different length peptides from human proteins. For each MA donor-dataset or SA

allele-dataset, a set of random negatives were included following a flat distribution of lengths 13–21, taking 5 times the number of peptides of the most abundant peptide length on the positive dataset. The flat distribution of the negatives helps the neural network to learn the natural length preference of the data, while the selection of 5 times the most abundant length will generate a ratio of $\sim 1:10$ positive to negatives, which we have previously benchmarked and found optimal (14). Although this approach will introduce some noise to the model, as it is possible that by chance some random peptides will bind to the specific MHC allele, this probability is very low and at the most will diminish the model performance.

Five-Fold Partitioning

All the data combined (SA and MA) were clustered into 5 partitions using a Hobohm algorithm with a common motif of 9 amino acids to perform cross-validation as previously described (27). The artificial neural network architecture consists of an ensemble of 150 independent networks varying; the seeds for weight initialization (10), a different number of hidden neurons in the hidden layer (20, 40, 60), and the 5 different partitions used for cross-validation. An average of the ensembles is used for the final predictions.

NNAlign_MAC Architecture

NNAlign_MAC algorithm integrates the basis of NNAlign_MA (22), an extension of NNAlign (27, 31), with peptide context information (PCI) (14, 23).

In short, NNAlign_MA (22) is a neural network framework capable of taking a mixed training dataset composed of SA data (peptides experimentally tested on a single MHC molecule) and MA data (peptides experimentally tested in cell lines expressing multiple MHC alleles), to fully deconvolute the specific MHC restriction of all MA peptides, while learning the binding specificity for all the MHCs alleles. The algorithm is trained in two steps. In a first step or pre-training (set-up here to 20 iterations), the neural networks are trained with SA data. After these initial iterations, the model manages to learn the first pattern for all MHC class II alleles. This is possible due to the pan-specific algorithm used here (that introduces relevant MHC amino acid positions known to participate in the interaction with the peptide in the binding groove (8). Based on this initial learning, the algorithm annotates the MA data according to the learnt binding rules. In a second step, those newly tagged MA peptides, now converted into SA with a specific MHC allele association, are included in a new training cycle of the network. As more data is included, the binding core for each MHC-II allele is revised. After each new training cycle, all the MA peptides are re-annotated to SA data again. This process is iterated up to 400 training cycles, thus refining the process until convergence (22).

The input neurons of this model were fed with: the peptide sequence (tagged from different experimental sources, BA or MS); a binding affinity measurement in the case of BA, or a binary classification (1-0) for those peptides derived from MS; the allele information (either single or with all alleles expressed by the donor-dataset for MA); and the MHC pseudo-sequence (specific positions of the MHC protein sequence involved in

the MHC-peptide recognition). This training resulted in a pan-specific model with the power to infer binding specificities also for the HLA-DR molecules not included in the training datasets. Additionally, a separate set of input neurons encoded peptide length and peptide context information (PCI) as described elsewhere (14). PCI included 3 amino acids from both C and N peptide termini (previously named peptide flanking regions) and 3 amino acids both from upstream and downstream of the MS peptide protein sequence.

Cross-Validation Performance

After training both models (with and without PCI), the test sets were predicted, and an AUC 0.1 calculated for each MA-donor-dataset and reported in **Supplementary Table 3**.

NetMHCIIpan version 3.2 (5) prediction algorithm was employed in this work as a benchmark comparison to the NNAlign_MAC model. As it was not possible to re-train NetMHCIIpan with the same partitions used for NNAlign_MAC to report AUC 0.1, the following scheme was used. Each peptide in the NNAlign_MAC test set was predicted for all the alleles expressed by the given donor with NetMHCIIpan, and the lowest %rank score from all alleles was assigned to each peptide to perform an AUC 0.1 per donor (**Supplementary Table 3**).

AUC is a common performance measure for predictive models, which takes into account the relationship between true positive rates (TPR) and false positive rates (FPR) for different prediction thresholds. AUC 0.1 (area under the ROC curve integrated up to a false positive rate of 10%) is similar to AUC but focuses on the high specificity range of the ROC curve.

Logos

Sequence logos for binding motifs and context information were constructed applying the Seg2Logo (32) tool using Kulback-Leibler logos and excluding sequence weighting. Amino acids were grouped by negatively charged (red), positively charged (blue), polar (green), or hydrophobic (black).

Infliximab and Rituximab Performance Evaluation

MAPPs Profiles

Infliximab in-house MAPPs were gathered together removing peptide duplicates from the same donor and imposing a filter of a minimum of 12 amino acids to be an MHC-II binder, to build a MAPPs cohort. After filtering, 73 peptides were mapped to Infliximab protein sequences, stacking them, and counting the number of peptides covering each position. The profiles were normalized to have a maximum value of 1.

Additional Infliximab and Rituximab MAPPs peptides were collected from Hamze et al. (15). Filtering and profiles were generated in the same way as for the in-house MAPPs.

NNAlign_MAC Evaluation

For each HLA molecule present in the MAPPs cohort, 1×10^5 random peptides—with a flat length distribution of 13–21—were predicted using NNAlign_MAC, and the N-percentile score for each estimated. For each N, a score threshold per allele was defined to select HLA binders from the protein-drug of

interest to be included in the prediction profile. Subsequently, all the peptides were stacked in the protein-drug sequence and the number of peptides overlapping each sequence position was counted. After that, a so-called “allele promiscuity” calculation was applied, capping the count per allele to a maximum of 1 per position. For example, a protein sequence position, with 10 peptides mapped to it from 3 different alleles, will have promiscuity of 3. After max normalization, these values refer to the “Promiscuity score” in all the profile plots in the manuscript.

From the HLA binding profiles made for Infliximab in **Supplementary Figure 1**, and precision and recall curves for different N values (**Supplementary Figure 4A**), 1% Rank ($N = 1$) was found to be optimal.

MixMHC2pred Evaluation

MixMHC2pred version 1.2 method was downloaded from the GitHub repository to run locally for all overlapping 13–21mers from Infliximab protein-drug and all the alleles present in the MAPPs cohort and covered by the method (HLA-DRB1*04:03, HLA-DRB1*1302, HLA-DRB1*15:02, HLA-DRB3*03:01, HLA-DRB5*01:02 were excluded). The output column for regular %Rank was selected. An HLA binding profile was constructed for MixMHC2pred (**Supplementary Figure 2**), and 0.5%Rank was selected to compare with NNAlign_MAC. After the peptides’ selection, the profiles and the Promiscuity Score were generated in the same manner as for NNAlign_MAC.

NetMHCIIpan Evaluation

To calculate NetMHCIIpan infliximab binding profiles, binding profiles were constructed for % Ranks values of 1, 2, 5, and 10 (**Supplementary Figure 3**). The performance of NetMHCIIpan was consistently found to be very low and close to random for all % Rank thresholds for both protein chains with only one example (% Rank of 2, LC) demonstrating a positive correlation to the MAPPs profile. Given this, a value of %Rank of 2 was selected for this method. After the peptides’ selection, the profiles and the Promiscuity Score was generated in the same way as for NNAlign_MAC.

Performance Measures

Two types of correlations were used to compare predictions from NNAlign_MAC, MixMHC2pred, and NetMHCIIpan to experimental MAPPs profiles. First, the Spearman correlation coefficient (SCC) was used to correlate the profiles’ predictions to MAPPs per position in the protein sequence. Additionally, scatter plots were made to confirm the correlation after losing positional information that could bias our interpretation. The scatter plot correlation was measured both using SCC and Pearson’s coefficient correlation (PCC).

Additional measures, PPV and AUC0.1, were used to compare performance across the methods in **Supplementary Figure 4B**. To allow for minor inconsistencies between the predicted and actual positive peptides, we here adapted a relaxed definition of positives. This was done by assigning all predicted binders (as defined by the selected % Rank threshold) with a binding core that overlapped any of the “original” MAPPs peptides as positive. This set of peptides is termed the “expanded-core”

MAPPs peptides. Next, this set of expanded-core peptides is used to calculate AUC0.1 (area under the receiver operator curve integrated up to a false positive rate of 10%), and PPV values using the lowest % Rank score predictions over all the alleles expressed by the donor as prediction values for each of the peptides. PPV was calculated as the number of true positive predictions from the number of “expanded-core” MAPPs in the top N predictions, divided by N, where N is the number of positives in the “expanded-core” MAPPs dataset per donor. Precision and recall curves were likewise calculated using the “core” scheme for each of the different % Rank (**Supplementary Figure 4A**).

Bootstrap resampling was used to calculate p-values of the SCC correlations comparison among methods, or %Rank values. 10 thousand sampling iterations with allowed repetitions were picked at random for each comparison. The p-value was obtained by #losses/iterations, where losses reflect the number of times the SCC was higher for the challenging method over the other.

Evaluation of the CD4-T Cell Response

PBMCs from 6 out of 7 donors were seeded at 2×10^6 cells/well and stimulated with the different test and control peptides (**Supplementary Table 2**). For one donor, the number of cells was not sufficient to perform this assay. The next day, IL-7 was added. On day 4, part of the medium was changed and IL-2 and IL-7 were added. On day 7, cells were harvested, and rested overnight at 37°C. The next day, cells were counted and seeded in IFN- γ FluoroSpot plates (Mabtech). Cells were re-stimulated with peptide or left unstimulated overnight, in duplicates. On day 9, FluoroSpot plates were developed, according to the manufacturer’s instructions. Data, spot forming units (SFU), were acquired with a Mabtech IRISTM FluoroSpot/EliSpotReader. Raw data (SFU) were transferred to SFU per million, which were then transferred to Δ SFU per million. Δ SFU per million = Average SFU peptide condition/per million - Average medium condition/per million.

We defined a positive response when the two independent peptide measurements were 4 standard deviations higher to the average signal for the control. Raw data, averages and statistical calculations are included in **Supplementary Table 4**.

An additional dataset of T cell responses for Infliximab (30 epitopes from 21 donors) and Rituximab (14 epitopes from 16 donors) was collected from Hamze et al. (15).

RESULTS

Here, we aimed to develop a predictor for MHC class II antigen presentation and assessed its performance for prediction of protein-drug specific MAPPs readouts and T cell epitopes.

NNAlign_MAC Is Able to Predict Infliximab-Associated MAPPs in a Cohort-Based Approach

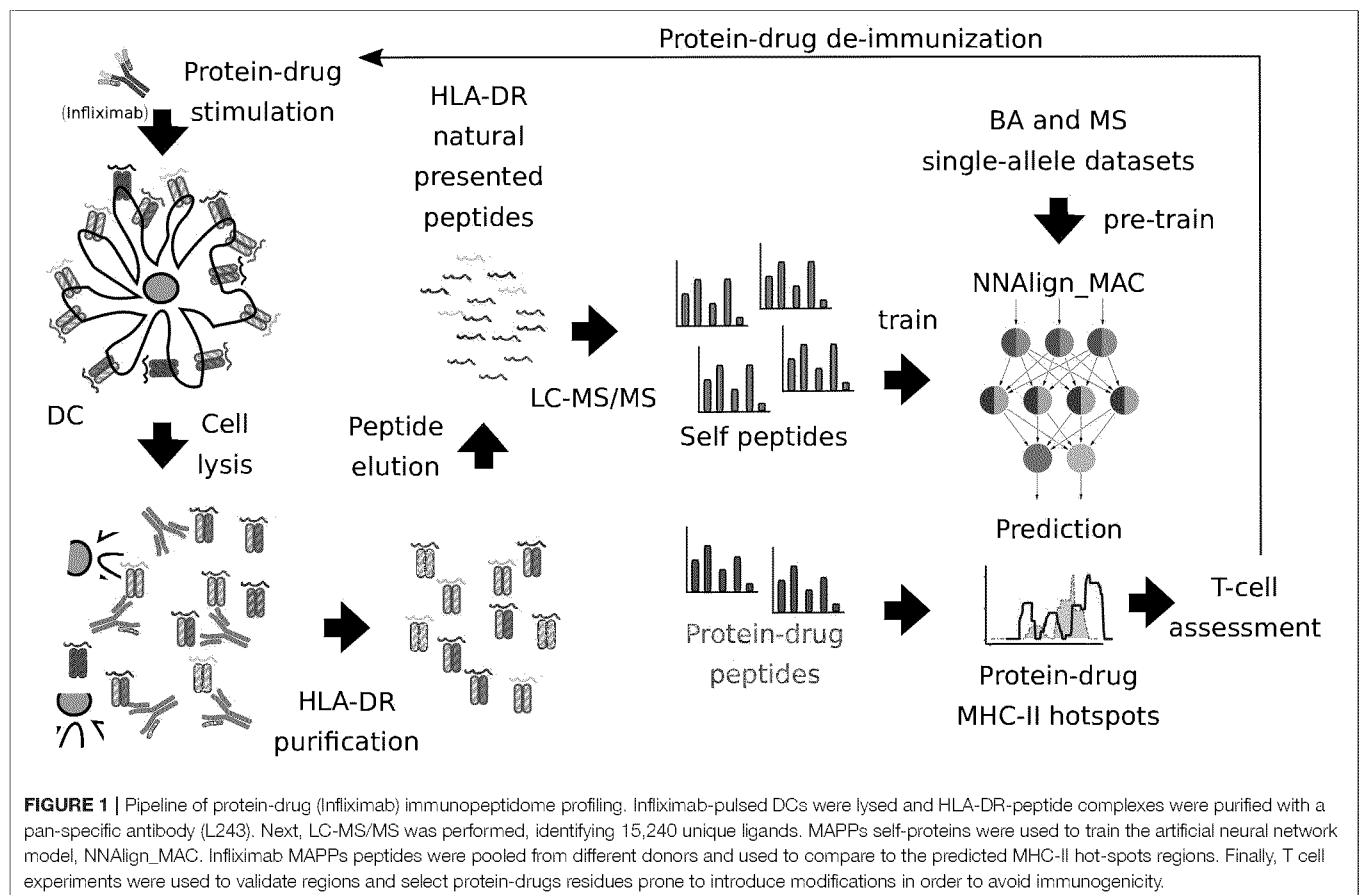
First, we sought to profile the MHC class II immunopeptidome of Infliximab (as a biotherapeutic prototype) to predict the immune response associated with it. For that purpose, we

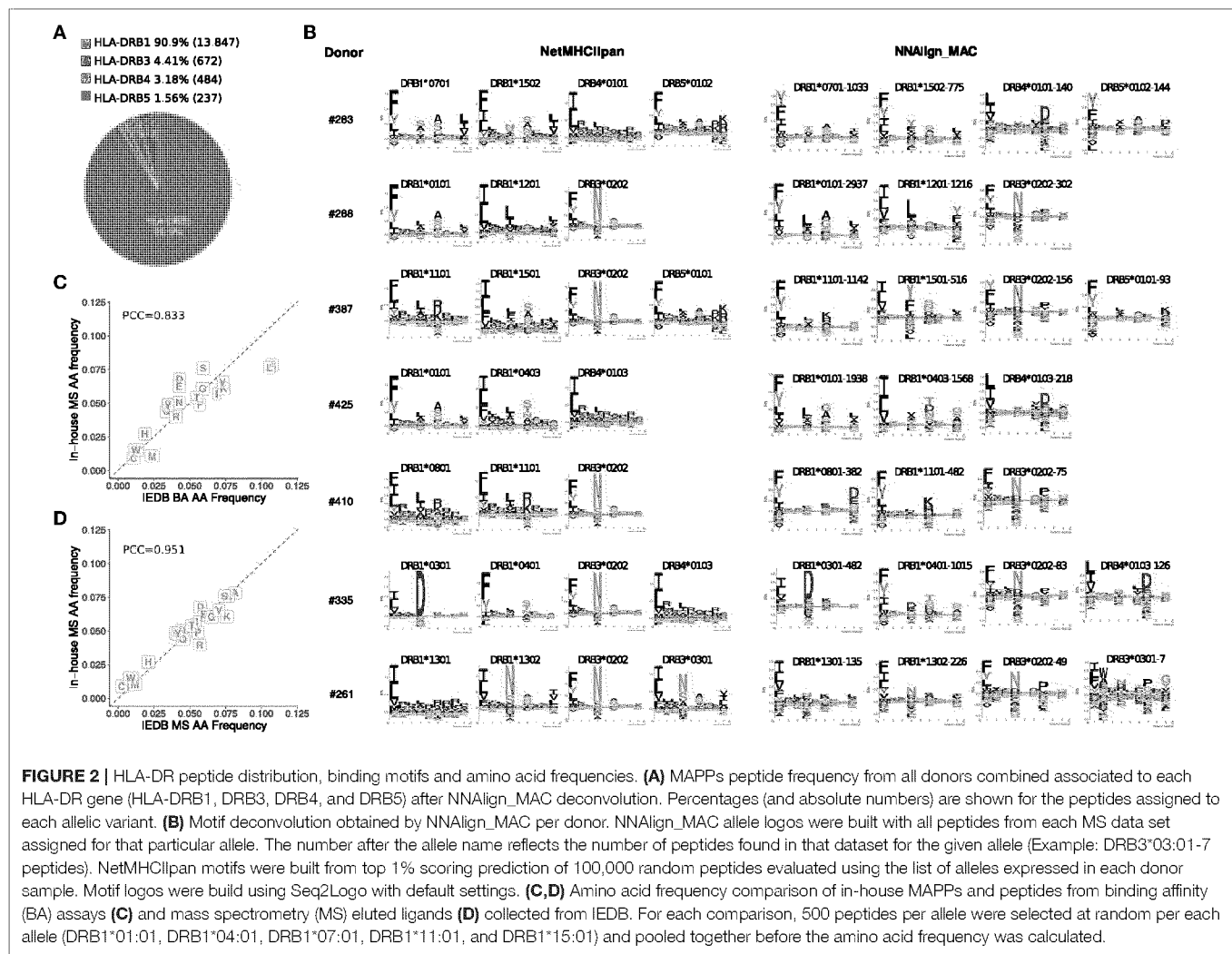
pulsed with Infliximab 7-donor monocyte-derived dendritic cells, expressing the most common world population HLA-DR alleles (**Supplementary Table 1**). Next, LC-MS/MS was performed, identifying 15,240 unique ligands. After removing ligands with a common motif to Infliximab and Rituximab protein sequences (see Materials and Methods), the remaining dataset was combined with single-allele BA and MS data collected from IEDB, to construct a dataset for training a model for HLA-DR antigen presentation prediction (**Figure 1**).

This training was performed using the NNAlign_MA machine learning framework allowing for accurate deconvolution of HLA-DR binding specificities and proper assignment of each MS ligand to its likely HLA-DR restricting molecule (22). Earlier work has shown this algorithm to be able to accurately perform this task, and at the same time to learn the rules for the MHC-II motifs present in the samples (22, 23). The algorithm used here was extended to include “peptide context information” (PCI) from the peptide flanking regions (PFRs) on both peptide termini, and from the protein sequence upstream and downstream the MS peptide sequence. The introduction of PCI was previously shown to significantly reinforce the learning of the rules of “natural processing” in the model (14). Evaluating the predictive power of models trained with and without PCI inclusion, confirmed this earlier observation (**Supplementary Table 3**). This benchmark also

confirmed a consistent and very pronounced gain in prediction performance of the NNAlign_MA method compared to the state-of-the-art method, trained with binding affinity measurements, NetMHCIIpan, for prediction of MHC eluted ligand data (**Supplementary Table 3**). We termed the NNAlign_MA model including PCI, NNAlign_MAC.

After deconvolution, each MS ligand was annotated to a specific allele expressed in the sample assessed. As expected, HLA-DRB1 due to the higher expression of those genes compared to HLA-DRB3, -4 and -5 (33), was assigned the highest proportion of ligands (~90%, **Figure 2A**). All the motifs obtained by NNAlign_MAC share a remarkable overall correspondence across cell samples expressing the same alleles, and to a lesser degree, also with the NetMHCIIpan motifs (**Figure 2B**). The HLA-DRB4*01:03 allele was shared by two donors, and the motifs obtained by NNAlign_MAC in these two, shared highly similar amino acid preferences (PCC = 0.924). Additionally, for some alleles, such as HLA-DRB1*08:01 and HLA-DRB4*01:03, the motifs from NetMHCIIpan and NNAlign_MAC, were however discordant (**Figure 2B**). Comparing the amino acid composition of the in-house MS data to that of MS data obtained from IEDB revealed a high consistency between the two MS datasets (PCC = 0.95) and a lower consistency to the BA data (PCC = 0.83), supporting the quality of in-house MS data, and suggesting that MS

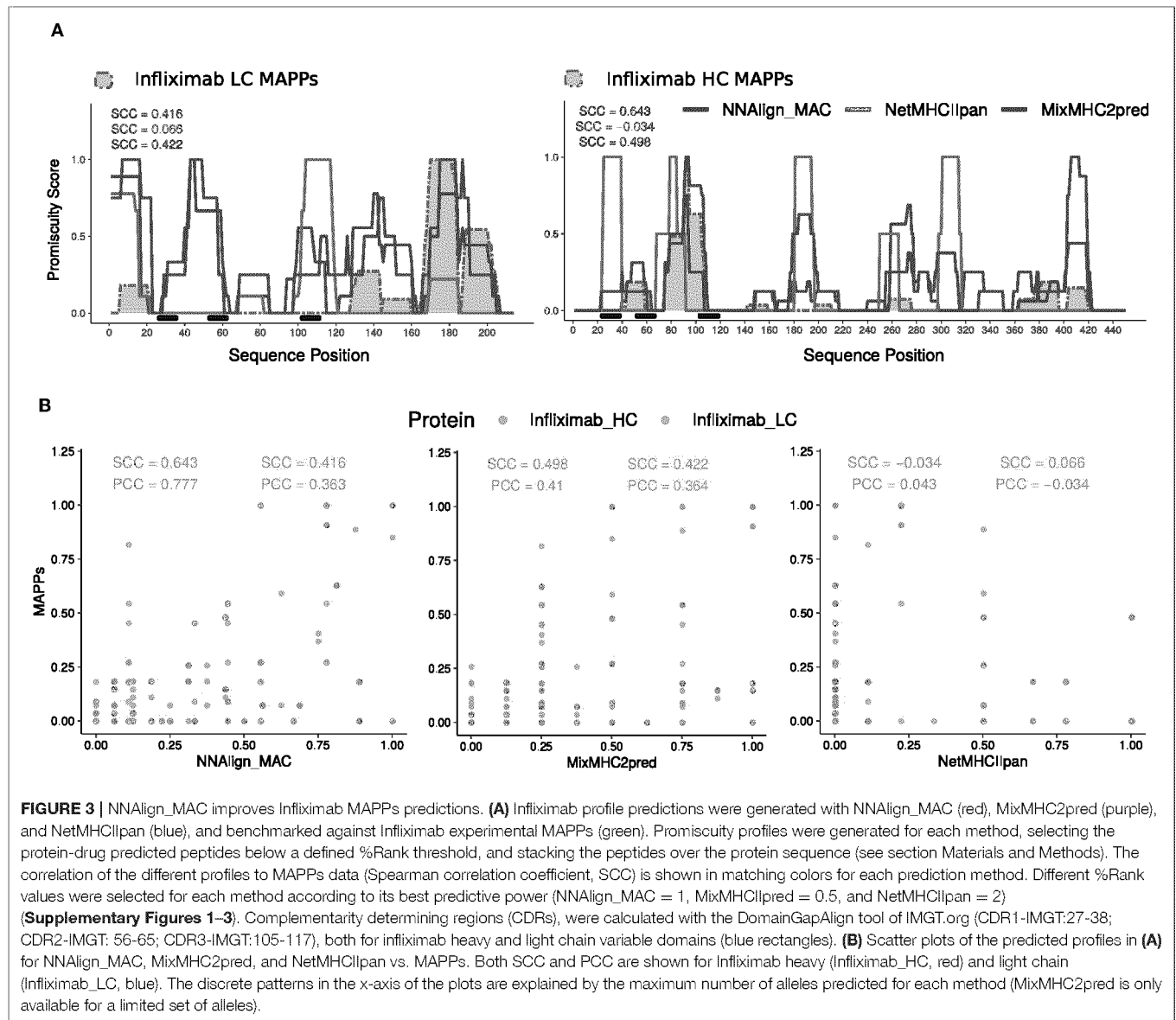




data may contain complementary information to BA data (Figures 2C,D).

Next, all MAPPs peptides unique to infliximab (73 peptides from 7 donors) were mapped to the heavy and light chains of the protein-drug and the count of peptides overlapping each amino acid position in the protein sequence was used to build a MAPPs profile (normalized to have a maximum value of 1) (Figure 3A). Later, infliximab sequences were (*in-silico*) digested into overlapping 13–21mer peptides, and the likelihood for MHC presentation predicted for each peptide using NNAlign_MAC, MixMHC2pred, or NetMHCIIpan for all the HLA-DR alleles present in the donor cohort (Supplementary Table 1). To define a threshold defining positive predicted peptides from each of the models, HLA binding-profile analyses were performed for different % Rank thresholds for each of the models (Supplementary Figures 1–3). Based on these analyses, a Rank threshold of 1% was selected for NNAlign_MAC, a 0.5% MixMHC2pred, and a value of 2% for NetMHCIIpan (detailed in Materials and Methods section, Supplementary Figures 1–3, 4A).

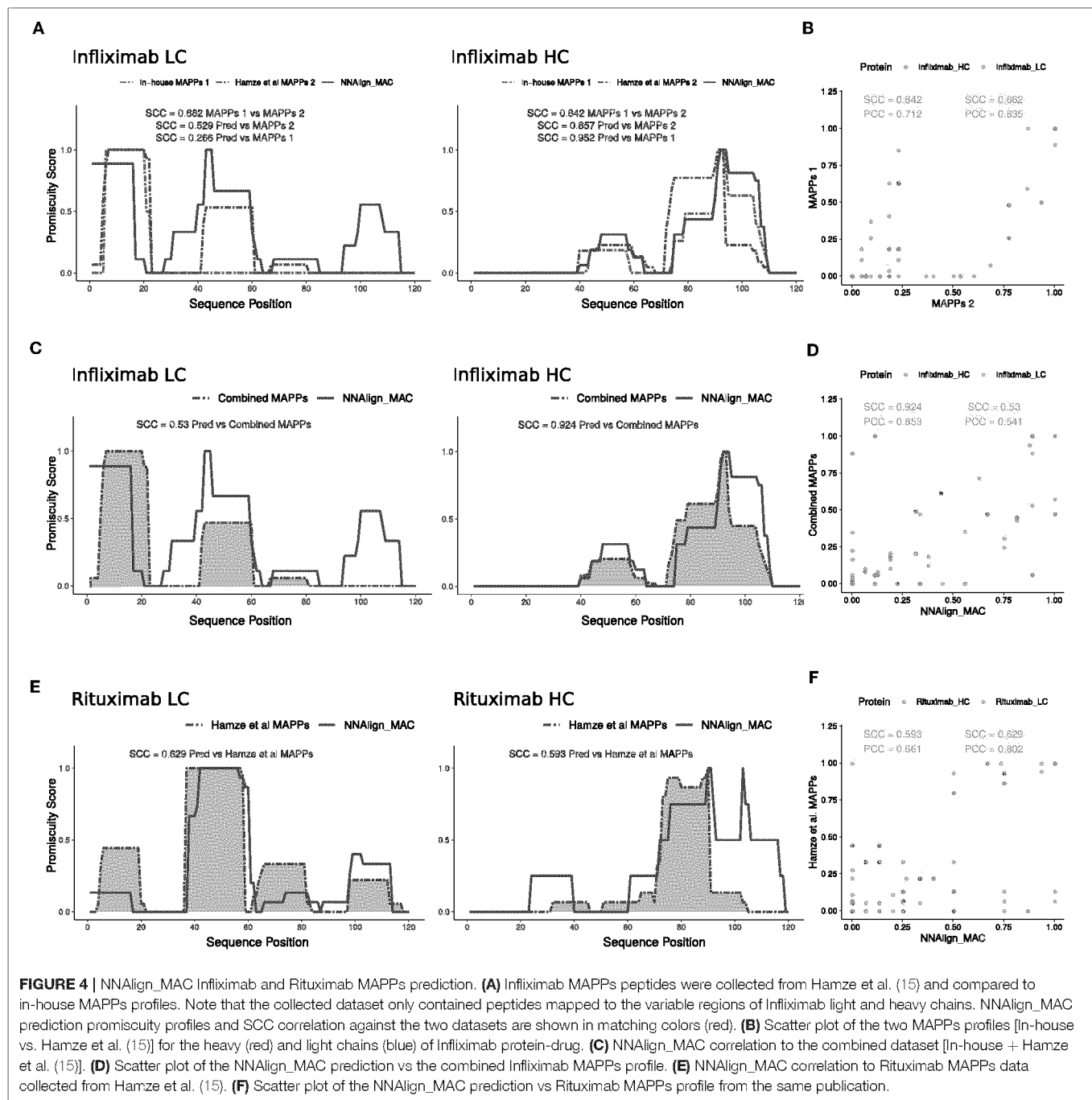
For each predicted HLA molecule, all the peptides with predicted values below the selected % Rank threshold were mapped to the Infliximab heavy and light protein sequences. Next, each position in the protein sequence was assigned a value of 1 if it was covered by one peptide or more and zero otherwise. Finally, these allele-specific binary peptide-maps were stacked constructing a “promiscuity profile” reflecting how many different alleles presented peptides overlapping a given protein position (detailed in Methods) (Figures 3A,B). This mapping was performed for each of the three prediction methods (Supplementary Figures 2, 3, respectively). Comparing the predicted profiles and experimental MAPPs demonstrated an improved power of NNAlign_MAC ($SCC = 0.416$ and $SCC = 0.643$) compared to NetMHCIIpan ($SCC = 0.066$ p -value $< 10^{-4}$ and $SCC = -0.034$ p -value $= 0.0004$) for predicting infliximab MAPPs data. And an improved power compared to MixMHC2pred for the heavy chain ($SCC = 0.498$ p -value $< 10^{-4}$), and a comparable power compared to the light chain ($SCC = 0.422$ p -value $= 0.802$). All regions in Infliximab covered by MAPPs were identified by NNAlign_MAC and



MixMHC2pred. In contrast, NetMHCIIpan failed to predict several of these regions (one prominent example being the region spanning positions 40–60 in the heavy chain).

Several protein regions, both in the light and heavy chain were predicted to have MHC-II ligands by NNAAlign_MAC even though no peptides were identified in the MAPPs assays. We hypothesized that this was due to the sensitivity limitations of the MAPPs assay. To inspect this conjecture, additional Infliximab MAPPs data, from 21 and 16 donors covering the variable heavy and light chain regions of Infliximab, respectively, were collected from a previous publication (15). First, we evaluated the correlation of this new MAPPs dataset to the in-house Infliximab dataset (including only the variable region of the antibody) and observed in both cases a high (though lower for the light chain compared to the heavy chain) correlation between the two datasets (SCC = 0.662

and SCC = 0.842 for the light and heavy chain respectively) (**Figures 4A,B**). Given that no HLA-allele information was available to us for the donors used in this study, we evaluated the ability of NNAAlign_MAC to predict the observed MAPPs using the alleles included in our in-house cohort, which have been selected covering the most frequent alleles in the world population (**Supplementary Table 2**) including only the variable regions of the protein (**Figure 4A**). Next, we combined the two Infliximab MAPPs datasets and analyzed the correlation of the NNAAlign_MAC predictions to this new extended infliximab MAPPs dataset (**Figures 4C,D**). We found a substantial (p -value = 0.0003, bootstrap) increase in predictive performance with the SCC increased from 0.266 to 0.53 for the light chain, while the performance for the heavy chain was conserved (SCC changed from 0.952 to 0.924, p -value = 0.883) (**Figure 4C**) (similar results were obtained for MixMHC2pred).



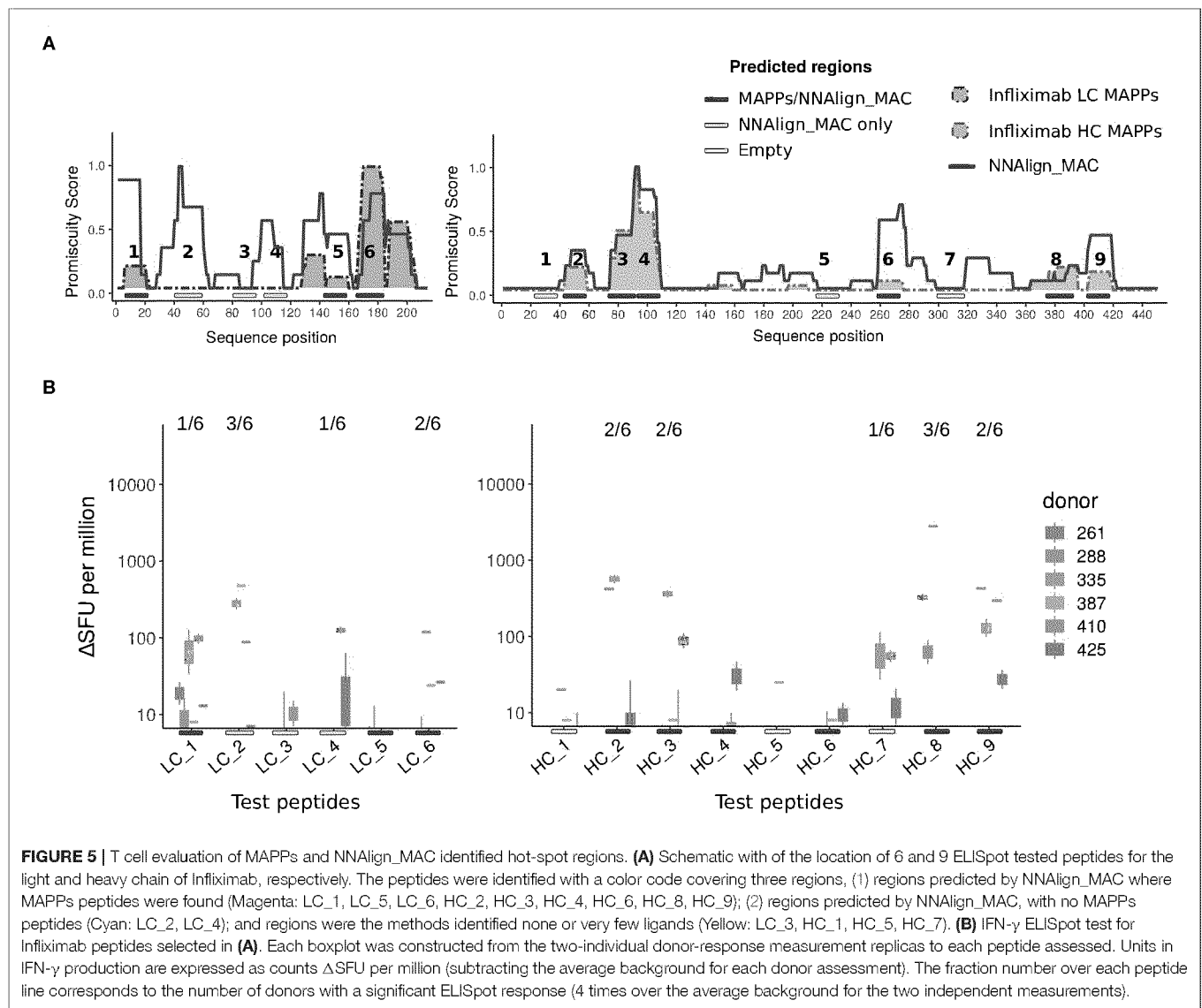
This observation suggests that the performance values of NNAalign_MAC reported in Figure 3A are lower bounds and that—at least some of—the additional peaks predicted by NNAalign_MAC represent regions with antigen presentation potential missed by the individual MAPPs assays.

As an additional proof of concept, we analyzed the correlation of NNAalign_MAC predictions to rituximab, an additional protein drug with MAPPs data collected from the above-mentioned publication (Figures 4E,F). The average SCC correlation considering heavy and light chains of both protein-drugs to the Hamze et al. (15)

MAPPs data was 0.652 for NNAalign_MAC, showing that the proposed method was able to predict most of the MAPPs regions.

NNAalign_MAC Is Able to Predict Infiximab-CD4 T Cell Epitopes

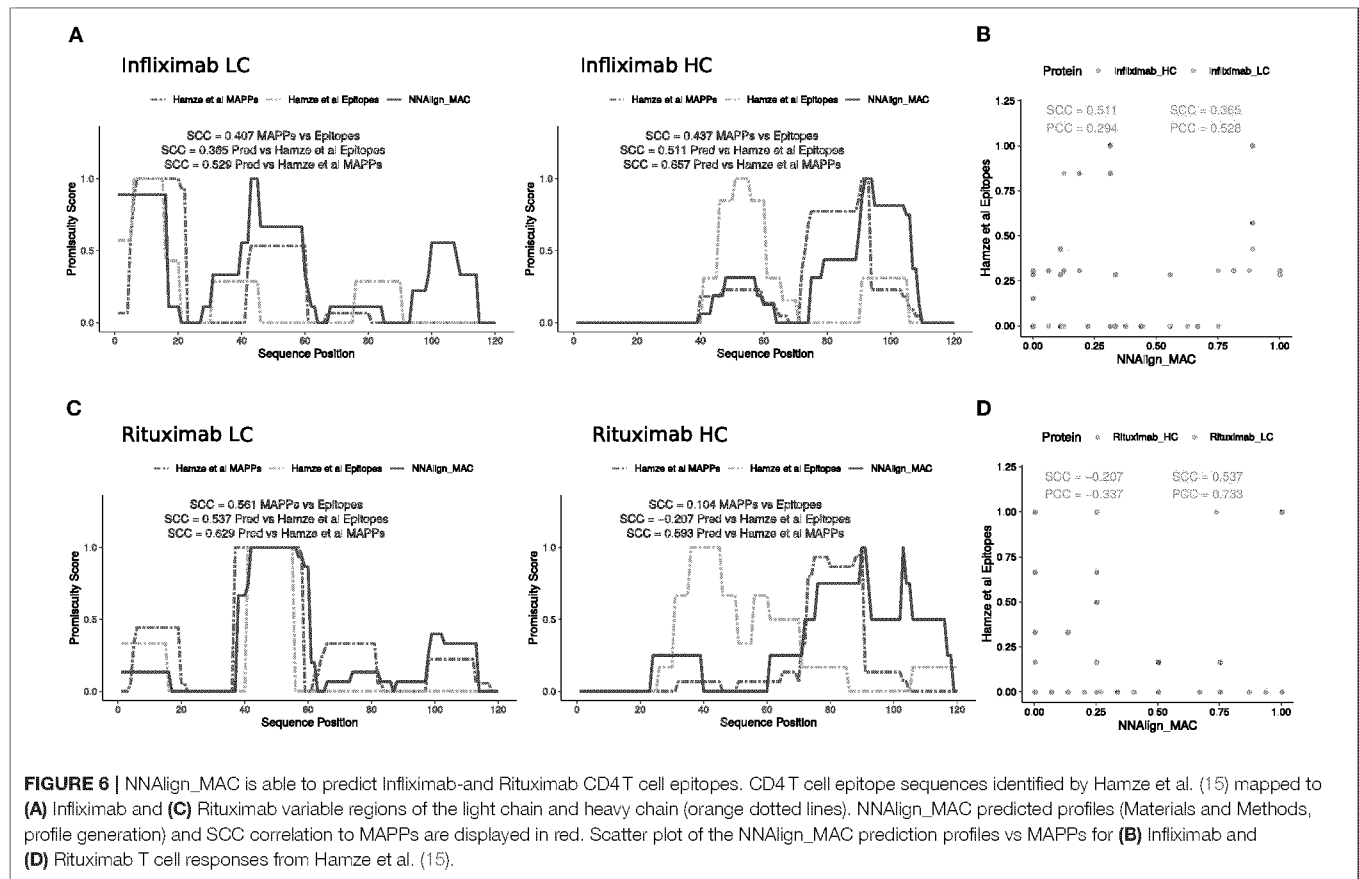
Next, we investigated if the peak regions predicted by NNAalign_MAC correlated with the location of CD4 T cell epitopes. For that purpose, 6 and 9 peptides respectively from Infiximab light and heavy chains were designed and assessed using ELISpot assays for CD4 T cell activation (for details refer to



section Methods). These infliximab peptides were selected from three categories: MAPPs regions—covering regions predicted by NNAAlign_MAC where MAPPs peptides were found (**Figure 5A**, Magenta: LC_1, LC_5, LC_6, HC_2, HC_3, HC_4, HC_6, HC_8, HC_9); NNAAlign_MAC regions—regions predicted by NNAAlign_MAC, with no MAPPs peptides (**Figure 5A**, Cyan: LC_2, LC_4); and regions were the methods identified none or very few ligands (**Figure 5A**, Yellow: LC_3, HC_1, HC_5, HC_7). In these assays, 67% (6/9) of the peptides spanning MAPPs positive regions were positive in at least one donor (all except for LC_5, HC_4 and HC_6, **Figure 5B**). Similar results were observed for the NNAAlign_MAC region peptides (**Figure 5B**). Here, both peptides (LC_2, LC_4) were positive for at least one donor (**Figure 5B**). Finally, one of the peptides, HC_7, selected from the “empty” region (yellow) was found to give a marginal response in one of the assessed donors (**Figures 5A,B**). These findings thus demonstrate a very high correspondence between the NNAAlign_MAC predictions, the location of observed T cell

epitopes, and further suggest that MAPPs potentially can miss relevant regions leading to immunogenicity.

As a final validation of the predictive power of the proposed prediction method, a set of CD4 T cell epitopes for Infliximab and Rituximab antibodies were collected from Hamze et al. (15). In this study, all 15mers spanning the light and heavy chain of the protein-drugs with an overlap of 10 amino acids were assessed in 15 healthy, 6 infliximab-treated donor, and 1 rituximab-treated donor, for T cell activation. Epitope profiles were constructed similarly to how MS profiles were built earlier by stacking the epitopes data over the light and the heavy chains of the protein-drugs and counting how many peptides overlap per each amino acid position. Next, as no complete allele information was provided for the tested donors, NNAAlign_MAC predictions were made for the alleles present in our in-house MAPPs dataset, and promiscuity profiles were built for the light and heavy chain variable regions as described earlier (**Figure 6**). Notably, NNAAlign_MAC was able to predict most of



the T cell immunogenic regions for Infliximab (Figures 6A,B) in a comparable fashion to experimental MAPPs. Analyzing the hotspots regions in 30–45 from the light chain, or 90–105 from the heavy chain, we observe that NNAIalign_MAC was able to predict those regions while the MAPPs experiment missed it (Figure 6A). In the Rituximab example, while both MAPPs (SCC = 0.561) and NNAIalign_MA correlations (SCC = 0.537) show high and comparable performance for the light chain (p -value = 0.81, bootstrap), both approaches demonstrated very limited predictive power over Rituximab heavy chain epitopes (Figures 6C,D).

DISCUSSION

Here, we have constructed a predictor, NNAIalign_MAC, for MHC class II antigen presentation trained on in-house MAPPs and data from the IEDB based on the previously developed NNAIalign_MA machine learning framework (22, 23) integrating context information and HLA binding promiscuity scores. The predictor was demonstrated to vastly improve in performance over NetMHCIIpan for the prediction of MHC antigen presentation hotspots in protein drugs. Moreover, our findings strongly suggest that the use of such prediction methods could effectively serve as a complement to MAPPs assays to improve the sensitivity for identification of hotspot regions enriched in MHC ligands and T cell epitopes.

One of the strengths of the NNAIalign_MAC algorithm lies in its ability to leverage information between multiple MAPPs datasets reducing noise and boosting performance in particular for alleles characterized by limited data (as exemplified by the clear motifs identified in the MAPPs data for the weakly expressed HLA-DR3, 4, 5 alleles). This combined with its pan-specific power (8) makes NNAIalign_MAC less sensitive to the critical limiting issues often associated with MAPPs assays including the requirement of a massive amount of biological material, the need for experimental replicates and repeated assaying over HLA diverse cohorts, and the non-trivial task of interpreting/mapping the raw MS spectral data to genomic templates.

We have here demonstrated the power of NNAIalign_MAC for two protein drugs infliximab and rituximab only. Further studies covering a broader set of proteins are needed to fully assess the gain in performance of prediction models trained on MS data for prediction of antigen presentation hotspots and T cell epitopes. Likewise, further studies are needed to assess if the complementary power observed in this study of *in-silico* predictions over MAPPs for hotspots identification remains valid when tested on a broader set of protein drugs. Moreover, additional methods for MHC-II antigen presentation prediction trained on MS data have recently been proposed (24–26). We showed an improved performance of NNAIalign_MAC in predicting Infliximab MAPPs data compared

to both NetMHCIIpan (5) and MixMHC2pred (25). Other methods have been recently published integrating MS MHC ligand data in the training. As for the method developed by Chen et al., MARIA (26), the comparison does not seem adequate in this scenario as its predictive power depends on the availability of protein expression levels, which makes limited sense in the context of protein-drugs. Another method, NeonMHC2 (24), only allows for to run max 20 predictions per day, making it impractical to include in a benchmark. Further evaluations remain to be conducted to benchmark the predictive power of these novel tools for the prediction of protein-drug MHC antigen presentation and immunogenicity. In conclusion, this work demonstrates that MS data can be used to train improved predictors for MHC class II antigen presentation, and showcase how such predictors can be used to effectively assess protein-drugs for the presence of MHC II hotspot and T cell epitope regions complementing the use of the conventional cost-intensive MAPPs assays.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on ProteomeXchange Consortium via the PRIDE partner repository (<http://www.ebi.ac.uk/pride>) with the dataset identifier PXD018303.

ETHICS STATEMENT

Donors IMXP00288 and IMXP00425 correspond to the protocol with national number B707201627607, IMXP00387 and IMXP00425 correspond to the protocol with national number B707201629385 and IMXP00335, IMXP00261 and IMXP00283 correspond to the protocol with national number

NL5791207516. The full name and affiliations of the ethical committee for protocol NL5791207516 is METC Isala, Gebouw Mondriaan, Kamer 0.47, Postbus 10400, 8000 GK Zwolle, Netherlands. The full name and affiliations of the ethical committee for the two other protocols is Comité d'Ethique Hospitalo-Facultaire Universitaire de Liège (707), C.H.U. Sart Tilman, Domaine Universitaire du Sart Tilman, B35, 4000 Liège 1, Belgium.

AUTHOR CONTRIBUTIONS

CB, BR, and MN conducted research and developed the computational pipeline. CB, CA, LJ, SP, EP, and MN analyzed the data. AJ and CA performed sample processing. MW, SC-M, and J-PG generated the MAPPs data. CA and JS performed T cell assays. CB, SP, EP, and MN designed the study. CA, MW, SP, and EP provided methodological details and reviewed the manuscript. CB and MN wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.01304/full#supplementary-material>

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Immunogenicity of Immunotoxins Containing *Pseudomonas* Exotoxin A: Causes, Consequences, and Mitigation

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Immunotoxins are cytolytic fusion proteins developed for cancer therapy, composed of an antibody fragment that binds to a cancer cell and a protein toxin fragment that kills the cell. *Pseudomonas* exotoxin A (PE) is a potent toxin that is used for the killing moiety in many immunotoxins. Moxetumomab Pasudotox (Lumoxiti) contains an anti-CD22 Fv and a 38 kDa portion of PE. Lumoxiti was discovered in the Laboratory of Molecular Biology at the U.S. National Cancer Institute and co-developed with Medimmune/AstraZeneca to treat hairy cell leukemia. In 2018 Lumoxiti was approved by the US Food and Drug Administration for the treatment of drug-resistant Hairy Cell Leukemia. Due to the bacterial origin of the killing moiety, immunotoxins containing PE are highly immunogenic in patients with normal immune systems, but less immunogenic in patients with hematologic malignancies, whose immune systems are often compromised. LMB-100 is a de-immunized variant of the toxin with a humanized antibody that targets mesothelin and a PE toxin that was rationally designed for diminished reactivity with antibodies and B cell receptors. It is now being evaluated in clinical trials for the treatment of mesothelioma and pancreatic cancer and is showing somewhat diminished immunogenicity compared to its unmodified parental counterpart. Here we review the immunogenicity of the original and de-immunized PE immunotoxins in mice and patients, the development of anti-drug antibodies (ADAs), their impact on drug availability and their effect on clinical efficacy. Efforts to mitigate the immunogenicity of immunotoxins and its impact on immunogenicity will be described including rational design to identify, remove, or suppress B cell or T cell epitopes, and combination of immunotoxins with immune modulating drugs.

Keywords: recombinant immunotoxins, neutralizing antibodies, anti-drug antibodies (ADA), B cell epitopes, T cell epitopes, moxetumomab pasudotox, LMB-100

INTRODUCTION

Protein and cell based therapeutic agents have great potential to treat many human diseases. However, because many of these contain non-self sequences, they often elicit an immune response that blocks their efficacy. Clinical trials with chimeric antigen receptor-T cells (CAR-T) (1), enzyme replacement therapy (2), monoclonal antibodies (3), antibody drug conjugates (ADCs),

immunotoxins (4), and viral based gene therapy vectors (5) have often failed to produce desired effects due to the formation of antibodies that neutralize the activity of the therapeutic agent.

Recombinant immunotoxins (RIT) are chimeric proteins that consist of a targeting element linked to a toxin. The targeting element is commonly an Fv portion of an antibody which targets a specific antigen on tumor or infected cells (6). RITs have been developed to treat a variety of indications, such as blood cancers (7, 8) solid tumors (9–11) graft-vs.-host disease (12), viral infections (13, 14), and autoimmune diseases (15). *Pseudomonas* exotoxin A (PE, also known as ETA) and diphtheria toxin are both favorable toxins for construction of RITs due to their high potency, expression and purification yields, ease of cloning, and relatively low non-specific toxicity compared to other toxins (16). Both toxins kill cells by catalyzing ADP ribosylation and inactivation of elongation factor 2, which results in arrest of protein translation, a fall in anti apoptotic proteins and apoptosis (11). Both toxins have been used as killing domains in antibody or cytokine targeted drugs and were approved for licensure by regulatory agencies. They represent “first in class” drugs for targeted toxins (17, 18).

Recently (September 2018), Moxetumomab pasudotox (Lumoxiti), whose pre-clinical and early clinical development took place in the Laboratory of Molecular Biology (LMB) at the U.S. National Cancer Institute and whose advanced clinical development took place at AstraZeneca, was approved by the U.S. Food and Drug Administration for the treatment of relapsed or refractory hairy cell leukemia. Lumoxiti is composed of an anti-CD22 Fv murine antibody fused to PE38, a 38 kDa truncated form of PE (Table 1) (26, 27). Encouraged by this success, major efforts are focused on developing PE based RITs against mesothelin and other proteins on solid tumors (20, 28–32).

PSEUDOMONAS EXOTOXIN A (PE)

PE is the most toxic virulence factor of the opportunistic pathogen *Pseudomonas aeruginosa* (33), a Gram-negative bacterium (34). *Pseudomonas aeruginosa* is ubiquitous in soil and water and generally infects only immunocompromised and elderly populations (34). This indicates that immune competent patients can efficiently mount an immune response and maintain an immune memory against *Pseudomonas aeruginosa* toxins. Indeed, the immunogenicity of the PE based moiety is a major hurdle in immunotoxin clinical development. PE is composed of three structural domains. A binding domain (I), a processing domain (II) and the catalytic domain (III). For RIT construction, the binding domain was replaced with antibody fragments.

METHODS TO ASSESS ANTIBODY RESPONSES AGAINST RECOMBINANT IMMUNOTOXINS

Clinical development of RITs has been ongoing for about three decades. Immune monitoring of ADA by enzyme-linked immunosorbent assay (ELISA) or neutralizing antibodies (Nab)

by neutralization assays (Nab assay) have changed in the past three decades as methods improved and as clinical development progressed. In early trials, ADAs were monitored using direct ELISA assays (28). A functional Nab assay was first reported in 1996 (20). The Nab assay entailed adding serum samples to two concentrations of immunotoxin and adding the mixture to sensitive cells. A sample was considered Nab positive if protein synthesis inhibition (20) or cytotoxic activity (7) was inhibited by 50 or 75%, respectively. Comparison of ADA positive patients and Nab positive assays revealed that all Nab positive samples are ADA positive but not all ADA positive samples are Nab positive (4). This indicates a higher sensitivity for the ADA assays and implies that some of the binding antibodies do not possess neutralization activity.

In the past decade, advancements in ADA monitoring methods and development of ultrasensitive assays have led to more specific and accurate monitoring approaches. Liang and colleague improved the ELISA assay by minimizing the impact of PE38 immunodominance on the ability to detect ADA against the murine antibody fragment. They tested each patient's sample in a bridging ELISA with biotin-Lumoxiti coated plates in three conditions: with the CD22 fragment, with the PE38 fragment or with both. A signal was obtained using the addition of a ruthenium labeled Lumoxiti (35) and the fragments in the three conditions competed for binding with the ADAs.

To determine the immunogenicity cut-point for Lumoxiti (the OD or neutralization activity at which a sample is considered positive) samples from normal donors are commonly used. Because many naïve donors have been exposed to PE and have pre-existing antibodies, sample manipulation was necessary to obtain a sensitive cut-point for immunogenicity monitoring. To overcome this problem, an irrelevant PE-immunotoxin was added to serum samples to occupy the pre-existing antibodies prior to evaluating samples for cut point establishment (36).

CLINICAL IMMUNOGENICITY OF IMMUNOTOXINS

Chemical Conjugates

The immunotoxin that was evaluated in a clinical trial (OV3-PE) (Figure 1A) contained a full length murine antibody (clone OV3) against an unknown antigen on ovarian cancer cells chemically conjugated to the PE protein (28). Formation of ADA against OV3-PE was evaluated by ELISA, which showed that 16/16 of the patients developed anti PE ADA within 14 days. Human anti-mouse antibodies (HAMA) were also detected in 75% of the patients.

In a clinical trial evaluating LMB-1 (Figure 1B), in which domain I of PE was removed and the remaining 40 kDa protein attached to an antibody to Lewis-Y, 33/39 of the patients developed ADA responses against LMB-1 3 weeks after the first cycle of treatment. The remaining 10% who did not make neutralizing antibodies after the first cycle, were further treated and developed neutralizing antibodies after subsequent

TABLE 1 | Immunotoxins tested in patients.

RIT name	Target	Antibody clone	Antibody format	Mouse or human	Clinical trial	References
D2C7-(scdv)-PE38KDEL	EGFR	D2C7	scdsFv	Mouse	NCT02449239	(19)
LMB-1	Lewis Y	B3	Mab	Mouse	NCT00001805	(20)
Oportuzumab Monatox	Anti-EpCAM	VB4-845	scFv	Humanized	NCT03258593	(21)
Moxetumomab Pasudotox (Lumoxiti)	CD22	Affinity matured RFB4	scFv	Mouse	NCT01829711	(22)
LMB-2	CD25	Anti Tac	scFv	Mouse	NCT00924170	(23)
MOC31PE	EpCAM	MOC31	scFv	Mouse	NCT02219893	(21)
SS1P	Mesothelin	SS1	scdsFv	Mouse	NCT00006981	(24)
LMB-100	Mesothelin	SS1	Fab	Humanized	NCT02798536	(25)

treatment cycles. ELISA assays indicated that eventually, 100% of the 38 patients made antibodies against the toxin moiety and 33/38 of the patients had HAMA against the antibody fragment (20).

Recombinant Immunotoxins

LMB-2 (structure similar to SS1P shown in **Figure 1C**), which is composed of a murine anti CD-25 Fv linked to PE38 (**Table 1**), was used to treat leukemia and lymphoma patients. Clinical evaluation showed several complete and partial responses; however, 10/35 of the patients developed Nabs, which prevented further treatment. Six of the patients developed Nabs after the first cycle of treatment. Three of the patients that developed Nabs also demonstrated immunogenicity related side effects including one anaphylactic reaction and other allergic grade 2–3 reactions (2/35) (7). These adverse events contra-indicated further treatments once Nabs are present.

The lowest immunogenicity rates were reported in early trials evaluating Lumoxiti for hematological malignancies; After the first treatment cycle, only 1/28 hairy cell leukemia (HCL) patients made Nabs and a total of 10/28 had Nabs throughout the entire phase 1 trial (38). Furthermore, of the 50 CLL patients that were treated with Lumoxiti, only two patients had a Nab response after four cycles of treatment (4, 39).

Phase II and III trials of Lumoxiti were monitored for presence of binding ADA rather than Nabs. In those trials, 65% of patients made ADA after two cycles (38). In a larger trial, 75% of patients had detectable ADA at the end of treatment (40). The difference in immunogenicity reports in the early trials is mostly explained by differences in monitoring methods; functional Nab assays are less sensitive than binding ADA assays.

The overall low rate of immunogenicity to Lumoxiti can be attributed to the immune status of the patients. Patients with HCL have usually been treated with Cladribine which kills immune cells in the bone marrow. Additionally, the leukemia cells infiltrate the marrow, causing immunosuppression. Furthermore, Lumoxiti targets the CD22 antigen which is highly expressed in the targeted cancer cells but also expressed in mature and immature B cells. It is likely that Lumoxiti kills some B cells that would mount an immune response against it.

A good example that exemplifies the importance of patients' immune status is that of LMB-2. Patients with hematological malignancies treated with LMB-2 had a relatively low rate of immunogenicity onset with 17% of the patients making

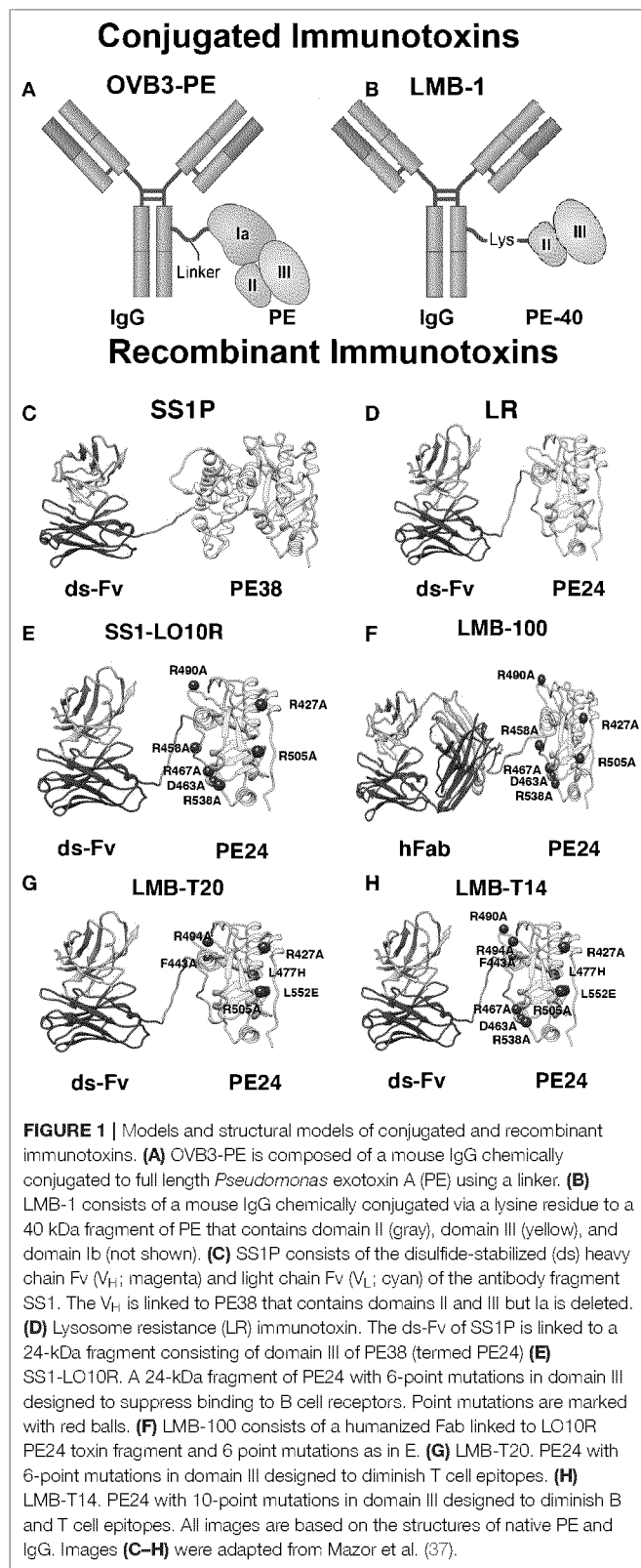
neutralizing antibodies after the first cycle (7). In contrast, melanoma patients who received LMB-2 and had a normal immune system demonstrated a high level of immune response; 92% of patients made neutralizing antibodies after the first cycle (41).

IMPACT OF IMMUNOGENICITY ON PHARMACOKINETICS AND CLINICAL OUTCOME

Generally, ADAs to therapeutic proteins have a risk of immune-related adverse events, including infusion-related reactions, allergic or anaphylactic reactions, delayed hypersensitivity, and autoimmunity (42). RITs show few of these responses. The only severe anaphylactic reaction reported occurred immediately after the first infusion of the RIT (7). Some patients reported grade 1, 2, or 3 skin reactions that were easily managed by a course of steroids [reviewed in (4)]. Neutralization and drug clearance are the main problems with RIT therapy, not immuno-toxicity. The low incidence of adverse side effects could be related to the relatively low doses administered and the small size (63 kDa) of the protein.

LMB-100 is a PE-based RIT engineered for decreased immunogenicity (**Figure 1F**). To study the impact of ADAs on LMB-100 levels, we analyzed immunogenicity and pharmacokinetic data from a clinical trial treating Pancreatic Ductal Adenocarcinoma with LMB-100 and nab-paclitaxel (25). Anti LMB-100 ADA were monitored using ultrasensitive methods to triage ADA positive and negative responses (screening assay). Patients with pre-existing antibodies were not excluded.

Using a cut point of O.D = 0.05, 9/20 had pre-existing antibodies. These low titers did not have much impact drug levels (**Figure 2A**). C_{max} in 19/20 patients was well above 100 ng/ml. In cycle 2, only 6/13 patients were ADA negative and their C_{max} well above 100 ng/ml. 7/13 of the patients were ADA positive still had effective blood levels (**Figure 2B**). Overall, more than half of patients receiving a second cycle of LMB-100 had detectable plasma drug concentrations. None of the patients received a third cycle of therapy due to toxicity of the nab-paclitaxel. However, post treatment ADA monitoring showed that 9/10 of the patients evaluated were ADA positive; most of them with a very high OD signal (25).



Altogether, a correlation was observed between the ADA levels and drug blood levels (Figure 2C). Using a five-parameter asymmetric sigmoidal curve fit, EC₅₀ has an OD = 2.8.

Therefore, it can be estimated that samples with an OD lower than 2.8 will predict an effective C_{max} and samples with OD > than EC₅₀ will not. Previously, patients with positive signals on ADA or Nab assays were excluded from clinical trials. However, this data indicates that a positive call on the ADA assay does not predict a low blood level unless the titers are very high (Figure 2).

Kreitman et al. reported that a minimum of three to five cycles of treatment was required to obtain major responses including durable complete remissions (43). In earlier trials, patients were not allowed to complete the therapy once they developed Nabs. This was to avoid immunological side effects unnecessary ineffective RIT drug administration. Kreitman and colleagues were able to observe a correlation between the timing of antibody formation and the outcome of the treatment (43). In the phase 1 study, 65% of patients made ADAs after two cycles based on ELISA results (38). Most patients (80%) who did not achieve CR had a positive antidrug antibody ELISA test (38). In a larger trial, patients with favorable responses (complete or partial responses) had lower antibody titers (<10,000), which probably improved their drug blood levels for more treatment cycles compared to patients with stable or progressive disease (40). Furthermore, when SS1P (Figure 1C) was combined with pentostatin and cyclophosphamide to lower T and B cells and suppress anti-drug antibodies, more treatment cycles could be given to most of the patients and major tumor responses were observed in several patients with advanced refractory mesothelioma (44). Altogether, these findings indicate that patients with low or delayed immune responses are likely to respond better and justifies the efforts described below to mitigate the ADA response.

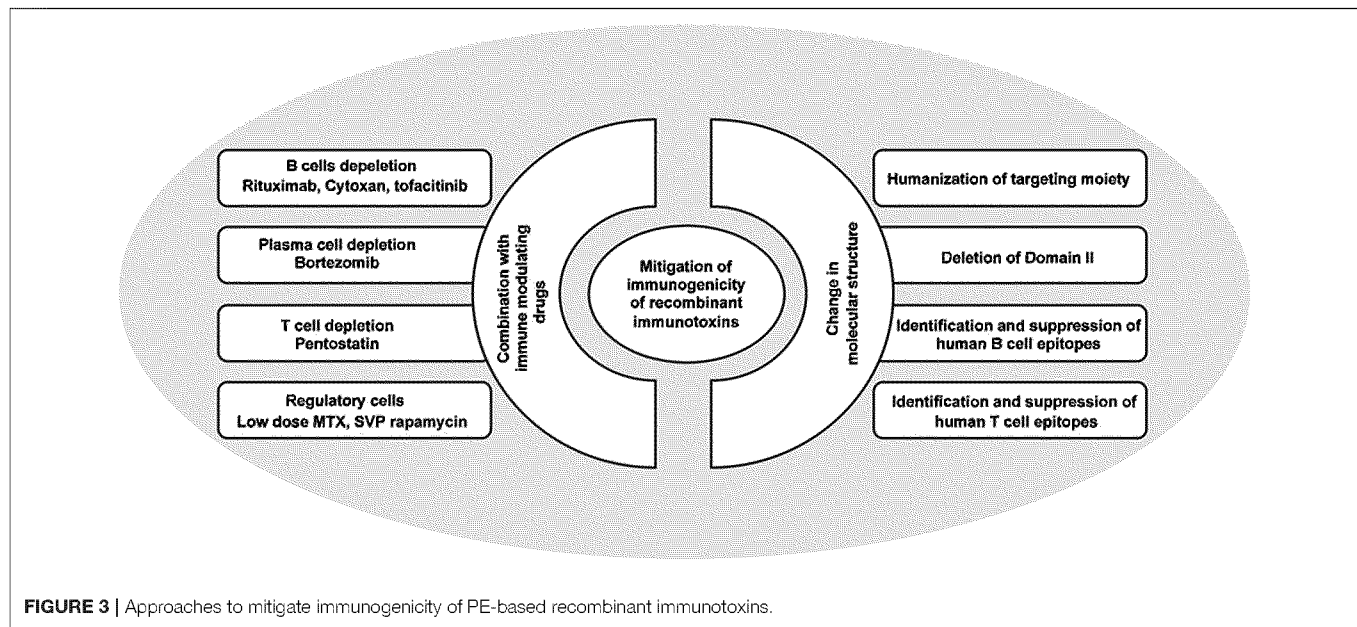
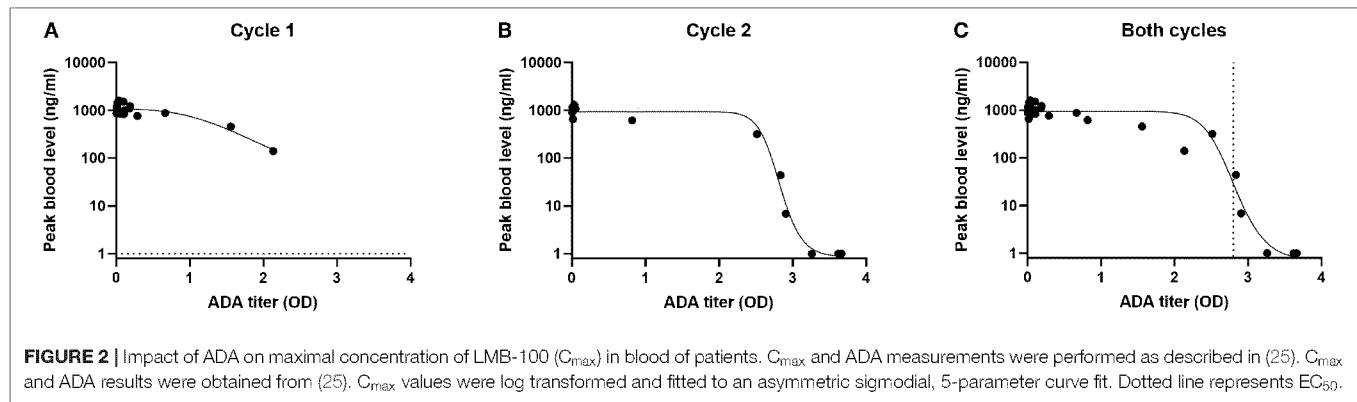
STRATEGIES TO MITIGATE THE IMMUNOGENICITY OF RITS

Combination With Immune Modulating Drugs

Combination approaches to mitigate immunogenicity of RIT include targeting of the B cells that form the adaptive immune response, targeting the plasma cells that produce high titers of IgG, or targeting the T cells that support a neutralizing immune response. In addition, recent approaches have targeted regulatory factors of the immune system that suppress the immune responses (Figure 3).

In 2004 five patients were pre-treated with rituximab to eliminate their B cells prior to LMB-1 administration. Binding ADA and Nab assay were used to monitor the development of human antibodies against LMB-1. Treatment with rituximab was effective in abrogating 99.9% of circulating CD20/CD19⁺ B cells in all patients (5/5). However, all these patients developed neutralizing anti-LMB-1 antibodies by day 21 of drug administration (45). This indicates that elimination of the peripheral B cells is not sufficient to eliminate the immune response.

To target both B and T cells, 10 refractory mesothelioma patients were treated with a combination of pentostatin and cyclophosphamide to kill B and T-cells. This combination



delayed the formation of neutralizing antibodies to SS1P by several cycles. Out of the 10 patients treated, only two made Nabs after the first cycle, and 6 patients made Nabs after the second cycle. One patient did not make any Nabs throughout six treatment cycles (44). The toxicity observed in the trial described above was similar to the known side effects of pentostatin and cyclophosphamide.

In a T cell leukemia clinical trial evaluating the efficacy of LMB-2 after both cyclophosphamide and fludarabine, a great decrease in immunogenicity was observed. This delay translated to higher drug blood levels for multiple treatment cycles and very good anti-tumor responses (60% of the patients achieved complete remission) (23).

Elimination of pre-existing antibodies and plasma cells is a major goal for RITs due to a high prevalence of pre-existing antibodies from environmental exposure to PE, as well as the need to have more than one treatment cycles. Bortezomib is a reversible proteasome inhibitor that showed high efficacy in targeting long and short lived plasma cells that have high rates of Ig production (46). Manning et al. found that combination of SS1P with Bortezomib was able to reduce ADA formation by 50% compared to SS1P with no immune suppression in mice.

Additional combination with pentostatin and cyclophosphamide reduced ADA formation by 88% (47).

Tofacitinib is a janus kinase 1 inhibitor that suppresses inflammatory responses. Treatment of mice with tofacitinib led to reduced numbers of CD127⁺ pro-B cells and reduction in B cell germinal center formation in mice spleens (48). Because normal Ig levels were still present during tofacitinib treatment, this agent specifically reduced ADAs.

Along with the immune depleting approaches, pre-clinical approaches to evaluate combinations with drug in low concentrations or encapsulated in nanoparticles have shown promising results. Low dose methotrexate (MTX) has been shown to reduce ADA formation against adalimumab [reviewed in (49, 50)] and against enzyme replacement therapy for infantile Pompe disease (51). Combination of low dose MTX with LMB-100 suppressed the formation of ADAs, maintained blood levels of LMB-100 and prevented its neutralization in immune competent mice. This did not compromise the immune response against a second antigen given after stopping MTX, suggesting contemporaneous immune tolerance (52).

To harness the immune modulatory properties of rapamycin, LMB collaborated with Selecta Bioscience that had encapsulated

rapamycin in PLGA-PEG synthetic vaccine particles (SVP-R). Combination of SVP-R with LMB-100 produced a specific and transferable immune tolerance, which prevented ADA and Nab formation against the RIT in naïve mice and in mice that model pre-existing immunity (53). This approach was quickly translated to a clinical trial combining the two agents to treat mesothelioma patients. However, the combination resulted in an unforeseen lung toxicity in this patient population and the trial was discontinued (clinicaltrials.gov T03436732).

Change in Molecular Structure

The two-unit structure of RITs which includes a targeting antibody unit and a toxin unit, and the variable immunogenicity properties of those two units (i.e., preexisting antibodies to the toxin or the presence of a murine fragment in the antibody) allows tailored mitigation to each unit based on its properties and what is known in the art as de-immunization (Figure 3).

MITIGATING THE IMMUNOGENICITY OF THE ANTIBODY DOMAIN

Immunogenicity of therapeutic monoclonal antibodies can be mitigated by increasing the content of the human sequence. Such antibody engineering includes framework humanization, chimerization, and use of mice with humanized germlines. Such approaches can reduce the common immunogenicity rate from about 40% in chimeric antibodies to 9% in humanized antibodies (54, 55). However, in some cases, the immunogenicity against the variable complementarity determining region domains (CDRs) may still cause ADA formation (55).

Most RITs have a murine antibody fragment (Table 1). The lack of humanization of these agents can be explained by the fact that their development began before approaches to humanize antibodies were readily available. Furthermore, the immunogenicity of the bacterial PE is a much bigger barrier than HAMA (56). Recently, LMB-100 a “second generation RIT” containing a humanized Fab instead of a mouse Fv has entered clinical testing (Table 1) (25). The humanization was done by combining framework regions in the CDRs of the mouse anti-mesothelin antibody SS1 and human Fab. To improve the binding to mesothelin and to stabilize the CDRs tertiary structure, some back mutations within the mouse parent residues as well as the human sequences were introduced (as described in patent WO2015051199). The new humanized antibody had comparable binding affinity to mesothelin, and LMB-100 showed comparable thermal stability and technical developability to that of SS1P (57).

MITIGATING THE IMMUNOGENICITY OF THE TOXIN

Identification of B Cell Epitopes

Antibodies and B-cell receptors bind to regions on the surface of a protein called B cell epitopes. These epitopes often cluster on the surface of the antigen and can control most immune responses (58). Roscoe et al. used synthetic peptides from PE38 to map the B cell epitopes in serum samples from

monkeys and humans treated with immunotoxins (59, 60). This approach identified linear epitopes but not discontinuous conformational B cell epitopes on the toxin. Onda and Nagata immunized mice with RIT and used a capture assay to isolate monoclonal antibodies that reacted with native PE38 in solution (61). They discovered seven murine conformational epitopes in PE38 and identified single point alanine substitutions that abolished binding to those antibodies. They constructed and characterized a novel de-immunized mouse RIT named 8M. This RIT retained excellent cytotoxic and anti-tumor activity and importantly, had a low immunogenicity response after injection into mice. These experiments established the first proof that removing B cell epitopes could greatly diminish immunogenicity (61, 62).

Human B-cell epitopes in domain III were mapped using phage display. These studies focused on domain III of PE, because it was found that most of domain II was not needed to make active immunotoxins and could be removed (63). B cells were isolated from 7 patients receiving immunotoxin therapy and phage Fv libraries was prepared from B cells that contained Fvs reacting with domain III of PE. This selected library should represent the antibody repertoire that can bind and neutralize RITs with domain III. Then an immunotoxin library was constructed. This library contained 36 mutant PE immunotoxin constructs, each with a single point mutation replacing large amino acids like arginine, glutamine and glutamic acid with alanine. Then, the phage library was panned against each mutant RIT in the mutant library, identifying point mutations that abolish binding (64). Seven major B cell epitopes were identified and subsequently silenced by converting a key residue in the epitope to alanine. The modified toxin was named LO10 (Figure 1E) (representing the initials of the last name of the two scientists developing it). The LO10 toxin (Table 2) has been used to make immunotoxins targeting both CD22 and mesothelin. LMB-100 contains the LO10 mutations and is the first “de-immunized” PE based toxin that has advanced to clinical development.

A similar approach was used to identify B cell epitopes in diphtheria toxin (71). Highly hydrophilic amino acids on the surface of the toxin were mutated, and the mutant constructs were injected into mice for screening. Constructs that did not activate the mouse immune system are speculated to be of low immunogenicity in humans as well (71). This approach, while simpler than the strategy used to generate LO10, suffers from the fact that mice and human have different self and non-self-selection, and immunogenic regions that activate a human immune system may not activate a mouse immune system.

Deletion of Domain II of PE38

Protease evasion can reduce processing of the protein in the endosome and late endosome and therefore, reduce peptide presentation by MHC II molecules and T-cell activation. Weldon et al. found that domain II of PE38 was very sensitive to lysosomal protease digestion and furthermore that 102/113 amino acids in domain II can be removed without loss of activity as long as the furin cleavage site (in amino acids 274–284) remained (72) (Figure 1D). Deletion of the majority of domain II had

TABLE 2 | Recombinant Immunotoxins that were mutated to decrease immunogenicity.

Drug name	Target	Toxin description	Activity			References
			IC50 (pM)	Relative activity (compared to PE38) (%)	Cell type	
Moxetumomab	CD22	PE38	3.4	100	CA46	(65)
LO10		PE24 with 5 point mutations to reduce B cell binding	0.9	378		(64)
LMB-T18		PE24 with 6 point mutations to reduce T cell binding	2.2	155		(65)
LMB-T19		PE24 with 10 point mutations to reduce B and T cell binding	3.4	100		Unpublished
SS1P	Mesothelin	PE38	47.5	100	KLM1	(66)
LMB-100/RG7787		Humanized Fab and PE24 with 5 point mutations to reduce B cell binding	9.9	480		
LMB-T20		PE24 with 6 point mutations to reduce T cell binding	13.1	363		
LMB-T14		PE24 with 10 point mutations to reduce B and T cell binding	27.9	170		
LMB-2	CD25	PE38	0.07	100	HUT102	(67)
LMB-2 T20		PE38 with 6 point mutations to reduce T cell binding	0.23	30		
LMB-142		PE38 with 9 point mutations to reduce T cell binding	0.69	10		
Tac-M18-PE24(T)		PE24 with C-C stabilizing linker and 6 point mutations to reduce T cell binding	0.7	10		(68)
LMB-75	BCMA	PE24	1.1	100	H929	(69)
		PE24 with 4 point mutations to reduce B cell binding	3.1	35		Unpublished
LMB-92						
LMB-103 (T20)		PE24 with 6 point mutations to reduce T cell binding	6	18		Unpublished
LMB-273 (T20)		PE24 with 5 point mutations to reduce T cell binding (excluding R494A)	1.1	100		Unpublished
HN3-PE38	GPC3	PE38	586.0	100	Hep38B	
HN3-mPE24		PE24 with 5 point mutations to reduce B cell binding	592.0	99		(70)
HN3-T20		PE24 with 6 point mutations to reduce T cell binding	766.0	77		
HN3-T19		PE24 with 10 point mutations to reduce B and T cell binding	1082.0	54		

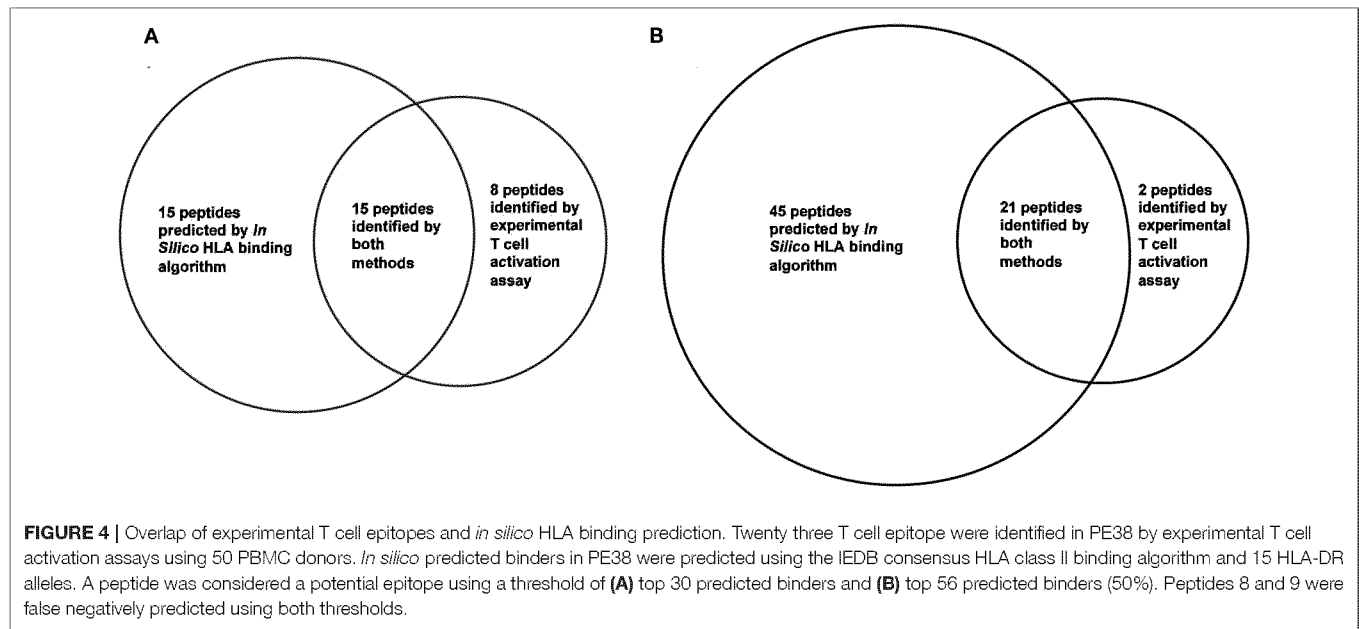
the additional benefit of deletion of the immunogenic B and T cell epitopes in that domain. RITs with the resulting mutant toxin (designated LR for lysosome protease resistance) or PE24 (Table 2) were tested in three strains of mice and showed a greatly decreased antibody response (73).

T Cell Epitopes

Elimination of B cell epitopes as described above should be effective in evading pre-existing antibodies. However, deletion of the immunodominant B cell epitopes cannot prevent B cells with low affinity B-cell-receptors from undergoing affinity maturation and class switching. These processes are supported by professional antigen presenting cells and helper T cells (58, 74). Unlike B cells, T-cell receptor specificity, does not change on antigen encounter. Once T-cell epitopes are eliminated, formation of new specificities is not expected (75). In a proof of concept study, the murine T cell epitopes in PE38 were mapped using a peptide library and IL2 ELISpot of immunized

mice spleens. Alanine scanning of each amino acid within 15 mer epitopes revealed single point mutations that can prevent the T cell response. A new RIT was constructed with several point mutations in PE38 that were effective on preventing anti PE antibodies and Nabs (76). Additional studies in BALB/c mice reinforced the identification of a subdominant murine T cell epitope in domain III (77). This study also showed that a slightly modified version of the de-immunized PE (A505H) using a different mode of administration and adjuvant has a significantly lower immunogenicity compared to PE24.

The human T cell epitopes in PE38 mapped using PBMCs from 50 donors that share similar HLA to the typical patient population in the western world. The PBMC were expanded with PE38 to allow antigen processing and presentation and enrich the T cells that recognize PE38 epitopes (78). The enriched T cells were re-stimulated with over-lapping peptides that span the sequence of PE38. T cell activation was monitored using IL-2



ELISpot (79). IL-2 supports T-cell activation, differentiation, and memory and is a less specialized cytokine than IL-4 or IFN- γ (80). Twenty-three peptides whose sequence overlap had positive responses and made up eight T cell epitopes (65). One of these epitopes, located in domain II, was present in 21/50 donors (79). The eight T cell epitopes identified in naïve donor PBMC were also identified using samples from 16 cancer patients previously treated with PE38 containing RITs and who had mounted an immune response to the protein. This supports the conclusion that PE38 has eight T cell epitopes and other regions of the protein are less immunogenic. Interestingly, similar assays using PBMCs from immunized HCL patients show several epitopes missing (65). Further work will address the absence of some epitopes in HCL patients and why cells recognizing these epitopes are absent in these patients.

HLA binding algorithms that predict the binding affinity of peptides to polymorphic HLA II molecules can be used to predict or narrow down peptides for potential T-cell epitopes. Overpredictions are expected for such algorithm predicted epitopes due to various factors involved in T-cell activation that cannot be predicted by the HLA binding, including antigen processing in the endosome, T-cell receptor binding, and T-cell activation. To compare the experimentally identified epitopes with *in silico* predicted epitopes, two primary HLA binding algorithms: Propred (81) and IEDB Consensus (82) were used to predict promiscuous binding to 15 common HLA DR alleles (83). Venn diagrams showing comparison of the predicted peptides using the *in-silico* analysis and the experimental approach is shown in **Figure 4**. The top 30 stringently predicted peptides had an overlap of 15 peptides with the 23 experimental peptides. This left 8 peptides (representing four epitopes) mis-identified by the analysis as negative. A less stringent threshold of 56 peptides (choosing 50% of the peptides as positive) had a much better precision and predicted 21 of the 23 peptides. However, the

epitope in peptides 8 and 9 was overlooked by the algorithm. While overpredictions are expected, underpredictions are not, and they such reduce the effectiveness of these computational tools for prediction of T-cell epitopes. HLA binding inhibition assays revealed that the missed epitope in peptides 8 and 9 was solely presented by HLA DP presentation molecules and not DR (84). The algorithm could not have predicted binding to this peptide, because the query was limited to DR alleles. Interestingly, re-analysis of the HLA binding prediction by adding 8 DP alleles still failed to recognize this epitope as a strong binder (84). This indicates that HLA binding algorithms cannot accurately predict all T-cell epitopes and should always be validated with experimental work.

The epitopes in domain II of PE38 were eliminated by deletion of the whole domain, except for the 11 amino acid furin cleavage site, which does not contain an epitope. To modify the epitopes in domain III, alanine scanning was used to identify amino acids that impact the T-cell epitopes. To ensure that the point mutation did not introduce a new T cell epitope PBMCs were stimulated with the mutant RIT and re-stimulated with the mutant peptides. Two of the epitopes (epitopes 2 and 6) were difficult to solve by alanine scanning, because the mutations caused loss in activity. To aid with that, Rosetta computational protein design methods was combined with an HLA binding algorithm to identify mutations that disrupt the binding to HLA II molecule and as the same time still maintained cytotoxic activity (85). Epitope 2 (in domain III) was not resolved using a single point mutation and required a combination of two-point mutations to diminish the T cell responses significantly (R494A and R505A). However, the cytotoxic activity was reduced 2- to 3-fold by one of these mutations (**Table 2**).

The six point mutations designed to remove or suppress T cell epitopes were combined into new RITs. LMB-T18 targets

CD22 (65), LMB-T20 (**Figure 1G**), targets mesothelin, HN3-T20 targets GP3 (70), and LMB-273 targets BCMA (69). Each protein contains the mutated toxin as shown in **Table 2**. Re-analysis of LMB-T20 for T cell activation showed that cryptic or new epitopes did not emerge as a result of altered antigen processing in LMB-T20 (86).

Interestingly, when the de-immunized toxin used to make a RIT that targets human CD25 to kill human T-cell malignancies, the deletion of domain II significantly impaired the cytotoxic activity (67, 68). The dependency on domain II for cytotoxic activity is receptor specific and probably attributable to a variable internalization pathway. To improve the cytotoxic activity of CD25-targeting immunotoxin, PE38 was de-immunized with three more mutations in domain II (**Table 2**).

B and T Cell De-immunized Immunotoxin

Intriguingly, two of the mutations intended to eliminate T cell epitopes are the same mutations that diminished binding to B cell epitopes. Both (R505A and R427A) have very high accessible surface area (150 and 142 Å, respectively) indicating these arginines are located on the surface of the molecule. Since B cell epitopes are known to contain bulky hydrophilic amino acids like arginine (87–89), it is not surprising that mutations that diminish T cell epitopes also diminish B cell epitopes. Other reports have shown that important epitopes may be shared by B and T cells (90–92), and a functional link between B and T cells that recognize overlapping peptides has been suggested (93).

To reduce reactivity with both B and T-cells, the mutations that eliminated T- and B-cell epitopes were incorporated into a single RIT that targets mesothelin (66). The final RIT (LMB-T14) (**Figure 1H**) has good cytotoxic and anti-tumor activity vs. human cell lines, patient-derived cells, and mouse tumor models. LMB-14 has reduced binding to serum from patients who developed antibodies compared to its unmutated parental immunotoxin. Unexpectedly, remapping of T-cell epitopes of LMB-T14 revealed that two mutations, that were introduced to eliminate conformational B-cell epitope, created a new T-cell epitope. This demonstrates the challenging balance between cytotoxic activity, B-cell and T-cell reactivity during de-immunization (66).

Translation of De-immunization Effort (Immunogenicity of LMB-100)

The effectivity of T cell de-immunization efforts has not yet been tested in clinical settings. However, LMB-100, a B cell de-immunized RIT, has been tested in a recent trial.

It is difficult to compare the immunogenicity rate in this study to previous ones due to significant variation in the immunogenicity monitoring assays. While the immunogenicity response against SS1P was mostly monitored using a functional Nab assay, immunogenicity response against LMB-100 was monitored using an ADA bridge ELISA. Furthermore, blood half time concentration cannot be compared due to differences

in dose, size and structure that can impact half time regardless of immunogenicity. Lastly, the clinical design of the SS1P study excluded patients who had elevated pre-existing antibodies to SS1P, presumably due to prior exposure to *Pseudomonas aeruginosa*, while 9/20 patients in the LMB-100 trial had pre-existing ADA.

To try and compare SS1P and its de-immunized counterpart, Alewine et al. compared the number of patients with effective RIT C_{max} levels (>100 ng/ml). They noted that more than half of patients receiving a second cycle of LMB-100 had detectable plasma drug concentrations. These results compare favorably with SS1P, for which more than 90% of the patients had undetectable drug levels by the start of cycle 2, after excluding patients with high preexisting antibodies (94, 95). This clearly indicates that LMB-100 de-immunization decreased the impact of immunogenicity. However, this improvement was only enough to allow one additional dose on the second cycle for most patients. Only a single patient was ADA negative after the completion of the therapy. We conclude that humanization of the antibody and silencing of the B cell epitopes (and some of the T cell epitopes) is helpful, but not sufficient to completely prevent an immune response. Future work is required to evaluate if the complete T cell de-immunized molecules (LMB-T20 and LMB-T14) are more effective in diminishing the immune response.

SUMMARY AND CONCLUSION

In this review, we described various methods to monitor the immune response against RITs and efforts made to minimize the immunogenicity response in patients by combination therapy or rational design. LMB-100 is the first humanized and de-immunized RIT that was rationally designed for reduced B cell epitopes and evaluated in patient. Although it showed lower rates of immunogenicity compared to its parental RIT (SS1P), formation of ADA and Nab was delayed but not eradicated. Future work will require evaluation of novel approaches like elimination of both the B and T cell epitopes or combination therapy of immune suppressive agents and the de-immunized RIT.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of Interest: Drs. IP and RM are inventors on patents describing how to make less immunogenic immunotoxins. These patents have all been assigned to NIH.

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T-Cell Dependent Immunogenicity of Protein Therapeutics Pre-clinical Assessment and Mitigation–Updated Consensus and Review 2020

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Immune responses to protein and peptide drugs can alter or reduce their efficacy and may be associated with adverse effects. While anti-drug antibodies (ADA) are a standard clinical measure of protein therapeutic immunogenicity, T cell epitopes in the primary sequences of these drugs are the key drivers or modulators of ADA response, depending on the type of T cell response that is stimulated (e.g., T helper or Regulatory T cells, respectively). In a previous publication on T cell-dependent immunogenicity of biotherapeutics, we addressed mitigation efforts such as identifying and reducing the presence of T cell epitopes or T cell response to protein therapeutics prior to further development of the protein therapeutic for clinical use. Over the past 5 years, greater insight into the role of regulatory T cell epitopes and the conservation of T cell epitopes with self (beyond germline) has improved the preclinical assessment of immunogenic potential. In addition, impurities contained in therapeutic drug formulations such as host cell proteins have also attracted attention and become the focus of novel risk assessment methods. Target effects have come into focus, given the emergence of protein and peptide drugs that target immune receptors in immuno-oncology applications. Lastly, new modalities are entering the clinic, leading to the need to revise certain aspects of the preclinical immunogenicity assessment pathway. In addition to drugs that have multiple antibody-derived domains or non-antibody scaffolds, therapeutic drugs may now be introduced via viral vectors, cell-based constructs, or nucleic acid based therapeutics that may, in addition to delivering drug, also prime the immune system, driving immune response to the delivery vehicle as well as the encoded therapeutic, adding to the complexity of assessing immunogenicity risk. While it is challenging to keep pace with emerging methods for the preclinical assessment of protein therapeutics and new biologic therapeutic modalities, this collective compendium provides a guide to current best practices and new concepts in the field.

Keywords: immunogenicity, protein therapeutic, T-cell, biologic, monoclonal, enzyme-replacement, anti-drug-antibody

INTRODUCTION

Immunogenicity of Biotherapeutics: Historical Context

Immunogenicity is a term that is used in the biotherapeutic industry to describe undesired immune responses to protein or peptide drugs. Immunogenicity is driven by components that are intrinsic to the product (such as protein sequences integral to the drug itself), to host cell proteins that hitchhike along with the drug as it is purified, or to factors such as excipients that are related to drug formulation. Immunogenicity is also dependent on engagement of the individual patients' immune system and genetic factors that may pre-determine and shape their immune response.

While immunogenicity is often measured in terms of “anti-drug-antibodies” or ADA, obtained from clinical samples, the role of T cells that recognize drug-derived sequences presented on highly variable Human Leukocyte Antigens (HLA), is critically important to determining the immune response of any given subject (Figure 1). Both the individual patient's HLA haplotype and their personal B and T cell repertoire contribute to their individual immune response, leading to a high degree of patient-to-patient variability. Due to inherent variability in the immune systems of each individual patient (and, due to the imperfect means by which this response is measured), they may have no ADA at all, or they may have binding or neutralizing ADA that may reduce the efficacy of the drugs.

The immunogenicity to a therapeutic protein can be associated with hypersensitivity related reactions. Type 1 hypersensitivity is accompanied by ADA of the IgE isotype. Both IgE and high IgG ADA titers may contribute to significant adverse effects including infusion reactions and/or anaphylaxis, although these types of adverse effects are uncommon. ADA-IgE complexes can bind and cross link the Fcε on basophils and mast cells, leading to IgE-mediated anaphylaxis. In addition, IgG ADA can complex with the therapeutic protein and these immune complexes can cross-link Fcγ receptors on neutrophils, releasing platelet activating factors that resemble histamine. Furthermore, large therapeutic-ADA complexes that fail to get cleared precipitate in the tissues like kidneys, synovial membrane and choroid plexus leading to tissue damage and organ failures (1, 2).

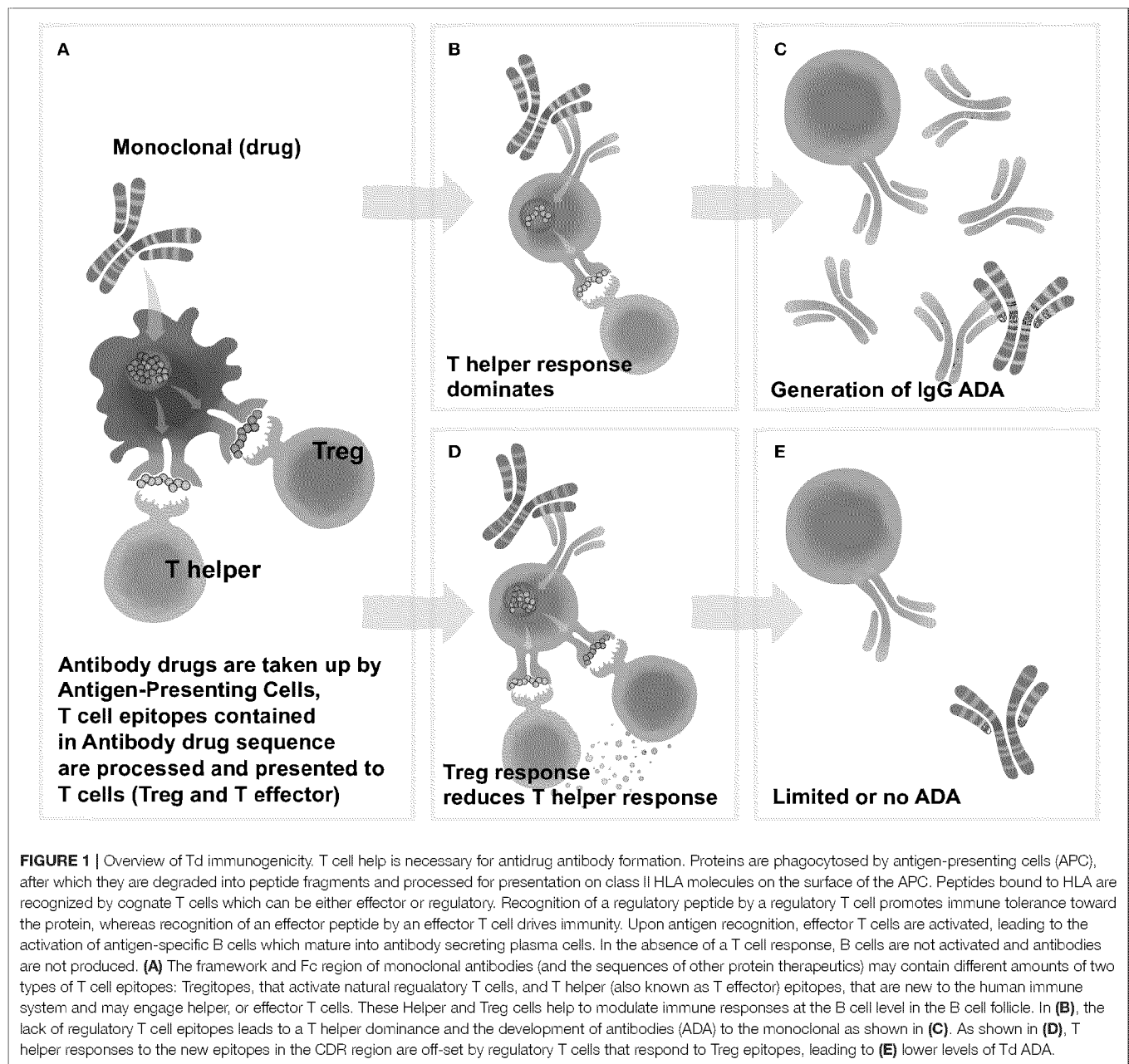
Abbreviations: AAV, Adeno-Associated Virus; ADA, Anti-Drug Antibodies; ADC, Antibody Drug Conjugates; APC, Antigen Presenting Cell; API, Active Pharmaceutical Ingredient; CAR, Chimeric Antigen Receptor; CDR, Complementary Determining Region; CPI, Check Point Inhibitors; CTL, Cytotoxic (Usually CD8+) T cells; DAMP, Danger Associated Molecular Patterns; DC, Dendritic Cell; EPO, Erythropoietin; HCP, Host Cell Protein; HLA, Human Leukocyte Antigens; IND, Investigational New Drug application; IgG, Immunoglobulin G; IIRMI, Innate Immune Response Modifying Impurities; ISPRI, Interactive Screening and Protein Reengineering Interface; GAA, Acid Alpha Glucosidase; MAPPS, MHC-Associated Peptide Proteomics; MHC, Major Histocompatibility Complex; moDC, Monocyte-derived Dendritic Cells; NLR, NOD-Like Receptors; PRCA, Pure Red Cell Aplasia; PRR, Pattern Recognition Receptors; RA, Rheumatoid Arthritis; TCR, T Cell Receptor; Td, T cell dependent antibody response; Thelper, CD4+ helper T cells; Ti, T cell independent antibody response; TLR, Toll Like Receptors; Treg, Regulatory T cells; Tregitope, Regulatory T cell epitope in IgG.

The most significant adverse events occur when ADA are cross-reactive with endogenous protein homologs. For instance, cases of pure red cell aplasia (PRCA) that were attributed to ADA developed unexpectedly after years of administration of recombinant erythropoietin (EPO) to patients without the development of any previous significant immunogenicity issue (3–6). These ADA were attributed to modification of the formulation and route of administration of EPO. PRCA was also recently observed during a clinical trial of a generic EPO developed by Novartis, and in this case was attributed to product aggregation induced by tungsten microparticles that were found in some lots of the drug product (4, 7). Other clinically significant adverse events related to ADA that cross-reacted with endogenous proteins include: neutralizing ADA caused by aggregates present in the formulation of human growth hormone (8), and ADA due to the presence of residual host cell proteins (HCP) in recombinant therapeutic products such as Factor VII (9).

These types of serious outcomes resulting from cross-reactive ADA have inspired the development of a wide range of *in vitro* methods for measuring the presence of ADA, which have been described in several white papers and regulatory guidance documents (10–17), including one on T-cell dependent immunogenicity published by our group in 2013 (19). In addition, methods for identifying drivers of immune responses to monoclonal antibodies and host cell proteins have also expanded and have been described in a number of publications (16, 20–29) and reviews (30) over the past few years.

As a result of these historical outcomes, regulatory agencies have asked drug developers to use a structured approach to measuring immunogenicity risk for biotherapeutics developers. For example, the European Medicines Agency (EMA) has published a “Guideline on Immunogenicity Assessment of Biotechnology-Derived Therapeutic Proteins” (17, 18) in which factors influencing the immunogenicity of therapeutic proteins were classified into helpful *patient-, disease-, or product-related* categories (see below). In addition to the EMA guidance, recent FDA guidelines for new drug products and generic versions of existing products have also suggested immunogenicity risk assessment approaches. See for example, the 2014 FDA guidance “Guidance for Industry: Immunogenicity Assessment for Therapeutic Protein Products” (31). This guidance highlights the contribution of T cell epitopes to immunogenicity and also mentions immune modulation attributed to regulatory T cells (22). Furthermore, many of the factors that might predispose a therapeutic protein to be immunogenic have been identified as “critical quality attributes” in the FDA-sponsored Quality-by-Design initiative (32) focused on manufacturing “process development.”

A recently published guidance for synthetic peptide drugs continues the regulatory guidance trend, expressly identifying the importance of T cell responses (33). Here, the Office of Generic Drugs at the FDA has suggested that immunogenicity assessment should extend to synthesis-related impurities, and asks peptide drug developers to evaluate whether impurities that may be co-purified with the active pharmaceutical ingredient (API) contain



T-cell epitopes. These recommendations extend to five generic drugs but could be expanded to other novel peptide drugs, and to new generic drugs that enter the generic development pathway.

For peptide or protein-based drugs, the primary amino acid sequence itself can be a strong determinant of immunogenic potential. Beyond the primary sequence, agency guidelines point to *patient-* and *disease-related* categories that may pre-dispose a particular individual to an immune response (34). Examples include immune deficiency and concomitant immunosuppressive treatments such as methotrexate, which may decrease immunogenicity, and autoimmunity, which may increase the risk of ADA. In contrast, *product-related factors*, i.e., factors intrinsic to the final drug product itself that contribute to

immunogenicity, may include modifications in the glycosylation profile (35–37), biophysical and biochemical attributes (10, 38–40), peptide manufacturing impurities and/or degradation products, or factors introduced during formulation (17, 28, 41, 42). Clearly, regulatory guidelines and updated preclinical immunogenicity risk assessment approaches are converging on a consensus, providing impetus for this review of the current state of the art.

Focus on Td Immunogenicity Assessment and Mitigation

While immunogenicity is measured by testing for ADA, the root cause is T-cell dependent (Td) immune response, whether

the driver is aggregates, host cell proteins, impurities, immune modulation due to target engagement or the sequence of the drug itself. Thus, **Td immunogenicity risk assessment** focuses on peptides known as T cell epitopes that may be derived from the sequence of the product (whether protein or peptide). Here we will focus on the biologic drug itself; host-cell proteins and other impurities that may be present in the drug product will be addressed in later sections.

Certain drug-derived peptides/epitopes may bind to human leukocyte antigen (HLA)/major histocompatibility complex (MHC) class II molecules, and the peptide/MHC complex is then presented to T cells on the APC cell surface (19, 22). More specifically, T cell epitopes that are processed and derived from the drug substance of the type known as *T helper* epitopes, are critically important to the development of ADA. The T helper epitopes are presented by a subset of HLA class II molecule (predominantly HLA DR but also DP or DQ) to CD4+ T cells which then provide the essential cytokines for B cell maturation and affinity maturation of the ADA. These interactions occur in the germinal center of lymphoid organs, where dendritic cells and B cells present T cell epitopes to T follicular helper cells and T follicular regulatory cells, which regulate the maturation of humoral immune response (43).

Just as identification of T helper epitopes is central to the process of immunogenicity risk assessment, removal of T cell epitopes; a process known as de-immunization, is key to Td immunogenicity risk mitigation. De-immunization is a process that is now entirely integrated into preclinical programs focused on mitigating Td immunogenicity risk. T cell epitopes that reduce immunogenicity, known as regulatory T cell epitopes, are equally important to immune responses to protein drugs that contain “human” components such as human-derived monoclonal antibodies, enzyme replacement therapies, and other human-origin biotherapeutics. Circulating regulatory T cells (Tregs) known as natural Tregs (nTregs) contribute to regulation of the human immune response and are also known to be epitope-specific (44, 45). The discovery of regulatory T cell epitopes known as Tregitopes in Immunoglobulin G (IgG) (46) improved risk assessment for monoclonals (47). The original IgG Tregitopes were published in Blood (22) others were published in Scientific Reports (48) IgG Tregitopes and “non-IgG” Tregitopes have also been identified in the patent literature (49). Discovery of regulatory T cell epitopes has now expanded beyond immunoglobulin, and is already improving the immunogenicity risk assessment of newer biotherapeutics as well.

A T-cell dependent immune response can drive an affinity matured anti-idiotypic response. Such a mature response driven by long term dosing can impact exposure, efficacy and safety as evidenced in enzyme replacement therapies and clotting factor proteins where immune response can not only lead to loss of exposure and efficacy but can have safety concerns due to cross reactivity to endogenous proteins or lack of other treatment alternatives. Some key examples of formation of neutralizing antibodies associated with loss of response are antibodies to FVIII/FVII and TNF inhibitors leading to loss of response (50–52). Safety concern key examples include development of IgE antibodies to cetuximab associated with

anaphylaxis (53), antibodies to EPO associated with pure red cell aplasia (54) and antibodies to MGDF/TPO leading to thrombocytopenia (6).

In summary, a T cell-focused approach to the mitigation of immunogenicity emerged by 2010, leading experts to codify existing Td approaches to immunogenicity assessment and mitigation in the first version of this Td immunogenicity “white paper” (19). Almost a decade later, new concepts have emerged, and new modalities are in the clinic, and it is time to update and review Td immunogenicity.

Definitions: T-Dependent Immune Responses to Biotherapeutics Self vs. Non-self

Before addressing Td immune responses to biotherapeutics in further detail, it is helpful to remember that immune responses to these drugs can be divided into two broad categories. The first category would include what are considered to be “foreign” proteins (foreign to the patient), and the immune response to these proteins is typical of responses elicited against pathogens, vaccines, or allotypic antigens. Blood factors such as Factor VIII fall in this category since they are developed for individuals who are lacking, in whole or in part, the endogenous counterpart. This is also true for replacement enzymes such as acid alpha glucosidase (GAA), for Pompe disease. The second category of biotherapeutics involves autologous proteins (“self”), and thus “immunogenicity” to these proteins suggests a breach of B and/or T cell tolerance, similar to the response elicited to autologous self-proteins in certain autoimmune diseases.

Self-tolerance is actively regulated by circulating regulatory T cells (**Figure 2A**). These T cells respond to sequences in self proteins such as immunoglobulin, that may be identical in HLA binding features to non-self epitopes, but respond differently to activation of their T cell receptor (TCR). For example, regulatory T cells secreting IL-10 in response to HLA DR-restricted T cell epitopes in IgG have been identified by Franco and Sette (55) in immunoglobulin-treated subjects with Kawasaki’s disease, and IL-10 responses (which may be due to Treg activation) have also been recorded in patients treated with infliximab to specific T cell epitopes derived from infliximab (56). Close inspection of peptide sequences eluted from antigen presenting cells that have been pulsed with monoclonal antibodies such as infliximab confirms the presence of many published and unpublished regulatory T cell epitopes known as Tregitopes, and these peptides do not elicit T cell responses (other than regulatory T cell responses) *in vitro* (57). See, for example, **Figure 2B** for an illustration of the location of Tregitopes (green) in two well-known monoclonal antibody drugs [infliximab (Remicade) and adalimumab (Humira)]; both monoclonals target TNF. Adalimumab (Humira) has fewer T cell epitopes and more Treg epitopes and is less immunogenic in the clinic (58).

Given that many self proteins such as monoclonals appear to contain regulatory T cell epitopes, the means by which breach of immune tolerance occurs is not as well-defined as the mechanisms for immune response to foreign proteins, but may include epitope mimicry, cross-reactivity of T cells, presence of

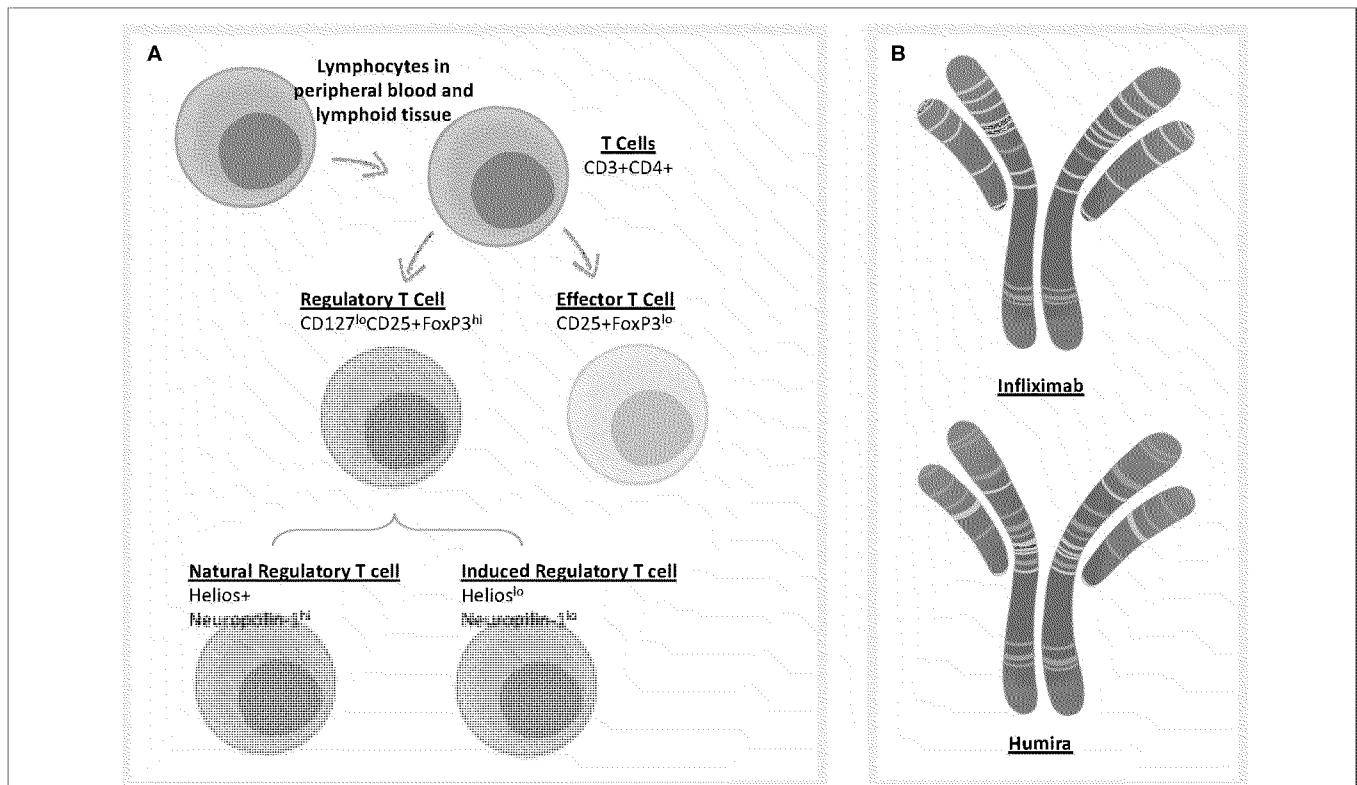


FIGURE 2 | Treg epitopes, T effector epitopes, and T cell types. **(A)** Categories of T cells that are involved in immune responses to biotherapeutics. CD4 T cells (also known as T helper cells) orchestrate T-dependent anti-drug antibody responses. One of the best methods to differentiate the types of T cells that respond to biotherapeutics is to use flow cytometry, in which the surface and internal markers are used to differentiate categories that correlate with function. There are two types of T cells that can respond to biologics, including Regulatory T cells or “Tregs” (characterized by low levels of the cell surface marker CD127 and high levels of the internal marker FoxP3); and Effector T cells that are CD25 intermediate, FoxP3 low. Regulatory T cells can be further divided into natural regulatory T cells that are trained in the thymus, and induced (iTReg) Tregs that can be induced in the periphery. Each cell type has characteristic cell surface markers. **(B)** Categories of T cell epitopes found in biotherapeutics. The framework and Fc region of monoclonal antibodies may contain different amounts of two types of T cell epitopes: Tregitopes, that activate natural regulatory T cells, and T effector epitopes, that are new to the human immune system and may engage helper, or effector T cells. Two types of anti-TNF monoclonals are shown, with colored lines representing the approximate location of Treg epitopes found in their sequence in green and T effector epitopes in yellow [analysis by Rob Ventura of EpiVax, using the ISPRI Toolkit (157)].

trace levels of innate immune activators such as toll-like receptor agonists (42, 59, 60), and/or aggregated proteins (61). Genetic variations in Toll Like Receptors (TLR); polymorphisms in co-stimulatory molecules, modifications to cytokine receptors, and more, are likely to be involved in “breach of tolerance.” Patients who have autoimmune diseases may have some of these genetic anomalies and can be considered higher risk for developing ADA (62–65).

T-Independent vs. T-Dependent

Beyond regulation by T cell responses, humoral immune responses such as ADA can be thymus independent [T cell independent, (Ti)] rather than Td in origin (66, 67). For example, B cells may be activated in a Ti manner when particular structural patterns, such as polymeric repeats or carbohydrate molecules, directly activate B cells via the B Cell Receptor (BCR). Ti activation of B cells can be distinguished from Td activation, as the antibodies resulting from Ti activation are limited in both isotype and affinity and if memory B cells are generated, they

are not long-lived (68, 69). In contrast, Td activation of B cells is characterized by class switching (IgM to IgG) and development of memory B cells that produce higher-affinity, more robust, and longer-lived antibody responses. The development of IgG-class antibodies following administration of a biotherapeutic generally indicates that the therapeutic is driving a Td immune response.

Td responses, by definition, are contingent upon T cell recognition of therapeutic protein-derived epitopes through the basic processes of protein antigen processing and presentation. Since human populations express a number of different HLA class II alleles, the interaction between antigenic epitope and HLA may exhibit a range of binding stabilities across the spectrum of HLA alleles expressed in the human population. This HLA genetic polymorphism and its consequent impact on the binding of specific peptides (HLA restriction) is the primary mechanism by which patient genetics (HLA haplotype) becomes a major determinant of immune responses to particular protein therapeutics.

Innate Immune Response

The innate immune system controls the initiation of Td immunity. Innate immune cells termed antigen presenting cells (APCs), upon activation in the periphery, migrate to the local lymph node where they can present drug-derived peptide antigens to antigen specific helper T cells in the presence of the proper co-stimulatory signals. Unlike the specific nature of the T and B cell receptors, cells of the innate immune system express germline encoded receptors termed pattern recognition receptors (PRRs) that recognize common microbial motifs (pathogen associated molecular patterns, or PAMPs). PRRs include several families of receptors such as Toll-like receptors (TLRs), RIG-1 helicases and C-type lectin receptors (42, 70).

In addition to recognizing microbial patterns, PRRs can also recognize a class of alarm signals called alarmins or danger associated molecular patterns (DAMPs) that are released in large quantities by stressed and dying cells to promote a localized inflammatory response. While DAMPs evolved to help combat pathogens, tissue damage, and stress that occurs during administration by a protein therapeutic can lead to DAMP-mediated inflammation and promotion of the adaptive immune response. Additionally, host cell and process derived impurities termed innate immune response modifying impurities (IIRMI) can stimulate the innate immune system through interactions with PRRs promoting adaptive immunity. *In vitro* and *in vivo* studies have shown that IIRMIs, even at trace levels, can break tolerance to therapeutic proteins and promote an unwanted immune response (42).

New modalities such as Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) therapies, characterize the intersection between innate and Td immunogenicity in drug development. Firstly, these new delivery systems, may engage natural Toll-like receptors that are activated by RNA or DNA, and, second, they may be delivering a payload that is inherently immunogenic, not only by driving T help and antibody generation but also by driving adaptive T cell responses that eliminate the transduced target cells.

NEW CONCEPTS: PATIENT- AND DRUG-SPECIFIC IMMUNOGENICITY

Careful observation of the field over the past 5 years has contributed to the emergence of important new concepts in Td immunogenicity. These include observations related to the immune state of the patient receiving the biotherapeutic and the mechanism of action, or target of the biotherapeutic. For example, treatments targeting cardiovascular disease subjects are generally less likely to be associated with increased ADA, whereas autoimmune disease subjects may present with a spectrum of immune dysfunctions that can lead to increased propensity for anti-therapeutic response. Alternatively, some populations of patients may have unusual HLA distributions that are linked to greater presentation of T effector epitopes derived from the drug sequence, leading to higher or lower levels of immunogenicity. Lastly, the mechanism of action of the drug itself may interfere

with, or promote, the activation of the immune system, leading to higher or lower risk of immunogenicity. Each of these topics is discussed in the next few sections.

Patient-Specific Determinants of Immunogenicity Disease Status

It is not uncommon to see one to two individuals per 100 that have higher baseline immune responses than others; these higher risk individuals may also have exaggerated immune responses to the delivery vehicle as well. The baseline immune status of a subject (including as described above, B and T cell repertoire as well as HLA haplotype) can influence their ability to mount an immune response to a biologic. Tsang et al. (71) have established that such differences can influence the outcome of immune responses to the therapeutic proteins through an in-depth analysis of immune parameters associated with PBMC, frequency of cell populations, serum levels of chemokines and proteins indicative of immune activation. Also as described above, biotherapeutics may be more immunogenic in autoimmune disease patients due to the underlying inflammatory status of the recipient patient's immune system.

In years past, drugs that targeted patients who have auto-immune diseases included anti-TNF agents, which had remarkably different immune profiles in selected patient populations. A systematic review by Thomas (72) illustrates the variability of biotherapeutics in the context of Rheumatoid Arthritis (RA): the most immunogenic were infliximab (25.3%), followed by adalimumab (14.1%) and certolizumab (6.9%). These rates of immunogenicity are significantly higher than those reported for the same drugs in patients who have ankylosing spondylitis, which may either reflect the immune status of the patients or the HLA-skewing of select auto-immune diseases.

Explanations for the increased level of ADA in RA and autoimmune patients vary, however, such patients may have defective regulatory T cells (73–76) or lack functional regulatory T cell cytokine receptors (IL-2, IL-10) (77–79). Perturbation in the function of regulatory T cells or of regulatory cytokines that are critical for Treg function, may dramatically decrease Treg response to drugs that contain Tregitopes, which include many of the monoclonals that are used to treat autoimmune diseases. Drugs such as methotrexate and TNF-inhibitors have been shown to restore Treg function, potentially reducing ADA once the drug is at therapeutic levels (80). This is one potential explanation for the observation that ADA tend to be higher in patients who have, active, flaring RA; and may also explain why ADA may disappear with effective anti-inflammatory drug treatment (81).

Clearly, the immune system can be modulated by anti-inflammatory treatments (see also Tolerance induction section). Clinicians and drug developers may benefit from collaboration so as to improve the proactive assessment of immunogenicity in the context of autoimmune disease. Collaboration will enable personalized treatments and better clinical decisions based on improved awareness and detection of immunogenicity risk factors.

HLA

ADA measurement has further limitations with lack of reliability during dosing timepoints. The most optimal way to support the translatability of the algorithm and T cell based predictive assays is to correlate with T cell responses in the dosed donors. Several recent studies have shown the direct association of a mature ADA response with presence of therapeutic specific T helper cells (82–84). The algorithms used to identify sequence-based risks can provide the first glimpse of HLA types in a population that would be at risk to bind the non-self epitopes in a protein. Based on the prevalence of the HLA types for a geographical location, the risk for immunogenicity in clinic can be modeled. Additionally, HLA typing of subjects being enrolled for clinical trials can help track if the ADA responses are associated with the HLA that were predicted to be at risk. Indeed, the past decade has been marked by a flurry of publications related to the association of certain HLA class II alleles with immunogenicity risk for selected biologic therapeutics. Buck et al. (85) demonstrated that HLA-DRB1*04:01 and HLA-DRB1*07:01 multiple sclerosis patients exhibit an increased risk for developing neutralizing antibodies to IFN β . A similar association of HLA-DRB1*04:01 and HLA-DRB1*15 carriage with a higher risk of ADA development to IFN β -1b and IFN β -1a, respectively, was identified by Link et al. (86). Increased risk of ADA development to infliximab was also observed for HLA-DRB-11, HLA-DQ-03, and HLA-DQ-05 carriers in rheumatoid diseases (87) and HLA-DRB1*03 inflammatory bowel disease patients (88) and two risk alleles (HLA-DRB1*03 and HLA-DRB1*011) and three protective alleles (HLA-DQB1*05, HLA-DRB1*01, and HLA-DRB1*07) were described for various anti-TNF α in rheumatoid diseases patients (88, 89). Larger databases of patient data may reveal greater numbers of HLA-associations and may also simply confirm that all HLA class II molecules, rather than just one or two, perform the critical function of presenting T cell epitopes to the immune system in drug-exposed subjects.

Microbiome

Recent studies have confirmed the long-standing hypothesis that the human gut is inhabited by microbiota that can have a strong impact on host immune responsiveness. On the one hand, the immune system, including T cells that may bear TCR for novel epitopes found in biotherapeutics may be tolerized to the commensal pathogens due to presence of the Toll-like receptors (TLRs) on the epithelial and lymphoid cells of the small intestine that suppress any inflammatory responses and maintain intestinal homeostasis (90). On the other hand, the microbiota in the gut can influence the differentiation of the Th cell subsets that maintain homeostasis. In addition, NOD like receptors (NLRs) can also recognize the microbial organisms and modulate the immune responses of T cells to avoid inflammation. If the therapeutic T cell epitope sequence contains sequences that resemble sequences from the genome of the microbiota, the risk of mounting an immune response may be higher (if T effector epitopes are conserved) or lower if regulatory T cell epitopes are conserved with the drug (91). The influence of gut microbiota in individuals from geographical

regions with a higher exposure to environmental pathogens vs. those from urban environments, and in individuals who have taken antibiotics prior to being treated with biologic therapeutics certainly deserves careful consideration by the immunogenicity risk assessment community.

Drug Function as a Determinant of Immunogenicity

With the emergence of immune-system-targeting biotherapeutics, it has become clear that the actions of the drug itself can also contribute to, or modulate immunogenicity. This was posulated to play a role in the activity of anti-TNF agents due to the impact of TNF on regulatory T cells, as described above. Improved Treg function as a result of anti-TNF therapy may lead to reductions in ADA to anti-TNF agents over the course of time (81, 92). Similarly, IL-2, a cytokine that is required for the function of regulatory T cells may not only induce a pro-regulatory environment but could also reduce the likelihood of ADA developing to the drug. This mechanism may contribute to the effectiveness of low-dose IL-2 therapy in autoimmune disease (93). Conversely, IL-2 is also capable of enhancing the function of effector T cell responses and has been used at high doses in the treatment of viral and oncological disease (94).

Teriparatide, a peptide drug, provides yet another illustration of target effects. It elicits cytokine release from T cells (e.g., TNF α , IL-1, and IL-6) as well as IL-2 (95, 96). Thus, Teriparatide may exert a direct effect (both pro-inflammatory and anti-inflammatory) on the immune system. And check point inhibitors, the newest class of biotherapeutics to hit the clinic, can directly interfere with immune response and contribute to immune response, potentially increasing immunogenicity as described in the next two sections.

Drug Target and Immunogenicity: Checkpoint Inhibitors

Some drugs, such as check point inhibitors (CPI), are used to enhance immune responses. As a result, checkpoint inhibitors have been proven to be successful in the treatment of aggressive cancers, and some of them are also more immunogenic than expected, potentially leading to loss of efficacy with continued treatments. One hypothesis is that their actions reduce the tolerizing effect of natural Tregitopes that may be present in the sequence of the checkpoint inhibitor drug and/or enhance effector T cell responses to foreign epitopes in the drug sequence. In line with the inhibition of immune inhibitory pathways, Treg depletion and a toxicology profile of decreased self-tolerance that is observed with CPI treatment, selected checkpoint inhibitors Atezolizumab (anti PD-L1) are associated with markedly higher ADA (39.1–48%) than would normally be expected given their fully human IgG framework.

The enhancing effect on immunogenicity appears to be especially salient when the drugs are used in combination. For example, the immune response to Nivolumab in monotherapy was 12%, however it was significantly increased to 24–38% when Nivolumab was dosed in combination with Ipilimumab (97). Combination therapies with checkpoint inhibitors like Pembrolizumab (Keytruda) and Nivolumab (Opdivo) and small

and large molecule T cell modulatory targets like CTLA 4, Lag3, TIGIT, GITR, etc. have not only shown improved efficacy as noticed by tumor regression and long term survival but may also have the potential for demonstrated synergistic immunogenicity when used in combination (98–103).

Despite these observations, some checkpoint inhibitor monotherapies have demonstrated standard rates of immunogenicity (1–10% ADA); for example (97). The reason for these differences is as yet unexplained, but may be due to differing degrees of “intolerance” specific to the actions of the molecule that is the target of the CPI, as well as attributes that improve processing and presentation of the drug itself, in the inflamed tumor or draining lymph nodes. Technical limitations of ADA assays (104–106) might also contribute to differences in ADA incidences in the immuno-oncology field. In summary, while checkpoint therapeutics may reduce tolerance to tumors, they also appear to enhance the likelihood of T-cell driven immune response of the biotherapeutics especially when administered in combination.

Drug Target and Immunogenicity: Anti-inflammatory Cytokine Inhibition

In contrast with CPI, certain anti-cytokine agents are known to be much less immunogenic than expected. One such drug is an anti-IL-6 biologic, known as Tocilizumab, a drug that is now widely used in RA and in other autoimmune diseases (107). Notably, IL-6 is required for T cell activation, thus, interference with IL-6 may reduce T engagement and thereby reduce ADA. Another example of a drug that may directly interfere with immunogenicity is Rituximab, which targets CD20 on developing B cells and reduces the formation of antibody secreting plasma cells, which may explain why ADA are not generally detected for this drug.

NEW MODALITIES

New means of delivering drugs such as via gene therapy (DNA, RNA) or encoded in a vector for delivery (108) may engage new types of immune response. For example, unexpected anti-drug CD8 T cell (HLA Class I-restricted) responses to biotherapeutics have been described recently. Specifically, therapeutic anti-CD19 CAR-T cells were destroyed by CD8 T cells that targeted murine sequence-derived T cell epitopes in the transgene, abrogating the efficacy of the CAR-T for several patients (109). Drugs that enter cells and are expressed by them (such as viral-vector mediated monoclonal antibodies) may be interfacing with cell mediated immune responses leading to unanticipated immunogenicity and, potentially, failure in the clinic.

Biologic Therapy by Viral Vector

Next generation viral and cell-based therapies are now being diverted from the gene therapy market to deliver modalities that target solid tumors directly. Additionally, antibody drug conjugates (ADC) which are antibodies or alternative scaffolds, delivering small molecules like toxins or cell inhibitors conjugated to antibodies, are being used to target tumors. In addition, viral mediated transduction of antibodies and cytokines

is being used to express the foreign transgenes in relevant cells, to enhance T-cell mediated killing. When viral vectors are used to deliver drugs, the impact is similar to a viral infection, engaging both CD8 T cell responses as well as CD4 T cell and antibody responses. Furthermore, the products may not be entirely pure, and thus hostcell proteins or impurities may be responsible for driving the immune response, not the sequence of the drug itself.

Gene Therapy

Immunogenicity to gene therapy can be challenging to address, and has the potential to limit efficacy. Viral-based delivery may be intrinsically immunogenic because they contain T cell epitopes that drive T-cell mediated elimination of transduced cells, as was the case with adeno-associated vectors (AAV) (110). Both pre-existing antibodies or T cell responses to the viral delivery vector can neutralize the delivery of the viral vectors, and some clinical studies have exclusion criteria based on pre-existing anti-vector antibodies. Switching to different viral isotopes or engineering of the viral vector surface proteins is further complicated by the tissue selectivity of the vector, which may also be required for effective gene delivery (111).

The transgene [the intended drug product, such as a monoclonal antibody or a replacement protein (blood factor, other) which will be expressed in the patient's body] can also be the target of immunogenicity, and immunogenicity is not limited to ADA, especially if the transgene is intended to replace a defective (or absent) gene, which may lead to recognition of the transgene as a foreign protein. T cell responses to the transgene can include HLA class I mediated CTL response to the intracellular product of the gene therapy, damaging the tissue that expresses the transgene and leading to loss of functional gene therapy product (112). Thus, consideration of both HLA class I and HLA class II-restricted epitopes is required for immunogenicity assessment of the gene therapy vector and its transgene product.

T Cell Specific Oncolytic Viruses

Oncolytic viruses are administered in combination with other therapeutic proteins like checkpoint inhibitors or immune modulatory targets to actively support tumor killing by activating the immune system (113). The efficacy of oncolytic viruses can however be impacted by development of neutralizing immune response to viral capsids as well as a virus specific T-cell response. Pre-existing immune response to the oncolytic virus can reduce the efficacy of the oncolytic virus due to neutralization by anti-viral antibodies, post-dosing (114, 115).

Chimeric Antigen Receptor (CAR)-T

Autologous T cells that have been transduced with genes that express anti-tumor-specific antigens such as CD19 have been demonstrated to have significant antitumor activity in hematologic malignancies. Even though cell therapies have gained approval by US and European regulatory agencies, there are considerable immunogenicity challenges that arise during the production and administration of these personalized therapies. Both humoral and cell mediated responses can occur against unique chimeric antigen receptor (CAR) components (108).

For example, immune response may target the CAR-T due to the presence of non-human sequences in the CAR construct and suicide domain components. Immunogenicity may also be generated by residual impurities such as viral proteins or other gene editing-related non-human proteins.

CD8+ T cell-mediated immune responses have been reported after anti-CD19 CAR-T cell infusion in some patients (116). These CD8 T cell response to CAR transgene limited CAR-T cell persistence and increased the risk of relapse. In the published study, five patients that had developed persistent leukemia or relapse after an initial infusion of anti-CD19 CAR-T received a second infusion of CAR-T cells, and for these patients, there was no expansion or persistence of CAR-T cells or demonstrable antitumor activity and infusion was followed by the loss of CAR T cell population. The loss was attributed to a specific CD8 T cell response to the CAR-T; a T cell line generated from one patient showed specific CD8+ restricted autologous CAR-T cell lysis which was shown to be driven by the murine portion of the CAR-T with a peptide ELISPOT (116).

Peptide Drugs: Novel, Generic, and Peptide Impurities

Over the last several decades, important advances in peptide synthesis has contributed to a major shift in the manufacturing of therapeutic peptide drugs and an expansion in the number of novel peptides entering clinical pipelines. As for monoclonals, blood factors, and recombinant enzymes, HLA-binding sequences that are present in peptide drugs may activate regulatory or effector T cells, and therefore, peptides can be immunogenic in clinical use. The transition from fully recombinant to synthetic peptide drugs has led to increased regulatory concern about synthesis-related impurities that may induce unwanted immune responses including ADA. Regulatory experience with selected generic peptides has contributed to the development of draft guidelines for generic peptide products that was recently introduced by the Office of Generic Drugs at the FDA (33).

Immunogenicity to peptide drugs is primarily related to peptide synthesis methods that can introduce peptide impurities that may be difficult to remove from the final drug formulation. These impurities may contain novel T cell epitopes that could contribute to T cell activation (and ADA). In some cases, impurities have been associated with anaphylaxis (117). Several classes of peptide impurities can be generated at each step of the peptide synthesis process including amino acid insertions and deletions, incorporation of diastereomeric amino acids, and oxidation of amino acid R groups. In addition, impurities can arise during storage. A thorough review of impurities in peptide drugs, and where they occur in the synthesis process can be found in D'Hondt et al. "Related Impurities in Peptide Medicine" (118). Analysis of these impurities can be performed with *in silico* tools and *in vitro* assays, similar to the process described below for biotherapeutics.

Relative to T cell dependent immunogenicity, new T cell epitopes may be introduced when unintended modifications to the amino acid sequence of the drug result in impurities

that contain new HLA-binding ligands or changes to the TCR-facing contours of existing epitopes. For example, a novel GLP-1 inhibitor that was in commercial development was discontinued after the number of patients with confirmed positive anti-drug antibody tests increased from 16% at week 12 to 39% at week 24 (117); up to 5% of patients also developed systemic allergic reactions.

COMPUTATIONAL IMMUNOGENICITY RISK ASSESSMENT

In silico Screening

Current practice of immunogenicity screening generally starts with an *in silico* assessment and then proceeds to HLA binding assays, T cell assays, and MHC associated peptide proteomics (MAPPs) as needed. Some groups (57, 83, 119) start with MAPPs and do not use *in silico* tools, however, MAPPs is resource-consuming and costly. Greater experience with and familiarity with available *in silico* tools is likely to lead to greater adaptation of these tools as the first step in immunogenicity assessment in the future. This section will briefly describe available tools and highlight improvements to these tools.

T Cell Epitope Prediction

As described in section Definitions: T-Dependent Immune Responses to Biotherapeutics, ADA responses develop due to an adaptive immune response, supported by T cells responding to linear peptide epitopes displayed by HLA on the surface of APCs. For biotherapeutics delivered via conventional (exogenous, i.e., intravenous, subcutaneous, even topical) routes, presentation through the Class II pathway to CD4+ helper T cells is most relevant, however, as also discussed above, CD8+ T cell response biotherapeutics delivered by viral vectors and cell therapies is a rising concern. Fortunately, T cell epitopes can now be predicted with a high degree of confidence (A separate manuscript describing the typical approach to *in silico* risk assessment in detail has been submitted to this issue and topic in *Frontiers*).

The core residues of a T cell epitope sequence that define the affinity and stability of binding to pockets of HLA DR, DP and DQ alleles are generally nine amino acids long. Despite this fact, due to the open ended conformation of the Class II HLA binding groove, and the stabilizing effect of "flanking" residues around 9-mer core sequences, peptides reported to bind to Class II HLA and to stimulate T cell response are most often longer in length, and most web-accessible T cell epitope mapping tools parse full protein sequences into overlapping frames of 9–15 residues and report a rank, score or predicted affinity for each frame. Methods to assess the immunogenic potential of a complete protein are available on several public and academic platforms (120, 121) in some cases paired with mathematical models based on hypothetical binding affinities and T cell precursor frequencies, or with MAPPs-determined peptidomes (122–125).

Publicly available websites for epitope scanning may appear and disappear, and can also be modified, often without notification, leading to changes in immunogenicity interpretations over time. For this reason, many mid to large-pharmaceutical companies import on-line algorithms

and operate them within their firewalls to reduce the risk of intellectual property disclosure. Others use web-based tools such as the secure-access commercial-grade ISPRI toolkit. Alternatively, companies may outsource immunogenicity prediction to commercial research organizations.

Some tools such as the commercial ISPRI platform use unique algorithms and knowledge to identify Treg epitopes in monoclonal antibody sequences and provide a statistical assessment of epitope content relative to random expectations and adjusted for selfness (i.e., tolerogenic potential) (126). Direct ranking of a new biologic drug products against other known non-immunogenic and immunogenic products is possible using a normalized “immunogenicity scale.” The toolkit also features novel algorithms to search for epitope that are “human-like” (see next section) and therefore less likely to engage activated T cells, and methods for deimmunization and tolerization that can be performed directly *in silico* (127).

Screening for Self-Ness

T cells recognize not only peptide sequences, but the complex of peptide bound in the cleft of an HLA molecule. In any HLA ligand, certain amino acids are in contact with the HLA molecule itself, while others are accessible to the TCR. If TCR-facing residues from a given epitope are conserved among multiple HLA-binding sequences from the human proteome, the epitope in question may activate T cells specific to these human proteins. This may lead to a regulatory response generated by natural Tregs or to a limited or null response due to T cell anergy or deletion during thymic selection.

For many HLA alleles, the peptide positions responsible for anchoring in the HLA binding cleft are known, and other residues have been reported to interact with the TCR. Algorithms such as JanusMatrix (128) can be employed to screen predicted epitopes derived from candidate therapeutics against the human proteome to distinguish the peptides that are more self-like, and thereby likely to be tolerated, from those that have limited human cross-conservation and are thereby more likely to be recognized as foreign by the human immune system. Therapeutic-derived epitopes that appear foreign are the most likely targets of anti-therapeutic T cell response.

Screening Against Relevant Peptide Libraries

Once T cell epitopes are identified, it is also possible to determine whether the epitope has been tested *in vitro* or *in vivo*. The Immune Epitope Database (www.iedb.org), a contracted endeavor from the US National Institute of Allergy and Infectious Diseases, has now curated 20,860 journal articles and direct submissions, cataloging nearly 622,105 peptidic epitopes (129). By screening novel sequences against this database, researchers can determine whether peptides related to the epitopes in products in development have been reported as MHC ligands, and whether the phenotype of T cell response is known, allowing for triage of well-understood sequences from unknown sequences of greater immunogenic risk. Furthermore, when risk signals are identified, proteomics databases that contain sequences eluted from antigen-presenting cells (130) can reveal

important relationships across tissues and disease states to inform careful monitoring during clinical studies.

Ranking Biologic Candidates by Immunogenic Potential

All other factors being equal, the greater the burden of T cell epitopes contained in a given protein, the more likely it is that the protein will induce an immune response. The comparison of one biologic to another is possible to accomplish by normalizing epitope content scores across HLA alleles and adjusting for sequence lengths, as is done on the ISPRI toolkit (47, 127). Regional epitope density can also drive immune responses. A detailed description of the global and regional approach to determining immunogenicity risk is described in detail in reference (47).

IN VITRO METHODS FOR ASSESSING IMMUNOGENICITY RISK

Extensive validation *in vitro* assays may be cost-prohibitive, thus current practice is to initiate the analysis with advanced *in silico* tools (127). Following *in silico* analysis, HLA binding and T cell assays can be performed or outsourced to commercial research organizations. These assays can be applied (i) at the very early stages of drug development to design *de novo* therapeutics with low predicted immunogenicity, (ii) at a later stage to de-immunize a clinical asset exhibiting high immunogenicity in First *in Human* studies, (iii) retrospectively after program termination, to decipher the mechanisms and immunogenicity risk factors underlying the high observed clinical immunogenicity. Clearly, for new (and generic versions of older) biologic drugs to be successful, immunogenicity risk assessment is most cost-effective if performed in the pre-clinical phase of development.

In vitro Assays

HLA Binding Assay

The first step in generating a T cell response is recognition of a peptide antigen presented on a HLA class II / MHC class II molecule to a T cell by an APC. Once a potential epitope is identified by *in silico* analysis, the prediction can be first validated through HLA binding assays, such as the assay described by Steere et al. (131), to assess the ability of a peptide to bind one or more HLA supertype alleles. Supertype alleles refers to families of HLA-DR alleles that share epitope binding motifs. By taking advantage of these supertype families, it is possible to perform binding assays on a relatively small number of alleles while covering >95% of the human population worldwide. A standard binding assay is described in Figure 3.

A key factor in generation of meaningful binding assay data is the design of the peptide sequence to be tested, and source of the test peptide. The core binding region of a class II peptide contains nine amino acids that sit within the peptide binding groove of an HLA molecule. This interaction is stabilized by flanking residues on either side of the core binding region and extend outside of the binding groove. When designing peptides for binding assays, it is important to properly center the binding motif within the

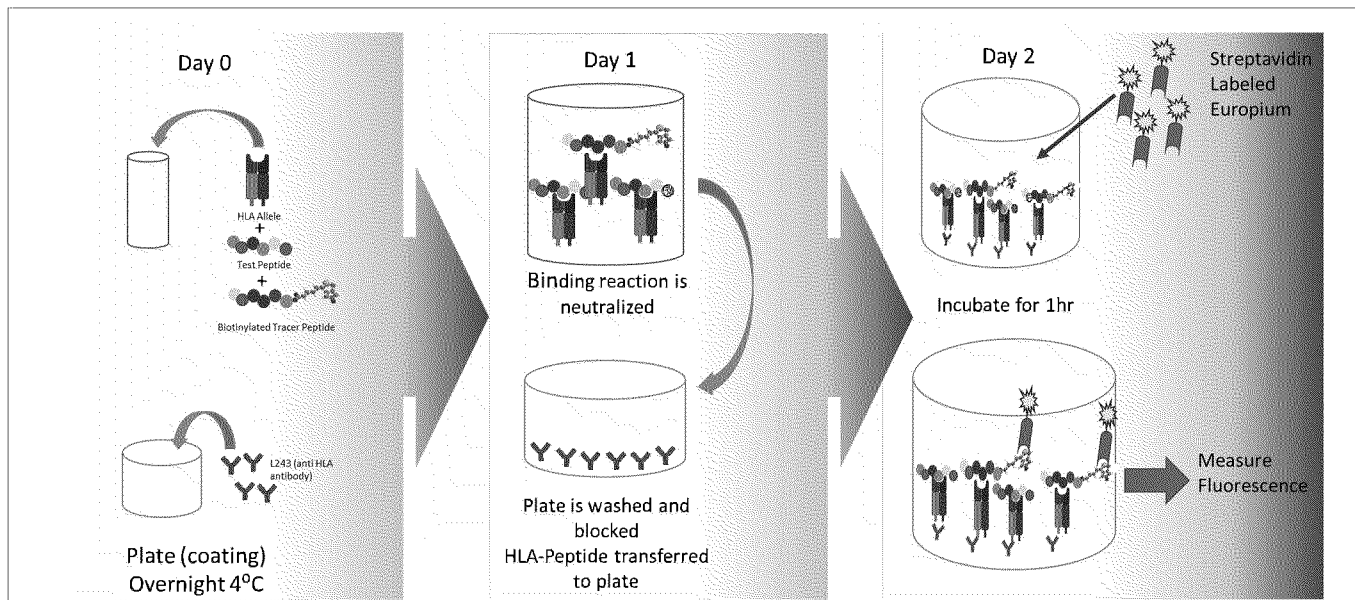


FIGURE 3 | HLA binding assays and optimal peptide design. In brief, the peptide of interest is incubated with an allele-specific labeled tracer peptide and a soluble HLA supertype monomer are incubated to equilibrium. The following day the binding reaction is halted and the mixture is transferred to assay plates precoated with a pan anti-HLA-DR antibody and incubated overnight. Following this incubation, the plates are developed and peptide binding is indirectly measured by time resolved fluorescence spectroscopy. By using a fixed concentration of the labeled tracer peptide and a range of concentrations for the test peptide, one can generate a multi-point dose ranging curve that enables the calculation of an IC₅₀ value which provides information not only about the ability of the peptide to bind HLA (yes/no) but also about the relative affinity of the peptide to a given HLA-DR supertype. Once can utilize the IC₅₀ values to divide peptides into categories based on their affinity for a given HLA allele, such as high, moderate, low, and non-binding. As new technology becomes available and accessible, it will be useful to look at the kinetics of the binding reaction as well.

peptide. Failing to do so can lead to the absence of binding despite the presence of an HLA binding motif. This is often seen in data generated by making use of overlapping peptides (83, 132).

The negative impact of improper centering of the T cell epitope in the peptide sequence (centered, with flanking residues on either side) is shown in Figure 4.

Peptide purity can also affect the outcome of a binding assay. Purity from some manufacturers can be as low as 60% due to the manufacturing process and the purity of the raw materials. Impurities within the peptides can lead to false positives and lead to faulty conclusions. Peptides for binding assays should be at a minimum 85% pure and should be ordered as net peptide. Spurious results can also be attributed to faulty synthesis. For example, non-binding peptides may have been synthesized on the same machine as earlier runs used to synthesize HLA binding peptides. This type of contamination can derail a drug development program, see for example, reference (133).

Ex vivo Assays

PBMC Assays

Peripheral blood mononuclear cells (PBMCs) isolated from whole blood are the most prevalent source of responder cells for *in vitro* cell based assays for immunogenicity prediction (19, 29). The PBMCs used in experiments can be freshly isolated from healthy volunteer or diseased individuals or thawed from a cryopreserved bank of material potentially covering an

appropriate representation of disease relevant or common well-documented HLA alleles. Due to the high throughput and ease of execution, PBMC assays using whole PBMCs, or CD8+ T cell depleted PBMCs remain the most commonly performed *in vitro* cell based assay for measuring the potential of immunogenicity (83, 119, 134–136).

In addition to typical biological products like protein, antibodies etc., product co-impurities including such as host cell proteins components, protein aggregates, synthesized peptide fragments, and others can also be evaluated in these assays. Multiple rounds of stimulation can be performed by replacing cell supernatants with fresh media spiked with the desired stimulant during extended culturing in order to expand populations of antigen specific T cells for further characterization (29, 119, 137). Schultz et al. recently reported success with a variation of the PBMC cell based assay that allows the enrichment of the number of CD4+ T cells prior to co-culture with irradiated syngeneic PBMCs in an effort to increase throughput and sensitivity (138).

The biological outcomes for T cell activation can be measured in these *in vitro* assays (both PBMC based and DC-T cell (see below) using a number of readouts. T-cell proliferation as assessed by thymidine incorporation and CFSE dye dilution are used frequently (7, 139, 140). Activation induced cytokine secretion may be measured using a focused (IL-2, IL-4, IFN- γ) or large multiplexed cytokine immunoassay panels and ELISPOT and are used as markers for T-cell activation and immunogenicity

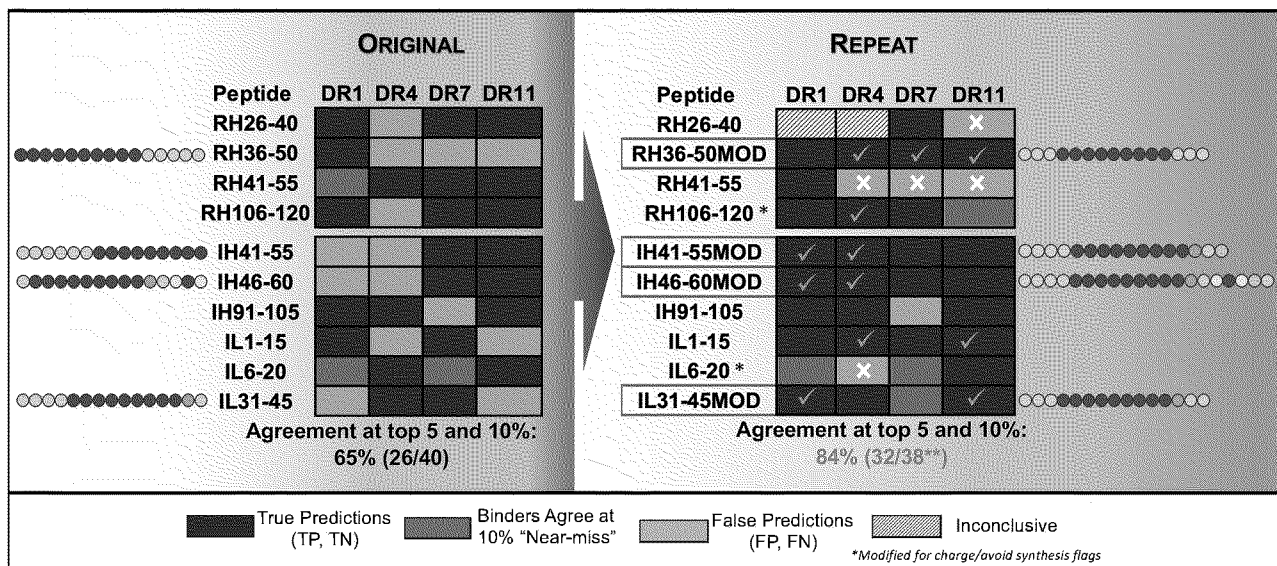


FIGURE 4 | Optimizing test peptides for the HLA peptide binding assay. HLA binding data for Infliximab peptides, published by (83) are shown as described in the publication and compared to *in silico* predictions. The peptides were re-synthesized with centered HLA binding motifs and the assays were performed using a seven-point concentration curve in a competition assay. *In silico* predicted core residues are shown in dark blue and flanking residues predicted to stabilize binding but not to interact with the binding groove are shown in gray. Residue positions in source protein are indicated next to the results for HLA binding assays to four HLA DR alleles (Columns). **(Left)** Shows the results for HLA binding of original (15mer, overlapping by 5) peptides tested *in vitro* and published data, as compared to *in silico* predictions. The agreement between predicted and published is only 65%. **(Right)** Shows repeat data with optimized peptides (MOD) with centered HLA-binding motifs, and repeat assays using a more sensitive assay (competition assays, see **Figure 2**) as compared to *in silico* predictions. Centering the HLA binding motif and using a more sensitive assay improved the agreement between *in silico* and *in vitro* assays to 84%. Assay performed by BJR, peptides synthesized at Twenty-first Century Peptides, Waltham, MA).

potential (136, 141, 142). Flow cytometry based detection of T cell responders allows a further characterization of the response in terms of intracellular cytokines, regulation of cell surface markers of activation, signal transduction events, and proliferation of specific T cell types (143, 144).

DC-T Cell Assays

In vitro co-cultures of monocyte derived dendritic cells (moDCs) and autologous CD4+ T cells are being increasingly used to evaluate immunogenicity potential of drug candidates and product CQAs. The DC-T cell or DC-PBMC methods pare the system down to the basic components of cell mediated immunity: CD4+ T cells interacting with an APC at relevant cell ratios, enhancing sensitivity as the total number of potential responder cells in the experimental system is much greater than the whole PBMC method. However, this method is time consuming and requires isolation and differentiation of monocytes into dendritic cells followed by an antigen loading/pulsing step which may be reagent, operator and material dependent.

Monocytes may be isolated from PBMC starting material using plastic adherence or isolation steps using magnetic bead separation methods. Differentiation and maturation of moDCs using cytokines or other factors is then performed (7, 57, 144–146), concurrently with the addition of the desired biotherapeutic, peptide fragments, or aggregates. The matured, pulsed moDCs are then typically combined in a co-culture with autologous, purified CD4+ T cells to allow for antigen

presentation and T cell activation depending on immunogenicity potential. The responses are measured as is performed for PBMC assays as described above. An advanced variation of the moDC-T cell system is the Modular Immune *In vitro* Construct (MIMIC®) model which is capable of reproducibly generating both antigen-specific innate and adaptive immune responses against biologic such as proteins, peptides, mAbs as well as novel modalities including nucleic acids (147, 148) has also been described for these purposes.

Flow Cytometry Analysis of T Cell Phenotype

Flow cytometry has become a valuable tool in the assessment of immunogenicity that allows for the characterization of an immune response down to the single cell level (149). As the instruments become more sophisticated by adding more laser and filter combinations as well as advances in staining and detection methods, a wealth of information can be obtained from a sample of patient's blood.

T cell epitopes have the capacity to be either immunogenic or tolerogenic. While it may be difficult to measure the expansion of Tregs in cell culture, the presence of Treg epitopes can be confirmed by co-incubation with effector T cells in the presence of immunogenic peptides. In this "bystander assay," activated Tregs inhibit the antigen-specific T effector response to the immunogenic peptides (150).

A standard bystander assay makes use of the immunologic memory toward antigens such as tetanus toxin, to which

the majority of the population has had previous exposure through vaccination or natural exposure. PBMCs are cultured for 10 days in the presence of inactivated tetanus toxoid and the Tregitope at varying concentrations. Cells are stained for analysis by flow cytometry (Teff cells are defined as CD3+CD4+CD25+FoxP3low, Treg cells are defined as CD3+CD4+CD127lowCD25+FoxP3hi) and proliferation can then be measured by CFSE dilution. In the presence of Tregitope, we have observed a reduced proliferation of effector T cells to tetanus toxoid compared to the tetanus toxoid alone (151).

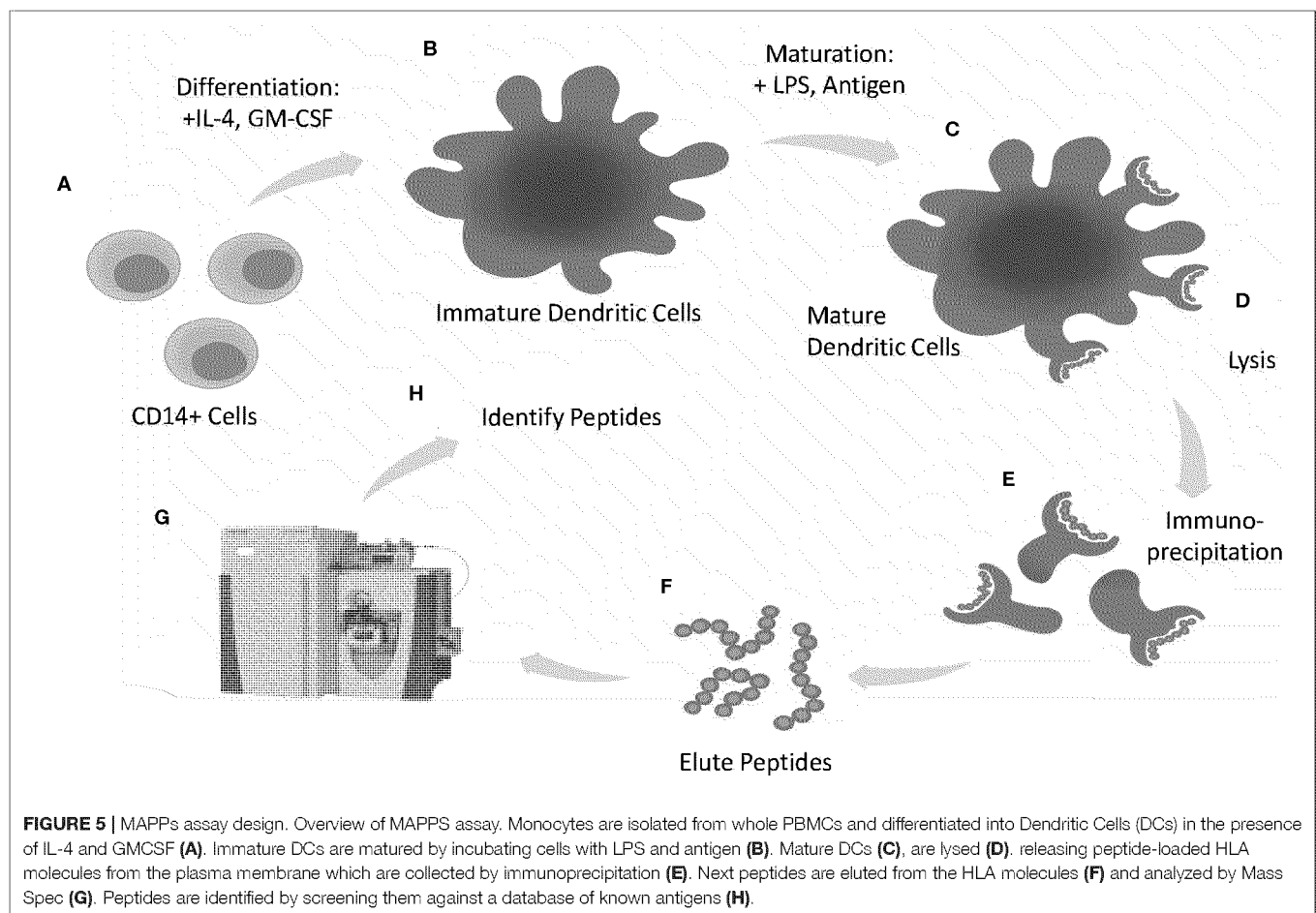
Proteomics

MAPPS Assays

In the early 1990s an additional method called MAPPS was first described (152). This assay has proved valuable in identifying processed peptides presented on the surface of antigen presenting cells by relevant HLA. Additionally this approach attempts to understand the variability in antigen processing contributed by enzyme cleavages in healthy and diseased subjects and sequencing of the peptide associated with HLA can provide confirmation/validation to the sequences identified by algorithms.

Recent advancements in LC/MS sensitivity and proteomics analysis have enabled HLA bound mapping assays to be utilized pre-clinically to map potential antigenic sequence contained within a biological therapeutic. Studies have shown that not all potential HLA binding peptides are processed and presented by APC due to a combination of partial unfolding HLA binding and cathepsin trimming. Additionally, editing functions of HLA DM and HLA DO further enhance selectivity of the peptides selected for presentation (153).

In these assays (presented as a schematic in **Figure 5**) antigen presenting cells are generated *in vitro* and incubated with the therapeutic protein of interest for 24 h followed by a cytokine/mitogen induced maturation step to upregulate HLA expression. After cell lysis HLA receptor peptide complexes are isolated by immune precipitation followed by an acid elution step to dissociate the peptide from the HLA complex and sequenced by LC/MS. Subtraction of endogenous peptides and mapping of the peptides to the therapeutic can be done using proteomics protein database algorithms. These assays are likely to point toward antigenic peptides that can be targeted for deimmunizing protein engineering. Furthermore, whole blood from relevant diseased state can provide insights into altered presentation as well as tolerance for recombinant replacement therapeutics.



A case study showing the use of algorithms, innate and adaptive phase outputs as well as MAPPs was applied to anti-IL-21 receptor ATR-107 (144). *In silico* analysis of the primary sequence predicted two overlapping CD4 T cell epitopes in the heavy chain Complementary Determining Region (CDR) 2, and one single epitope in the light chain CDR2. The MAPPs confirmed the epitope in LC CDR2 as a dominant peptide presented by DCs. ATR-107 induced DC activation as attested by an increased expression of cell surface activation markers and cytokine production, and specific proliferation of autologous CD4 T cells in co-culture conditions. As illustrated in **Figure 5**, the validation of *in silico* predictions using MAPPs can be reassuring for developers.

However, elution of a peptide in a MAPPs assays does not confirm whether the peptide drives T-cell dependent immune response (13). T cell responses may differ depending on the phenotype of the T cells that are responding to the sequence. Using MAPPs without additional tools that explore the phenotype of T cells that respond to the eluted peptides, may over predict immunogenicity.

The importance of individual epitopes driving immunogenicity was also reinforced in a recent demonstration by Cassotta et al. who conducted a MAPPs analysis of natalizumab immunogenicity, a humanized antibody directed against alpha4 integrins (82). Taking advantage of a combination of *in silico* and in cellular *in vitro* assays, in particular a MAPPs assay performed with B cells isolated from patient peripheral blood, the authors established that two multiple sclerosis patients treated with Natalizumab who developed neutralizing ADA mounted a T cell response against a CD4 T cell epitope located in the V region of the light chain.

MITIGATION OF IMMUNOGENICITY

Mitigation by Deimmunization and Tolerization

Deimmunization

Ideally, mitigation of immunogenicity starts with the engineering of molecules designed to exhibit a low risk of provoking unwanted immune responses in patients. This can be achieved by combining the deimmunization and tolerization processes. In the case of monoclonal antibodies, deimmunization encompasses two non-mutually exclusive approaches: ultra-humanization, which consists of grafting murine CDRs into antibody frameworks of human origin, and removal of T cell epitopes sequences identified through the combination of epitope prediction logarithms and *in vitro* confirmatory assays. For examples of mitigation strategies involving the removal of T cell epitopes see (127, 154–156).

Grafting of murine CDRs into human V regions often leads to a decrease or loss of affinity, which can be restored by introduction of murine amino-acids in the human framework at positions critical for drug-target interactions. These so-called back-mutations have the potential to introduce additional T cell epitopes, hence the necessity to apply an iterative and timely deimmunization strategy to exhaust the possibilities of epitope

removal as the sequence of the molecule is refined to reach the desired predicted efficacy. In this context, the Augmented Binary Substitution technology could prove an effective combinatory approach but needs further exploration (157).

Tolerization

Complementary to the removal of deleterious CD4 T cell epitopes is the introduction of T regulatory sequences, a process also known as tolerization (126). This is of particular interest in the case of replacement therapies, where removal of T cell epitopes might affect the function of the drug, or in the case of gene therapy to counterbalance the activation of the cytotoxic response induced by capsid antigenic determinants. Indeed, prophylactic administration of an AAV-derived capsid protein fused to Tregitopes was found to reduce viral capsid-specific CD8 T cell responses with a concomitant increase in Treg numbers (158). To date, the demonstration of the expected reduced immunogenicity of de-immunized and/or tolerized molecules relies on *in vitro* and *ex vivo* assays or re-clinical models (127, 154, 155, 159). De-immunized versions of high immunogenicity monoclonal antibodies have yet to reach the clinic, as biotherapeutics developers have focused instead on developing new, less immunogenic molecules that have a longer patent life and greater freedom to operate.

Treatment-Induced Tolerance

Efforts to mitigate the risk of ADA development often focus on reducing therapeutic protein's intrinsic immunogenicity, with the exception of the well-established immune tolerance induction protocols for hemophilia A and B patients who develop inhibitors to recombinant clotting factors. ADA development to monoclonal antibody-drugs can also lead to loss of response and drug switching, even in the case of fully humanized molecules. In this context, various approaches to inducing immune tolerance to biotherapeutics have been envisaged and reviewed elsewhere (160). ADA responses to other lifesaving therapeutic proteins, such as enzyme replacement therapies, have compromised treatment efficacy and even caused death. In the case of gene therapy, development of ADA to the transgene and the viral vector remains major obstacles to treatment success: patients with pre-existing neutralizing antibody response to the viral capsid are not eligible for treatment, and patients who develop treatment-induced humoral immunity will not be eligible for re-dosing.

While removal of T helper epitopes that drive T helper immune responses may reduce T helper immune responses, in a process called deimmunization (127), identification and augmentation of Treg responses by preservation of Treg epitopes or introduction of Treg epitopes such as Tregitopes into the protein sequence is now referred to as "immune engineering" or "tolerization" (126). This *in silico* approach enables the introduction of regulatory T cell epitopes to reduce the potential for immunogenicity.

Alternatively, immune tolerance induction regimens can be undertaken using available drugs that target the major players of the immune cascade that leads to ADA development, by either inhibiting deleterious effector responses or activating

tolerogenic pathways. The former can be realized by interfering with T and B activation mechanisms or by depleting immune cells with immunosuppressive agents such as cyclophosphamide or methotrexate, anti-CD3, anti-CD20 antibodies, proteasome inhibitors, or a combination of multiple depleting agents. Several such approaches are already in use, including concomitant methotrexate to diminish T cell-mediated immunogenicity (161, 162).

In Pompe disease and in the context of tolerance induction for inhibitors to FVIII therapy, current regimens combine multiple agents such as Rituximab (to eliminate antibody-secreting B cells) and IVIG (to bind and remove antibodies or to induce tolerance). Methotrexate is added in Pompe disease, and this regimen has been successful in establishing tolerance to alglucosidase alpha in high risk Pompe disease infants (163).

Other methods under consideration include concomitant administration regimen of rapamycin in a nanoparticulate form (164–167) or co-administration with Tregitopes (48, 168). Infusion of *in vitro* expanded T and B cells engineered to express antigen-specific receptors was also shown to control development of inhibitors in a pre-clinical model of hemophilia A (169).

Most immune tolerance induction approaches are still at an early stage of development, and the long term impact of these interventions remains unknown. However, the demonstrated value of the tolerizing regime that have reached the clinic is an incentive to pursue the evaluation of immune tolerance induction as a mean to mitigate unwanted immunogenicity of biotherapeutics.

FUTURE DIRECTIONS

ADA Assay Standardization

Comparing immunogenicity of therapeutic proteins across clinical studies has proven difficult due to the lack of ADA assay standardization and harmonization. For a given therapeutic protein, variability in critical assay parameters such as sensitivity and drug tolerance can lead to dissimilar estimation of clinical incidence across laboratories. In this context, the IMI-funded ABIRISK consortium (Anti-Biopharmaceutical immunization: prediction and analysis of clinical relevance to minimize the risk) generated monoclonal antibodies to serve as standards in ADA assays. Such universal standards could be used to benchmark assay sensitivity and drug tolerance, monitor routine assay performance, and validate antigenicity equivalence of comparator products in biosimilars ADA assays. Additionally, the immunogenicity assessments with such standards can help inform the clinician on dosing strategies if loss of efficacy is observed (170). Monoclonal neutralizing antibodies of various isotypes and affinity specific for rituximab, natalizumab, infliximab, adalimumab, or Interferon beta were generated from B-cells isolated from patients immunized with the respective therapeutic proteins, as previously described (171). Production scale-up and further characterization using ABIRISK validated ADA assays are on-going. Ultimately, all antibodies will be openly available at the National Institute of Biological Standards and Controls (NIBSC).

New Modalities and Immunogenicity Risk Assessment

As discussed in section New Modalities, new modalities such as cellular and gene therapies have shown immunogenicity in the clinic. The mechanisms by which these modalities can elicit immune response are complex due to the high level of engineering, intracellular expression, introduction of engineered gene products, as well as complex delivery systems. Modified Immunogenicity risk assessment tools and assays developed primarily for protein therapeutics can be used to minimize immunogenicity risk of these novel therapeutics (Figure 6).

Specific Cell Lines/Soluble TCRs

Novel *in vitro* assays that rely on the ability of antigen presenting cells displaying the processed peptides in the context of HLA class I/II to interact with T cell repertoires are proving to be useful for further defining the antigen specificity and immune response propagation (172, 173). Additionally, use of engineered B-cell lines expressing class I and class II HLA can support a high-throughput prediction of intracellular processing and presentation of potential antigenic epitopes. One example would be to use a competitive approach where soluble T cell receptors recognizing an HLA-reference peptide complex are used to detect presentation of potential immunogenic epitopes by mono-allelic antigen presenting cell lines (Merck, unpublished data).

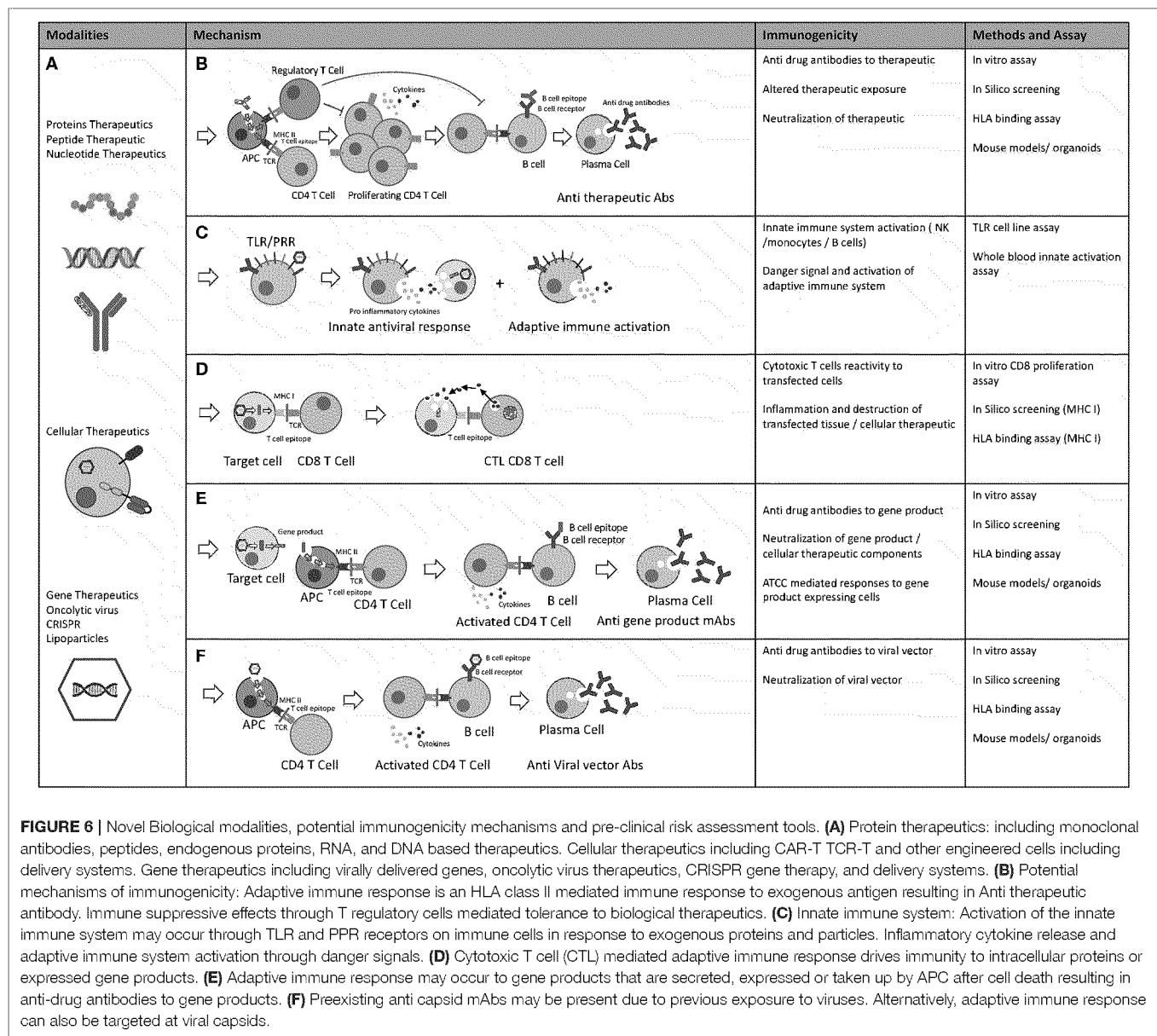
Modeling

As described above, a suite of *in silico* and *in vitro* tools can be deployed early in development to guide protein engineering and design drug candidates with predicted low immunogenicity. However, the tools will assess product-related risks, in particular sequence-based risk, but won't inform other factors pertaining to immunogenicity such as patient- and treatment-related factors. The overall immunogenicity risk relies on the weighting and integration of the different risks, some of which are empirical, some theoretical. Immunogenicity Quantitative Systems Pharmacology (QSP) simulators could simplify and homogenize this integration (174). They incorporate biotherapeutics, physiologically-based pharmacokinetic, and mechanistic models of immune responses to simulate large scale clinical trials and predict immunogenicity incidence. The impact of critical variables such as HLA genotype, combination therapies, dosing regimens and route of administration on ADA incidence, as well as ADA impact on drug Pharmacokinetics (PK) can be modeled. QSP simulators are still in development, requiring a greater set of empiric input data and refinement of parameters related to the immune system such as kinetics of antibody development (174). Once validated, QSP simulators could give rise to personalized management and mitigation of immunogenicity.

DISCUSSION

Immunogenicity-Focused Organizations

Faced with the challenge of accurately performing an immunogenicity risk assessment as well as measuring and determining the clinical relevance of ADA, pharmaceutical companies, biotech and contract research organizations



joined forces to progress the field by addressing the gaps. Scientific non-profit associations were created, such as the European Immunogenicity Platform (EIP, <https://www.e-i-p.eu/>). The purpose of the EIP is to stimulate exchanges between immunogenicity experts, encourage, and lead interactions with regulatory agencies, share knowledge and state-of-the art in immunogenicity field with the broader scientific community and training courses on practical and regulatory aspects of immunogenicity.

The ABIRISK consortium mentioned above represents another collaborative approach to contributing to the advancement of immunogenicity sciences. Clinical and basic research academic centers worked with industrial partners on a 6-year research project, addressing some of the main questions and practical hurdles related to unwanted immunogenicity,

such as the value of existing predictive tools, ADA assays, harmonization and standardization, clinical relevance of detected ADA, identification of patients' risk factors, and predictive markers (175–178).

A spin-off initiative emerged from this extensive collaboration across laboratories in Europe, the United-States and Israel. BIOPIA (<https://ki.se/en/cns/biopia>) is a non-profit effort of European laboratories with expertise in biopharmaceutical pharmacokinetics and immunogenicity in many diseases, which aim to raise awareness about immunogenicity and advocate integration of drug levels and ADA testing as a means to improve patient's management. The website provides information about ADA and drug level testing with the goal of helping clinicians with the implementation of routine, clinical testing for immunogenicity and drug levels. Similar efforts are underway in

the United States, under the umbrella of the Therapeutic Protein Immunogenicity Community as part of the AAPS (American Association of Pharmaceutical Scientists). The Immunogenicity risk assessment and mitigation (IRAM) working group has initiated a survey to characterize performance and harmonize methods for risk assessments including algorithms and *in vitro* assays through member surveys. The future focus is on adapting the current tools and developing innovative assays to answer questions around novel modalities and next generation therapies.

Regulatory Perspective on Immunogenicity

The recent FDA guideline proposes a risk-based approach to assess immune response to a therapeutic protein and its impact on safety and efficacy on a case-by-case basis (33). There is also a recommendation that the risk-based strategy be developed early in development, preferably after humanization and in parallel with other developability efforts. The early assessment would enable a more robust understanding of the liabilities due to structure and sequence. A continuous evaluation of the risk through the different stages of drug development can guide the bioanalytical strategy for clinic as described below. This would include risks due to changes in process development, manufacturing, formulation, and device.

Integration of Risk Assessment Into the Preclinical Pipeline

Briefly, the immunogenicity risk assessment should take into account potential therapeutic benefits and weigh those against the potential impact of immunogenicity taken into account patient population and indication as well as previous experience with therapeutic target.

Early assessment of biologic candidates allows ranking based on least probability of identified risk. There is also room for deimmunization/sequence optimization which could involve removing a few amino acids to remove the epitope or inserting regulatory sequences to drive a suppressive T cell response. Furthermore, the risk-based strategy should include any liabilities due to post-translational modifications that are a consequence of process related changes associated with expression, purification, etc. as well as formulation/excipient induced aggregation or degradation.

The knowledge of early pharmacology of the therapeutic protein including on and off -target engagement and consequent activation of the immune pathways should also be a consideration during development of the risk-based strategy. This is especially relevant for therapeutic proteins (TPs) targeting immune modulatory pathways. The pre-clinical toxicology studies could provide an insight into the safety

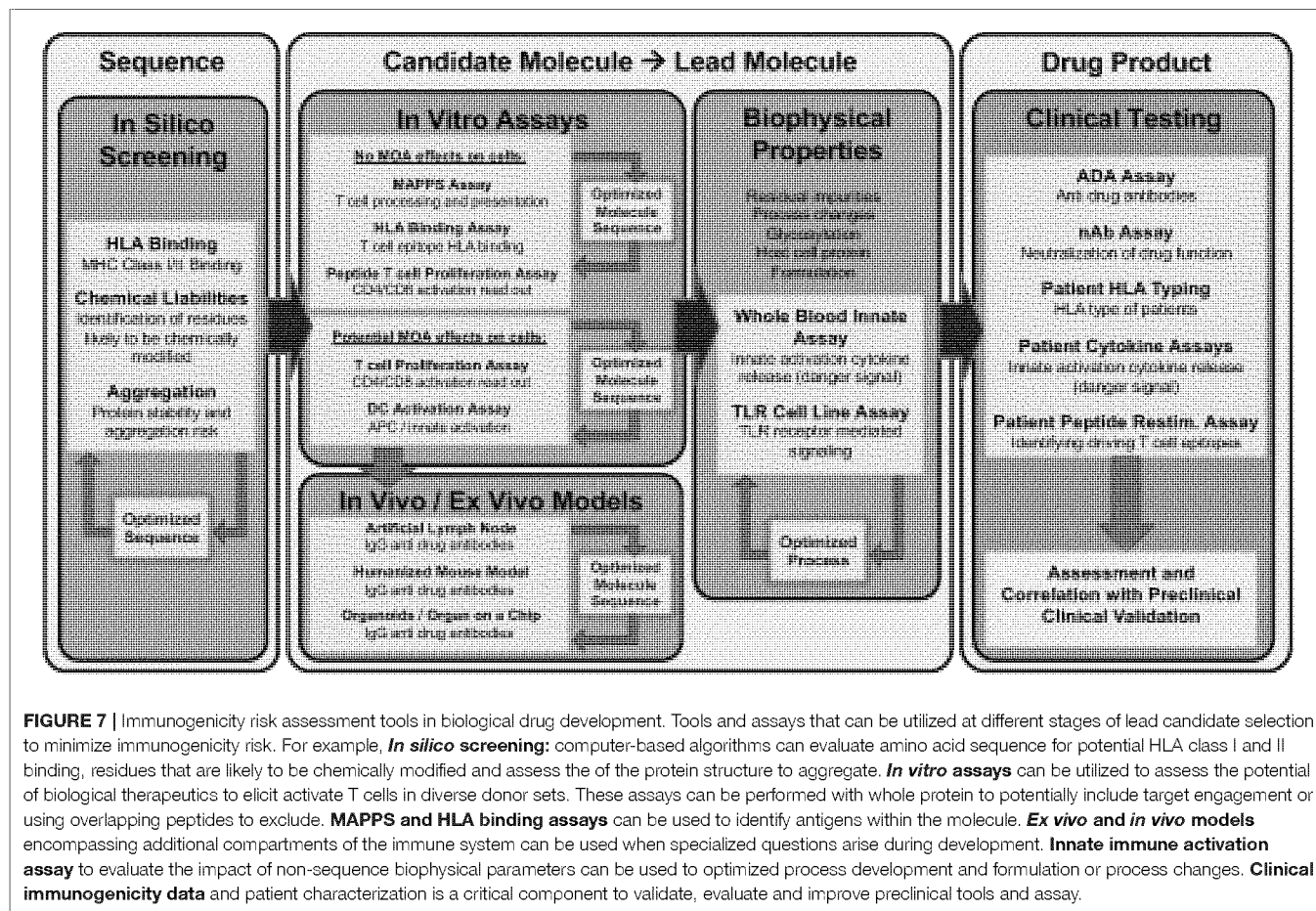


FIGURE 7 | Immunogenicity risk assessment tools in biological drug development. Tools and assays that can be utilized at different stages of lead candidate selection to minimize immunogenicity risk. For example, **In silico screening**: computer-based algorithms can evaluate amino acid sequence for potential HLA class I and II binding, residues that are likely to be chemically modified and assess the of the protein structure to aggregate. **In vitro assays** can be utilized to assess the potential of biological therapeutics to elicit activate T cells in diverse donor sets. These assays can be performed with whole protein to potentially include target engagement or using overlapping peptides to exclude. **MAPPS and HLA binding assays** can be used to identify antigens within the molecule. **Ex vivo and in vivo models** encompassing additional compartments of the immune system can be used when specialized questions arise during development. **Innate immune activation assay** to evaluate the impact of non-sequence biophysical parameters can be used to optimized process development and formulation or process changes. **Clinical immunogenicity data** and patient characterization is a critical component to validate, evaluate and improve preclinical tools and assay.

associated concerns related to on and off target liabilities, especially when the pre-clinical and clinical targets have homology. The risk-based strategy can also benefit, where there is previous clinical experience such as proteins with similar targets that are already in the commercial phase. Additionally, if there is enough clinical experience around the TP in one disease indication, the outcome of the studies related to any safety and efficacy can also be summarized for the investigational new drug application (IND) being developed for the new IND. **Figure 7** provides an overview of the sequence to product stage of development and tools and their outputs to address key attribute relate questions.

FIVE YEAR VIEW

As a result of advances in immunogenicity risk assessment methods as well as derisking efforts pertaining to both the product (primary sequence and formulation) as well as improved understanding of the patient factors that may contribute to development of ADA, most biotherapeutics developers are integrating the assessments into pharmacokinetics/pharmacodynamics, safety and clinical efficacy outcomes to better understand the risk of a new product or biosimilar. Ongoing consideration should be given to use of emerging technologies (novel *in silico*, *in vitro*, and *in vivo* assays) for use during development (designing of new sequences, lead selection, de-risking of identified liabilities, or comparison of biosimilar prioritization). These methodologies also provide an estimation of risk, including prior knowledge of individual risk (HLA type) disposition for clinical immunogenicity. *In vivo* studies in animal models are not currently recommended for immunogenicity due to differences between animal model and human HLA. Instead, *in vitro* assays are preferred for evaluating risk of cell-mediated immune responses. MHC-related immune response variation can be expected when transitioning from one model species to another, or to human. T cell epitopes bound by MHC in mice, non-human primates, and other model species are frequently different than those bound by humans. Testing for immunogenicity *in vitro*, with human PBMC samples that are

selected to provide broad coverage of human MHC, is how most pre-clinical studies with biologics circumvent this concern.

Within 5 years, it is expected that much of the risk-assessment will be performed first *in silico* before moving to (limited) *in vitro* and *in vivo* models. This is due to the fact that most drug companies performing comprehensive pre-clinical development generate literally thousands of potential candidates for a single target. *In silico* analysis gives a good first pass approach to immunogenicity, enabling detailed inspection of certain molecular features using *vitro* methods where required. The accuracy of computational tools will increase with increasing results available to public review.

Machine to machine interfaces, enabling the integrated and high throughput screening of multiple candidates for the same target, will simultaneously improve the pre-clinical selection of candidates for clinical development. Drug developers will need to become familiar with available tools as the sheer volume of candidates that are expected to be screened will be impossible to manage without automated *in silico* analysis pipelines. It is also likely that the breadth of *in silico* analysis (and *in vitro* validation) will begin to encompass HLA class I immunogenicity assessment and *in vitro* assays. This is due to the introduction of novel modalities and viral vectors, which interface with the class I pathway.

The field of immunogenicity risk assessment has matured and will continue to evolve as new modalities are introduced into the clinic.

AUTHOR CONTRIBUTIONS

VJ, FT, JG, SM-K, BR, ST, and AD conceptualized this review, composed, and edited the manuscript document. Figures were created by JG, BR, and FT. All authors contributed to the article and approved the submitted version.

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Using Serum Metabolomics to Predict Development of Anti-drug Antibodies in Multiple Sclerosis Patients Treated With IFN β

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Background: Neutralizing anti-drug antibodies (ADA) can greatly reduce the efficacy of biopharmaceuticals used to treat patients with multiple sclerosis (MS). However, the biological factors pre-disposing an individual to develop ADA are poorly characterized. Thus, there is an unmet clinical need for biomarkers to predict the development of immunogenicity, and subsequent treatment failure. Up to 35% of MS patients treated with beta interferons (IFN β) develop ADA. Here we use machine learning to predict immunogenicity against IFN β utilizing serum metabolomics data.

Methods: Serum samples were collected from 89 MS patients as part of the ABIRISK consortium—a multi-center prospective study of ADA development. Metabolites and ADA were quantified prior to and after IFN β treatment. Thirty patients became ADA positive during the first year of treatment (ADA+). We tested the efficacy of six binary classification models using 10-fold cross validation; k-nearest neighbors, decision tree, random forest, support vector machine and lasso (Least Absolute Shrinkage and Selection Operator) logistic regression with and without interactions.

Results: We were able to predict future immunogenicity from baseline metabolomics data. Lasso logistic regression with/without interactions and support vector machines were the most successful at identifying ADA+ or ADA– cases, respectively. Furthermore, patients who become ADA+ had a distinct metabolic response to IFN β in the first 3 months, with 29 differentially regulated metabolites. Machine learning algorithms could also predict ADA status based on metabolite concentrations at 3 months. Lasso logistic regressions had the greatest proportion of correct classifications [F1 score (accuracy measure) = 0.808, specificity = 0.913].

Finally, we hypothesized that serum lipids could contribute to ADA development by altering immune-cell lipid rafts. This was supported by experimental evidence demonstrating that, prior to IFN β exposure, lipid raft-associated lipids were differentially expressed between MS patients who became ADA+ or remained ADA-.

Conclusion: Serum metabolites are a promising biomarker for prediction of ADA development in MS patients treated with IFN β , and could provide novel insight into mechanisms of immunogenicity.

Keywords: immunogenicity, anti-drug antibodies, multiple sclerosis, metabolomics, cholesterol, machine learning

INTRODUCTION

Multiple sclerosis (MS) is a progressive neurological disease driven by a combination of inflammatory and neurodegenerative processes. There is currently no cure, but a variety of disease-modifying therapies are now available (1). Many of these are biopharmaceuticals which can elicit an undesirable immune response (immunogenicity) leading to the production of anti-drug antibodies (ADA). The therapeutic consequences of ADA include accelerated/delayed drug clearance, neutralization of bioactivity, cross-reactivity with the endogenous protein and hypersensitivity reactions. Consequently, ADA can compromise treatment efficacy (2–6) and safety (7), and are a clinically significant problem for the treatment of MS.

Beta interferons (IFN β) have been used to treat MS for more than 20 years (8), reducing relapse rate by $\sim 33\%$ (9). Although drugs that are more effective are now available, IFN β is still used first line due to its favorable safety profile. However, depending on the formulation, IFN β can induce ADA at rates varying from up to 30% with subcutaneous injection of IFN β -1b (Betaferon/Extavia) or IFN β -1a (Rebif), $<5\%$ with intramuscular injection of IFN β -1a (Avonex) and $<1\%$ for PEGylated IFN β -1a (Plegridy). The type (IFN β -1b or -1a), route of injection, dose, and frequency of administration all influence the intrinsic immunogenicity of the drug (10).

Numerous studies have demonstrated that persistent high titers of neutralizing antibodies (nAbs) can significantly reduce and even negate the therapeutic benefit of IFN β treatment (11). At the cellular level, IFN activity can be inferred from the induction of IFN-response genes such as MXA, and nADA have been shown to inhibit MXA induction in a titer-dependent manner (12). The clinical relevance of low nAbs titers and binding antibodies (bAbs) is less clear, but could include immune complex formation and complement activation (13) and increased IFN β efficacy by lengthening its half-life (14).

It can be difficult to detect loss of efficacy because disease activity is infrequent and can be asymptomatic, and time spent on an ineffective treatment places patients at risk of accruing irreversible neurological damage. Therefore, it is highly desirable to identify patients at high risk of developing immunogenicity prior to therapeutic intervention so that their treatment strategy can be tailored accordingly (15).

However, our understanding of the biological parameters that contribute to an individual's risk of ADA development remains

limited. To date, a small number of genetic and immunological parameters have been associated with ADA risk, including human leukocyte antigen (HLA) class II alleles (16), HLA and non-HLA associated single nucleotide polymorphisms (17), and NOTCH2 expression on monocytes (18). Thus, there remains an unmet demand for a predictive biomarker of immunogenicity against IFN β , and a better understanding of the mechanisms underpinning ADA development is required.

In recent years machine learning (ML) approaches have been applied to clinical problems in MS, including computer-aided diagnosis, neuroimaging analysis and prediction of disease trajectories (19–21). The majority of models have been based on clinical information, but ML-generated serum lipid signatures have also successfully been used to identify (22) and stratify (23) MS patients. Circulating lipids are dysregulated in MS, and have been associated with disease progression (24–28). Indeed, circulating lipids can profoundly influence immune cell behavior (29–32). However, serum lipids have not previously been studied in the context of immunogenicity.

In the present study, serum metabolites and lipids were quantified using an established nuclear magnetic resonance spectroscopy platform (Nightingale Health). A variety of supervised ML methods were applied, including random forest (RF), support vector machine (SVM) and lasso (least absolute shrinkage and selection operator) logistic regression which have all been proved effective for analysis of metabolomics data (22, 33–36). K-nearest neighbors (kNN) was also included for contrast, as in side by side comparisons it has proved inferior to other algorithms (22, 34). Finally decision trees were also implemented due to the ease of interpretation and visualization, although it is acknowledged they are prone to overfitting. Overall, SVM, RF and logistic regression with/without interactions could all predict future ADA status at baseline or month 3 with F1 score (a measure of accuracy) > 0.735 and specificity > 0.83 . Thus, we present a new approach to personalized prediction of ADA development utilizing a combination of serum metabolites and clinical information.

MATERIALS AND METHODS

Patient Cohort

A prospective cohort of MS patients was recruited across six European countries as part of the Anti-Biopharmaceutical Immunization: prediction and analysis of clinical relevance

to minimize the RISK consortium (ABIRISK consortium; www.abirisk.eu/). Patients were diagnosed with relapsing remitting multiple sclerosis (RRMS) or clinically isolated syndrome (CIS) according to the revised McDonald criteria 2010 (37). Ethical approval for this study was obtained from the ethics committee of the University College London Hospitals National Health Service Trust, London, United Kingdom (18/SC/0323 and 15/SW/0109), Medical Ethics Committee of the General University Hospital in Prague (125/12, Evropský grant I.LF UK-CAGEKID), Ethikkommission der Fakultät für Medizin der Technischen Universität München, München, Germany (project no. 335/13), Ethikkommission Nordwest- und Zentralschweiz, Basel, Switzerland (project no. 305/13), and Ethikkommission der Medizinischen Universität Innsbruck, Innsbruck, Austria (UN2013-0040_LEK). All participants provided written informed consent in accordance with the Declaration of Helsinki.

Demographic and clinical information were also recorded, including sex, age, ethnicity, body mass index (BMI), smoking status, type and dose of IFN β , and expanded disability status score (EDSS) at baseline and 18 months post treatment (Table 1). Smoking status was categorized as never smoked, quit, or current smoker. Patients were on one of four IFN β formulations: Avonex, Rebif, Betaferon, or Extavia. These were categorized as follows: intramuscular IFN β -1a (Avonex), subcutaneous IFN β -1a (Rebif) and subcutaneous IFN β -1b (Betaferon/Extavia). The dose and frequency of treatment varied between individuals, therefore dose of IFN β per week was also calculated (dose per administration $\times$ frequency of administration).

Separation of Serum and Peripheral Blood Mononuclear Cells

As part of the ABIRISK consortium standard operating procedures were implemented at all sites. Samples were collected prior to IFN β treatment (M0), and after 3 (M3) and 12 (M12) months of treatment. Peripheral blood samples were non-fasting.

To separate serum from clotted blood, BD SST vacutainers were allowed to coagulate for at least 30 min before centrifugation at 1,500 g for 10 min at 4°C with full acceleration and brake. Serum was aliquoted into screw-capped cryovials and stored at -20°C or below.

To separate peripheral blood mononuclear cells (PBMCs), whole blood was collected into vacutainers containing sodium heparin, and centrifuged at 400 g for 10 min at room temperature (acceleration 5, brake 3) to separate the plasma fraction. Plasma was decanted and heat inactivated (56°C for at least 35 min) before centrifugation at 2,400 g for 15 min (acceleration 9, brake 9). The remaining blood was diluted 1:1 in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with L-glutamine (Sigma) and layered onto 15 mL Ficoll-Paque PLUS (GE Healthcare) using SepMate tubes (StemCell Technologies) as per the manufacturer's instructions. Cells were washed twice in cold RPMI and resuspended in heat-inactivated autologous plasma with 10% dimethylsulfoxide (Sigma-Aldrich) at a density of $\sim 1 \times 10^7$ cells/mL and cryopreserved in liquid nitrogen until use.

TABLE 1 | Cohort characteristics.

		ADA- (n = 52)	ADA+ (n = 30)	P-value
Sex n (%)	Female	37 (71)	19 (63)	0.4635 ^a
	Male	15 (29)	11 (37)	
Age years	Mean (SD)	34.6 (9.3)	37.9 (9.8)	0.1265 ^b
Ethnicity n (%)	Caucasian	52 (100)	30 (100)	n/a
BMI	Median (IQR)	23.7 (6.8)	24.6 (7.2)	0.2941 ^c
Smoking n (%)	Non-smoker	32 (61.2)	16 (53.3)	0.6007 ^a
	Quit smoking	8 (15.4)	4 (13.3)	
	Current smoker	12 (23.1)	10 (33.3)	
Type of IFN n (%)	Avonex	21 (40)	0 (0)	<0.0001 ^a
	Rebif	27 (52)	9 (30)	
	Betaferon/Extavia	4 (8)	21 (70)	
ADA status	nAbs ⁺ bAbs ⁺	0 (0)	28 (93.3)	n/a
	nAbs ⁺ bAbs ⁻	0 (0)	2 (6.67)	
Country n (%)	Austria	6 (11.5)	3 (10.0)	n/a
	Czech Republic	27 (51.9)	13 (43.3)	
	Germany	6 (11.5)	7 (23.3)	
	Spain	10 (19.2)	1 (3.3)	
	Sweden	1 (1.9)	2 (6.7)	
	Switzerland	2 (3.8)	4 (13.3)	
EDSS at M0	Median (IQR)	2.0 (1.5)	1.5 (2.0)	0.2198 ^c
Change in EDSS	Median (IQR)	0 (0.5)	0 (0.63)	0.4902 ^c

Baseline demographic and clinical characteristics were compared between patients who did or did not develop anti-drug antibodies (ADA) to interferon treatment within 1 year. Statistical comparisons were made using ^achi-squared, ^bun-paired two-tailed t-test or ^cMann-Whitney U. bAbs, binding antibodies; BMI, body mass index; EDSS, expanded disability status score; IQR, interquartile range; M0, month 0; nAbs, neutralizing antibodies.

ADA Detection

Serum was tested for both binding (bAbs) and neutralizing (nAbs) ADA. bAbs were measured with an enzyme-linked immunosorbent assay (ELISA) (38) and nAbs were detected by a cell-based luciferase reporter gene assay (39).

As the test for bAbs is less sensitive than the one for nAbs, patients were classified as ADA positive if they were positive for bAbs and nAbs, or were bAbs- but had a nAbs titer ≥ 320 U/mL within 12 months of starting treatment (40) (Table 1). Patients were considered ADA negative if they were negative for both assays. Patients with missing data or negative for bAbs and with a nAbs titer < 320 U/mL were excluded from this analysis ($n = 7$).

Serum Metabolomics Analysis

Measures of 228 serum biomarkers were acquired with a well-established nuclear magnetic resonance (NMR)-spectroscopy platform (Nightingale Health) (41, 42). These included both absolute concentrations, ratios, and percentages of lipoprotein composition. For this study, we have excluded the percentages from analysis leaving 158 metabolite measures (Supplementary Table 1). Serum lipids measured included apolipoproteins (Apo) and (very) low density ((V)LDL), intermediate density (IDL) and high density (HDL) lipoprotein particles of different sizes ranging from chylomicrons and

extremely large (XXL), very large (XL), large (L), medium (M), small (S), and very small (XS).

Predictive Models

Please consult **Figure 1** for a schematic outlining the data analysis pipeline. RStudio (The R Foundation, Vienna, Austria) (43), Orange 3.24.1 (Bioinformatics Lab, University of Ljubljana, Slovenia) (44) and MATLAB (The MathWorks Inc., Natick, USA) were used for machine learning analysis.

Six different supervised learning algorithms were implemented: *k*-nearest neighbors (kNN), support vector machine (SVM), logistic regression with and without interactions, decision trees, and random forest classification. The outcome of the learning algorithms was to predict whether an MS patient is likely to develop ADA in response to IFN β treatment. Predictive models were generated from metabolite concentrations prior to IFN β exposure (M0) and after 3 months (M3).

Missing Data

Features with >10% missing data were excluded (glutamine and glycerol). Remaining missing values ($n = 6$ M0, $n = 7$ M3) were imputed using *k*-nearest neighbors with $k = 5$.

Homology Reduction

Many of the metabolites measured are biologically interdependent, and therefore highly correlated. To reduce homology, if two features had a correlation co-efficient > 0.95 then the feature with the greatest mean absolute correlation with the remaining features was removed (**Supplementary File 1**). This left 60 metabolites at M0, and 59 metabolites at M3.

Data Scaling

Metabolite concentrations were centered on the mean and scaled to the standard deviation.

Predictors

The independent variables included in the models were either the full data set (Lasso logistic regression $\pm$ interactions and networks) or the homology reduced dataset (60 and 59 metabolites at M0 and M3, respectively), as well as the cohort information (sex, age, BMI, smoking status, country of sample, baseline EDSS, IFN β type, and dose). Ethnicity was not considered, as all participants were Caucasian. The type of IFN β was significantly associated with 12 month ADA status, in agreement with other studies (10, 45) (**Table 1**). Full lists of the predictors contributing to each model are included in **Supplementary File 2**.

kNN

K-nearest neighbors is a non-parametric classification algorithm which assigns the class of an unknown observation based on the class of a number (*k*) of similar observations in the feature space (46). The default value of $k = 5$ was used in this analysis.

SVM

Support vector machine is a supervised classification method which creates a hyperplane to optimally separate data into two

classes (47). As this data set was not linearly separable, the radial basis function kernel was used. Values for *C*, epsilon, and gamma were tuned using the R Package *e1071* (48). The parameters were set to $C = 4.5$, epsilon = 0.1, gamma = 0.015 for the M0 model and $C = 2$, epsilon = 0.2, gamma = 0.01 for the M3 model.

Decision Tree

Decision trees are a form of supervised machine learning which outputs a flowchart-like structure, which classifies incidents according to their features. These are built using forms of impurity measures, such as information gain and entropy (49). In an effort to prevent overfitting, decision trees were limited to a depth of 4 and subsets of 5 or less were not split further.

Random Forest

Random forest (RF) is a statistical classifier (machine-learning algorithm) that assigns observations into classes (ADA-/+) by creating a set of decision trees, or “forest.” Only a small random sample of predictors are candidates for selection at each node, so the created trees are decorrelated. Ensembling these uncorrelated trees offers a natural way of reducing the variance of the model. Importance was quantified by the Gini index, which represents the total variance across the two classes, the purity of each node and the quality of each split. The optimum number of variables randomly chosen at each node ($m_{try} = 8$ and $m_{try} = 11$ for M0 and M3, respectively), have been tuned with the function “tuneMTRY” (package “RFmarkerDetector”), with respect to the Out-of-Bag errors. The package “randomForest” function (package “randomForest”) produced RF models which ensemble 1,000,000 trees (50–53).

Logistic Regression With/Without Interactions

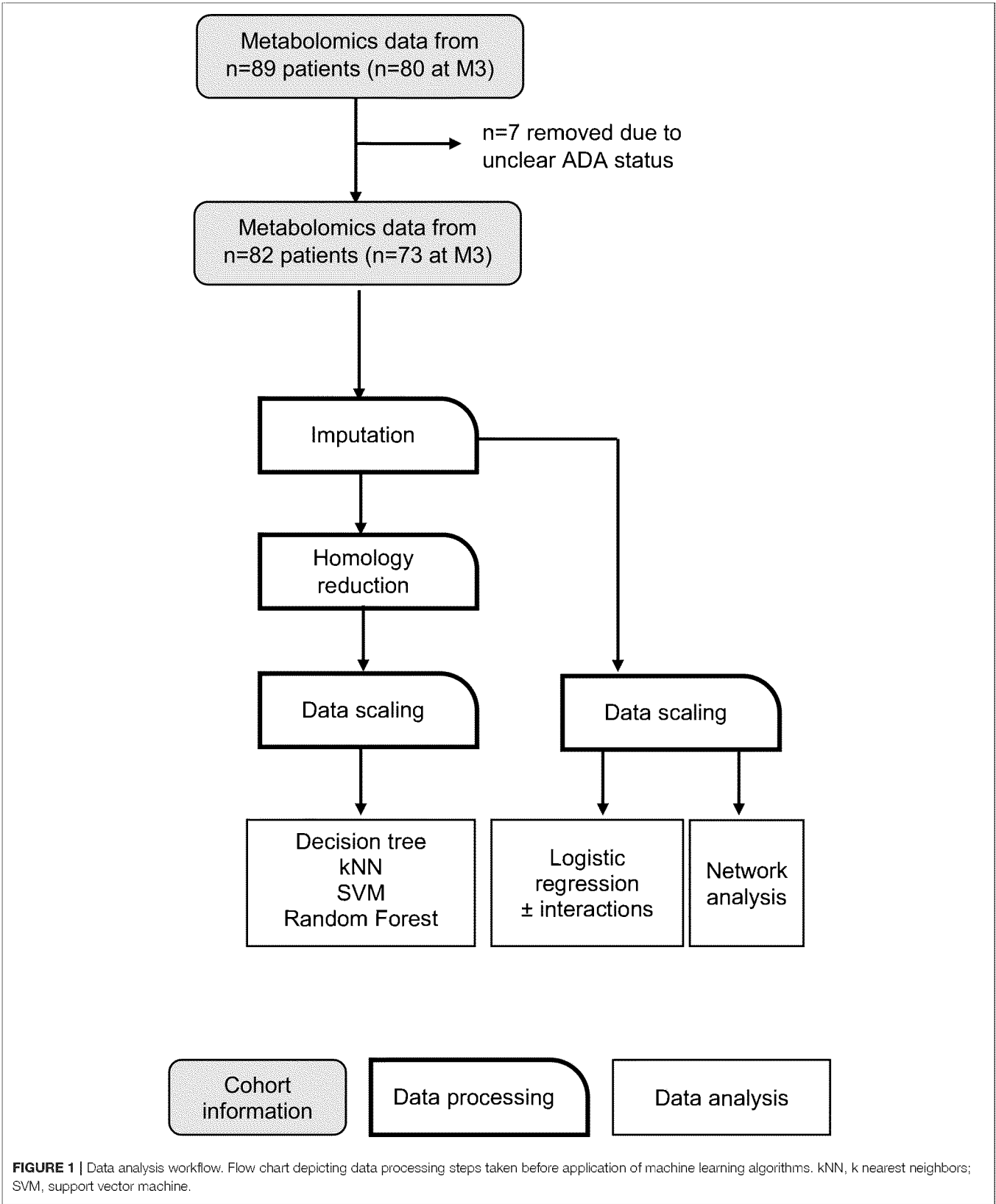
The least absolute shrinkage and selection operator (lasso) method uses the absolute value of the co-efficient as a penalty to shrink less important features to zero. The strength of shrinkage is determined by tuning the regularization variable lambda (λ). Logistic lasso regression with interactions was conducted with the R package *glmnet*. All 158 metabolites were included. Categorical predictors were coded as dummy variables with the following treated as the reference class: sex—male, smoking status—never smoked, treatment—Avonex, country—Spain. Age, BMI, baseline EDSS, and dose/week were treated as continuous variables. $\ln(\lambda)$ was tuned to -2.7 for the logistic regression without interactions (M0 and M3), and -2 (M0) or -2.2 (M3) for the logistic regression + interactions.

Model Performance

Ten-fold cross-validation was used to evaluate model performance. The following performance metrics were calculated from the confusion matrices: (1) F1 score—a weighted average of precision (positive predictive value) and recall (sensitivity), (2) specificity—the true negative rate, and (3) classification accuracy (CA)—the proportion of correctly classified cases.

Logistic Regression

To assess the association of ADA development with NMR metabolomic biomarker data, logistic regressions were performed for each individual serum metabolite, adjusted



for sex, age, BMI, smoking status, treatment type and dose, EDSS, and country of sample origin (**Supplementary File 3**). Standard deviation-scaled odds ratios $\pm 95\%$ confidence intervals were visualized in a forest plot using the R package *foresplotNMR* (Nightingale Health Ltd) as exemplified in Ahola-Olli et al. (54) (**Supplementary Figure 1**).

Network Analysis

Metabolite network diagrams were created with the R package high dimensional undirected graph estimation package [huge (55)]. Graphical lasso (glasso) was used to estimate the sparse inverse covariance matrix, with the stability approach to regularization selection (StARs) (56). The metabolites contributing to each predictive model have been superimposed onto the network diagrams. Where appropriate, variable importance was determined by ranking mean decrease in Gini (RF) or information gain (SVM). kNN is excluded as this model performed poorly relative to the others.

Quantification of Cholesterol and Glycosphingolipids by Flow Cytometry

Flow cytometry staining was performed as previously described (57–59). In brief, 1×10^6 PBMCs were stained with Zombie (BioLegend) fixable viability dye for 30 min at 4°C, then labeled with antibodies to surface markers in Brilliant Stain buffer (BD Biosciences) for 30 min at 4°C. Subsequently samples were stained with 25 µg/mL cholera toxin B subunit FITC conjugate (CTB-FITC) (Sigma-Aldrich), fixed for 1 h in 2% paraformaldehyde, and stained for 2 h with 50 µg/mL filipin complex from *Streptomyces filipinensis* (Sigma-Aldrich) before reading the samples on a BD LSRFortessa X-20 cytometer using BD FACSDiva software. Compensation was performed using anti-mouse IgGκ/negative control compensation particles set (BD Biosciences) or OneComp eBeads (ThermoFisher Scientific), with the exception of viability dyes and filipin which were performed with single stained and unstained cells. Data was analyzed using FlowJo (Tree Star).

Antibodies for surface markers: CD45RA-BUV737 (clone HI100, BD Biosciences, 584442) CD27-APC (clone M-T271, BioLegend, 356409), CD4-AF700 (clone OKT4, eBioscience, 56-0048-82), CCR7-BV421 (clone G043H7, BioLegend, 353207), CD69-BV510 (clone FN50, BioLegend, 310936), CD8-BV711 (clone RPA-T8, BioLegend, 301044), CD3-BV785 (clone OKT3, BioLegend, 317330), CD25-PE (clone M-A251, BioLegend, 356104), CD127-PE-Cy7 (clone A019D5, BioLegend, 351320).

Statistical Testing

Statistical tests were performed in Microsoft Excel and GraphPad Prism version 8.3.0 for Windows (GraphPad Software, San Diego, USA). Data was assessed for normality and analyzed with parametric or non-parametric tests as appropriate. Details of statistical tests are given in the figure legends. $P < 0.05$ were considered statistically significant.

RESULTS

Serum Metabolites Can Be Used to Predict Future ADA Development

Metabolites were quantified in serum from MS patients both before IFNβ treatment (month 0–M0) and after 3 months (M3). Patients were classed as ADA positive (ADA+; nAbs+, bAbs+/-) or negative (ADA-; nAbs-, bAbs-) based on their ADA status at M12. Several ML models were applied to this data in order to develop a model to predict ADA status (**Figure 1**). All models were adjusted for sex, age, body mass index (BMI), smoking status, type of IFNβ and weekly dose, country, and baseline expanded disability status score (EDSS).

At M0 all models were better at predicting ADA- individuals (specificity) than ADA+ (F1 value) (**Table 2A**). Overall the logistic regression (LR), LR+i (**Table 3**) and decision tree performed comparably when predicting ADA+ cases, correctly identifying 21 out of 30 (70%) (**Figure 2A**). On the other hand, the SVM performed better for ADA- cases, with excellent specificity (0.981, **Table 2A**), only misclassifying one ADA- patient (**Figure 2A**). Overall the tree had the best performance, with an F1 score of 0.788 (**Table 2A**, **Figure 2B**). Seven lipid measures featured in more than one model (**Figure 2C**), which were all significantly elevated in the patients who went on to develop ADA (ADA+) (**Figure 2D**). Three of these lipid metabolites (M-VLDL-CE, TG/PG, and XXL-VLDL-FC) represent clusters of highly correlated metabolites, particularly measures of VLDL composition (**Supplementary Table 2**, **Supplementary File 1**), indicative of broader differences in metabolite expression.

TABLE 2 | Comparison of predictive model performance.

Model	F1	Precision	Recall	Specificity	CA
A					
kNN	0.510	0.619	0.433	0.846	0.695
Tree	0.778	0.875	0.700	0.942	0.854
RF	0.678	0.690	0.667	0.827	0.768
SVM	0.735	0.947	0.600	0.981	0.842
LR	0.764	0.840	0.700	0.923	0.842
LR + i	0.764	0.840	0.700	0.923	0.842
B					
kNN	0.571	0.636	0.519	0.826	0.712
Tree	0.741	0.741	0.741	0.848	0.808
RF	0.764	0.750	0.778	0.848	0.822
SVM	0.745	0.792	0.704	0.891	0.822
LR	0.808	0.840	0.778	0.913	0.863
LR + i	0.808	0.840	0.778	0.913	0.863

(A,B) Performance statistics for five predictive models based on serum metabolites at M0 (A) and M3 (B). The models used were k-nearest neighbors (kNN), decision tree (Tree), random forest (RF), support vector machine (SVM), and logistic regression (LR) with and without interactions (i). The classification accuracy (CA) represents the proportion of correctly identified cases, in contrast to specificity, which is the true negative rate. F1 is the weighted average of the precision and recall (see Methods). Statistics are rounded to 3 decimal places.

TABLE 3 | Predictors for lasso logistic regression with interactions.

M0 features	M3 features
(Intercept)	(Intercept)
Treatment (IFN β -1b)	Treatment (IFN β -1b)
bOHBut: TG/PG	AcAce: EDSS
M-HDL-TG: XS-VLDL-CE	bOHBut: His
TG/PG: XS-VLDL-PL	Lac: Val
BMI: Treatment (IFN β -1b)	Val: Smoking (Quit)
	VLDL-D: Country (Germany)
	XXL-VLDL-TG: Treatment (IFN β -1a sc)
	BMI: Treatment (IFN β -1b)

List of features selected by the lasso regressions with interactions at month 0 (M0) and M3 (M3). Interacting features are separated by a colon (:). Where predictors are categorical (treatment, smoking status, country) the specific category is shown in brackets. BMI, body mass index; EDSS, expanded disability status score; sc, subcutaneous administration.

The association between individual metabolites and future ADA status were examined by performing logistic regressions on a per metabolite basis (Supplementary Figure 1, Supplementary File 3). No significant associations were detected demonstrating the importance of accounting for the dependence between metabolites.

IFN β exerts widespread effects on the immune system. Since the response to IFN β treatment can also influence the development of immunogenicity, similar models were constructed based on serum metabolite concentrations at M3. The best performing models at this time point were again the LR and LR+i (Table 3, Supplementary File 2), which had the highest F1 and specificity values, and lowest total number of misclassifications (Table 2B, Figure 3A). As at M0, all of the models were better at predicting ADA- (specificity) than ADA+ (F1 value) (Table 2B). Four metabolites featured in multiple models (Figure 3B), but in contrast to M0 few of these were differentially expressed when comparing ADA- to ADA+ (Figure 3C, “all”). However, when patients were stratified by treatment type more differences were revealed. Glucose (Glc) levels differed in patients treated with IFN β -1b, whereas XXL-VLDL-FC was raised in ADA+ patients treated with subcutaneous IFN β -1a (Figure 3C). The LR+i also selected the interaction between subcutaneous IFN β -1a and XXL-VLDL-TG (Table 3), which is highly correlated with XXL-VLDL-FC (Supplementary Table 2). Indeed, XXL-VLDL-FC is highly correlated to many other VLDL measures (Supplementary Table 2), 12 of which were also found to be significantly associated with ADA status on a per metabolite basis (Supplementary Figure 1, Supplementary File 3). Only XXL-VLDL-FC was selected by multiple models at both time points (Figures 2C, 3B), with a greater concentration in ADA+ patients (Figures 2D, 3C). This suggests that a cluster of interconnected VLDL lipids may be persistently associated with an increased risk of developing ADA.

The majority of metabolites were not predictive at both time points, suggesting it could be beneficial to implement predictive models both before and after exposure to IFN β . We examined

the longitudinal concordance in predictions for each model (Supplementary Table 3). The logistic regressions generated the same predictions at both timepoints for all but one patient. The decision tree had the highest rate of discordance, particularly in the positive class (38%), coinciding with a reduction in performance at M3. In contrast The RF had a high discordance rate in the ADA- class (21%). This demonstrates that different models have different advantages—some are better at predicting positive cases or negative cases, or are better at M0 or at M3.

Metabolite Interactions and IFN β Response Differ Between ADA+ and ADA- Patients

In addition to examining individual metabolite concentrations we compared metabolite networks in ADA- and ADA+ before and after IFN β treatment (Figure 4). The metabolite networks were more tightly clustered in ADA- patients at both time-points. A number of metabolites had very different positions depending on ADA status. For instance, Unsat (Figure 4, i) and MUFA/FA ratios (Figure 4, ii) had more connections in ADA+ patients, whereas at M3, L-HDL-TG (Figure 4, iii) lost its relationship with the main metabolite cluster in ADA+ patients.

In both patient groups IFN β treatment considerably altered the shape of the network (Figure 4, compare M0 vs. M3 in ADA+ and ADA- patients). Therefore, we examined the response to IFN β in more detail. In total 29 metabolites were differentially regulated between ADA- and ADA+ during the first 3 months of IFN β treatment (Figure 5A). Some of the metabolite increases induced by IFN β in ADA- patients were inhibited in ADA+ (Figure 5B). Other metabolites were more suppressed in ADA+ compared to ADA- individuals (Figure 5C). This suggested that IFN β had an enhanced lipid-lowering effect in ADA+ patients.

Plasma Membrane Lipid Rafts Are Dysregulated in MS Patients Who Develop ADA

Serum lipids can modulate immune cell function by altering the composition of plasma membrane lipid rafts; glycosphingolipid and cholesterol enriched microdomains that regulate cell signaling by regulating the lateral mobility of membrane proteins (Figure 6A). Before exposure to IFN (M0) plasma membrane cholesterol was higher and glycosphingolipids were lower in CD4+ T cells isolated from ADA+ patients (Figure 6B). This could suggest that differences in serum lipid composition, for example the observed changes in M-HDL-TG or XXL-VLDL-FC, could generate an immune cell phenotype that predisposes an individual to immunogenicity.

DISCUSSION

Serum metabolites are attractive candidate biomarkers in MS, and have already been shown to have diagnostic (22, 23, 60) and prognostic (27, 61, 62) potential. Furthermore, they are relatively inexpensive to measure, and a blood-draw is less invasive and time-consuming than a lumbar puncture or MRI scan. We measured serum metabolites at an unprecedented level of detail and, using a combination of ML models, we demonstrated

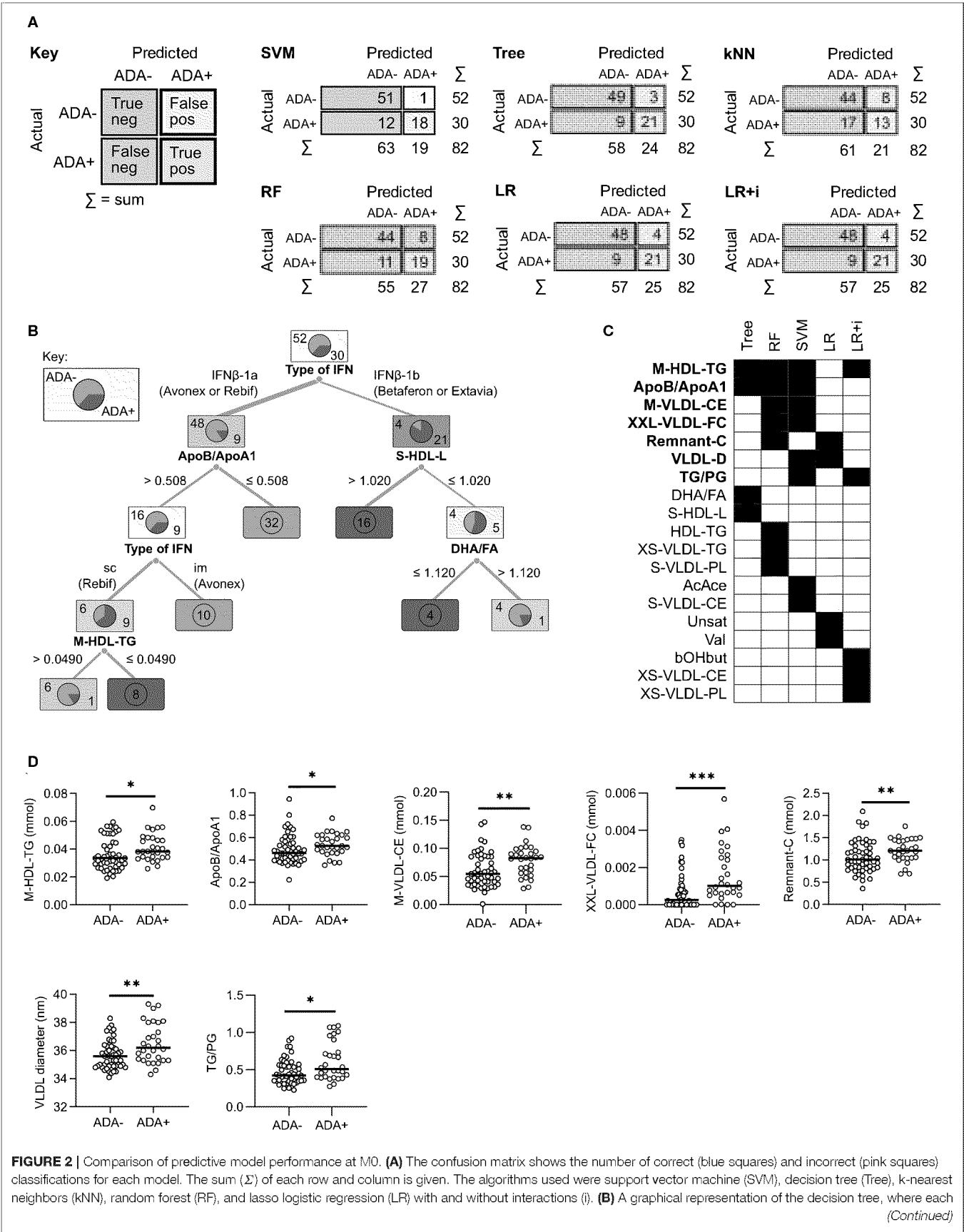
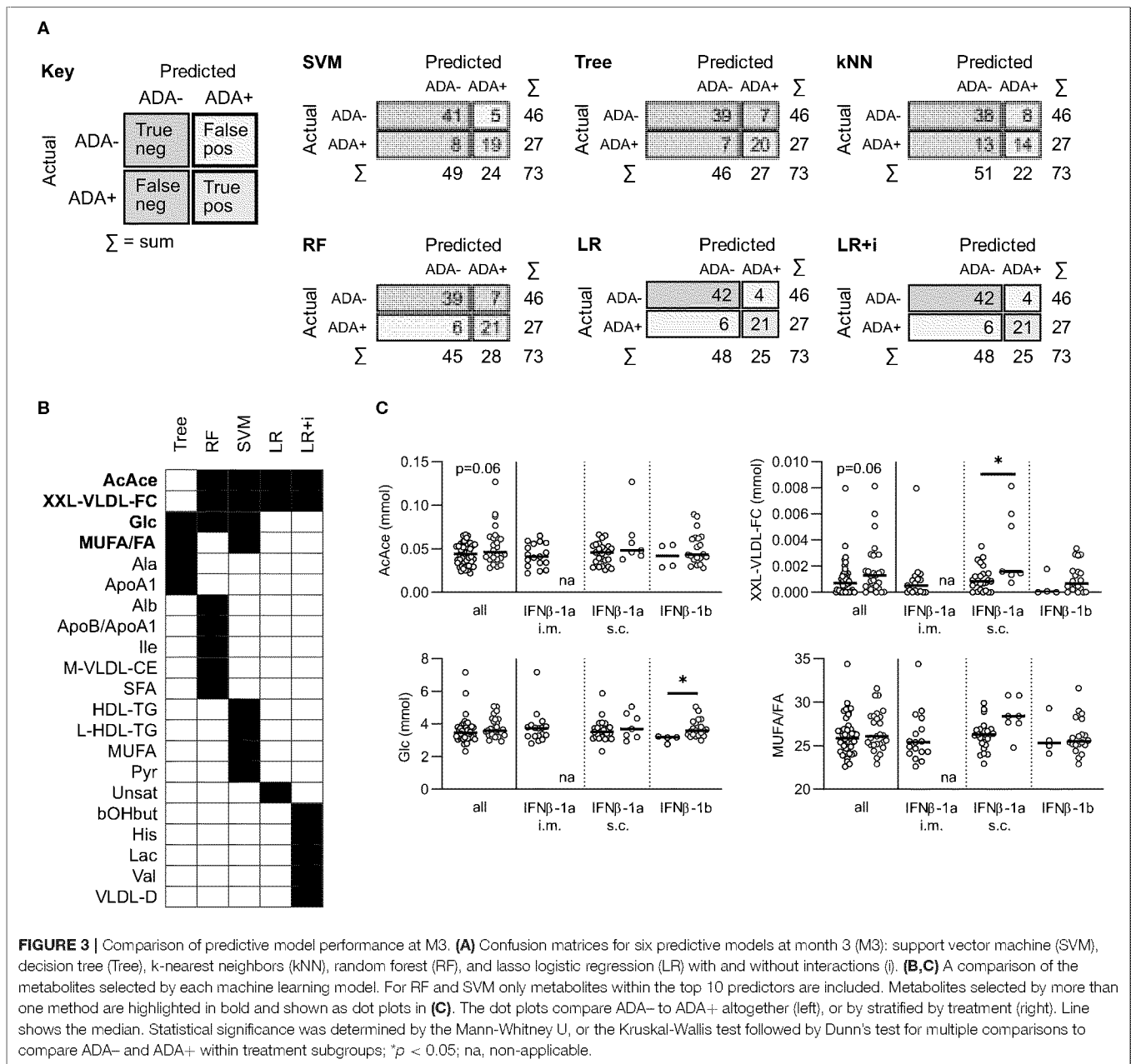


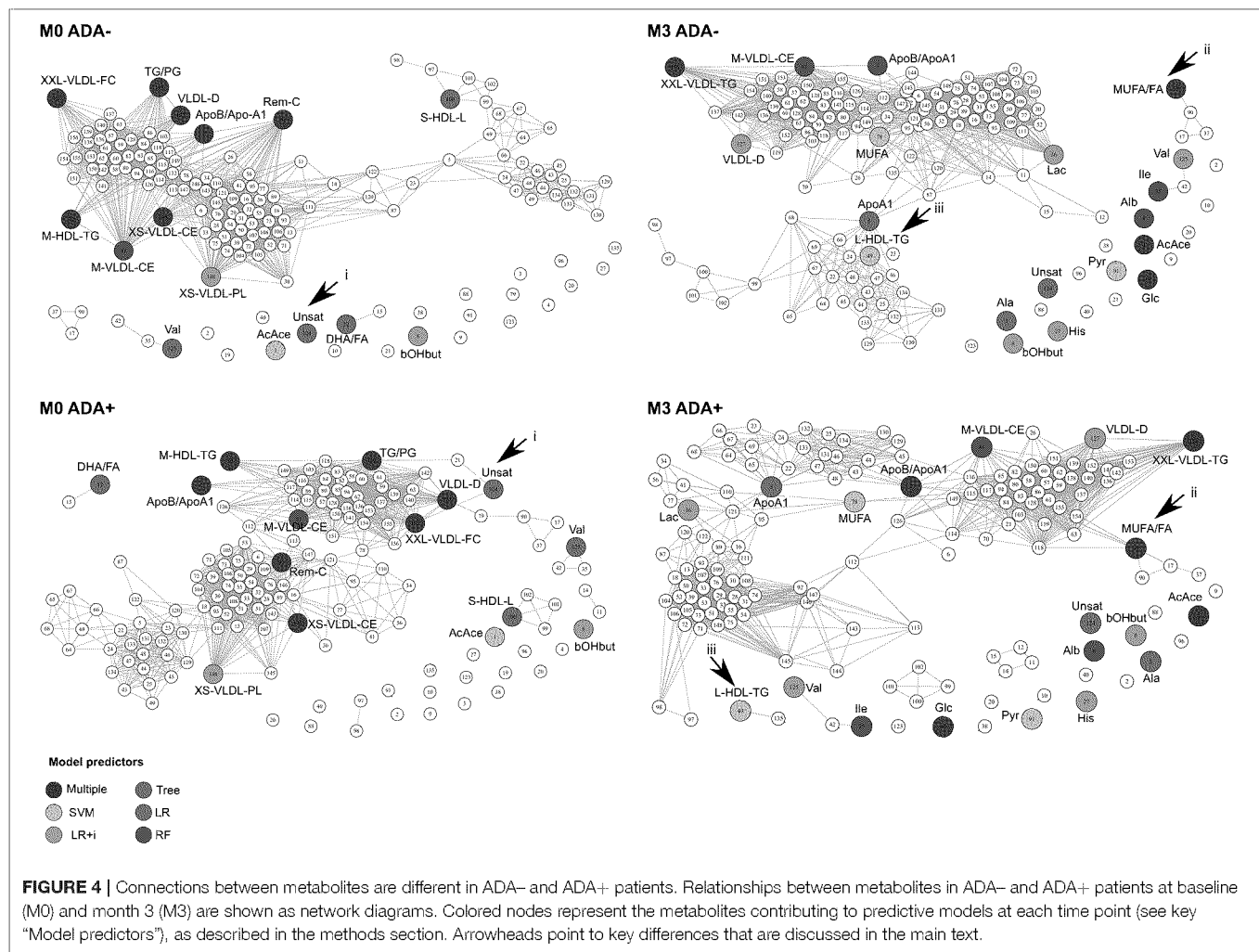
FIGURE 2 | square shows the proportion of patients who stay ADA negative (top left, blue) or become ADA positive (bottom right, red). The numbers on the branches represent cut-off concentrations (mmol) or ratios (ApoB/A1 and TG/PG). **(C,D)** A comparison of the metabolites selected by each machine learning model. For RF and SVM only metabolites within the top 10 predictors are included. Metabolites selected by more than one method are highlighted in bold and shown as dot plots in **(D)**. Line shows the median, and significance was determined by Mann Whitney U; * $p < 0.03$, ** $p = 0.01$, *** $p = 0.0003$. im, intramuscular; sc, subcutaneous.



that a subset of serum lipid metabolites could predict ADA development against IFN β in MS patients.

Future ADA status could be predicted before commencing IFN β treatment, with four out of six models achieving F1 score > 0.73, specificity > 0.92, and classification accuracy > 0.84. The decision tree achieved the most correct predictions at baseline. Although they are the easiest to interpret, decision trees are

prone to overfitting and tend to be unstable. Consequently, we conclude the logistic regression models are the best choice for the classification of both classes (84% CA), whereas SVM is the best choice for identifying negative cases (98% specificity). We suggest that an ensemble model, combining several ML approaches, is more likely to prove optimal. In this way, models which were better at predicting positive cases could be combined



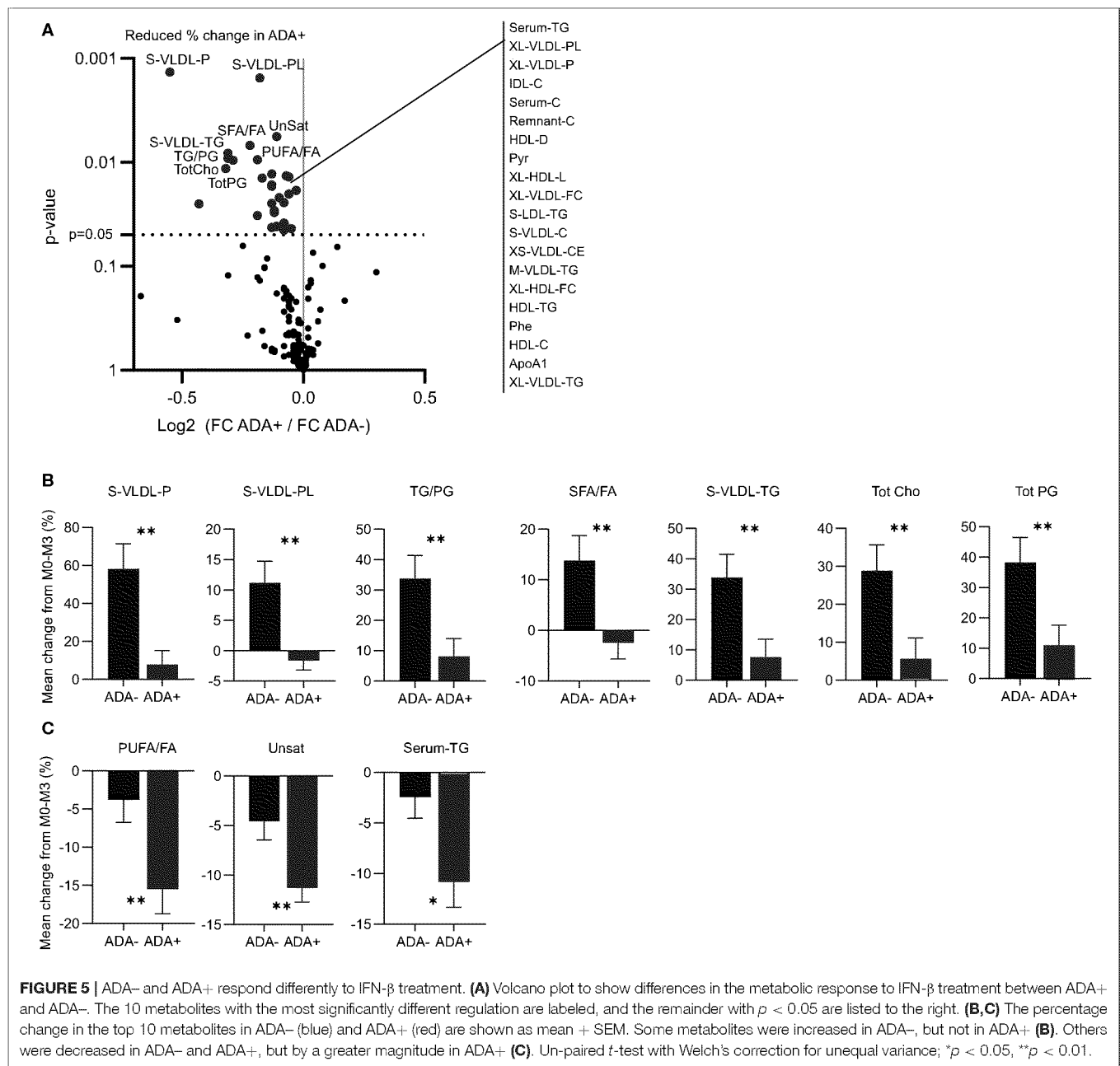
with the models better at predicting negative cases to achieve superior performance. Clinically acceptable thresholds for model performance must be carefully considered, based on the medical, psychological and financial implications of incorrect predictions. In many cases existing tests using conventional biomedical techniques can be used as a benchmark. However, there is currently no method to predict ADA against IFN β before starting treatment. It could be beneficial to investigate how MS patients feel about the risks of an incorrect result in the context of ADA prediction, and false positives or false negatives could be penalized accordingly.

We also produced models based on metabolite concentrations at M3 as IFN β activates the immune system and effects systemic lipid levels (63, 64). Both the immune and metabolic responses to IFN β treatment could influence the probability of ADA development. Model performance was comparable between M0 and M3, with the LR, LR+I, SVM and RF all achieving F1 score > 0.74, specificity > 0.84, and classification accuracy > 0.82. Overall the LR models had the most correct predictions at this timepoint. However, the contributing metabolites were dissimilar. This is unsurprising, as IFN β had widespread effects

on metabolite concentrations, which were likely to overwrite baseline differences. Interestingly, IFN β inhibited a number of metabolites in ADA+ patients, suggesting a difference in IFN response. However, we cannot currently decipher to what extent the differences in IFN-response are truly related to ADA development, to patient intrinsic factors, or to the unequal distribution of treatment types between classes.

A limitation of our analysis was that our cohort received different types of IFN β , which had different probabilities of inducing ADA development. Our sample size was insufficient to perform a comparison of only ADA- and ADA+ patients who were exposed to the same treatment. Indeed, when we examined the predictive metabolites at M3 several were only differentially expressed in patients on a particular IFN β type. Therefore, any future validation of this work should be performed on a per treatment basis.

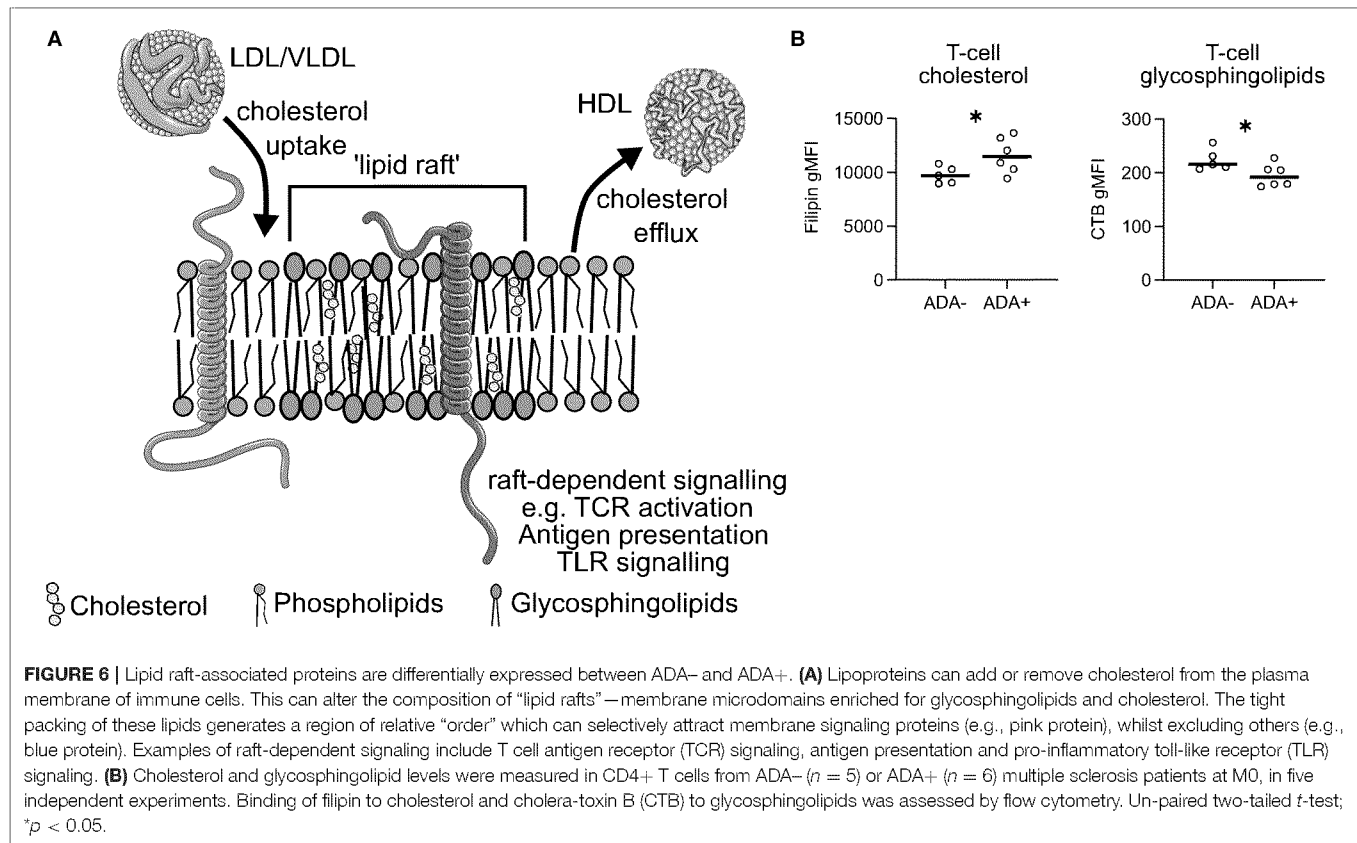
Notably, in this study nobody treated with intra-muscular IFN β -1a (Avonex) developed ADA. Despite this, one patient treated with Avonex was predicted to be ADA+ by SVM, RF and the decision tree at M3. This suggests that the metabolic profile outweighed the type of treatment in this case. Thus, although the



type of IFN was an important factor, the serum metabolites added an additional layer of personalized information. In addition to making predictions, the differences in metabolite concentrations and relationships identified here could be involved in the mechanisms driving ADA production. Excess cholesterol in the membrane leads to enhanced pro-inflammatory signaling in both macrophages (65, 66) and T cells (67), and we provided preliminary evidence that plasma membrane cholesterol is elevated in T cells isolated from ADA+ patients. Many of the lipids measured could influence T-cell cholesterol levels, including M-HDL-TG which featured in all of the models generated at baseline. Elevated triglyceride content of HDL is

associated with its dysfunction and reduced capacity to support cholesterol efflux (68, 69). Therefore, the increased concentration of M-HDL-TG in patients who later became ADA+ could lead to abnormal cholesterol transport, and a predisposition to a pro-inflammatory immune response.

From a therapeutic perspective, it is possible that combining IFNβ treatment with an intervention to modify specific metabolites could protect against ADA development. In terms of lipid modification, there have already been several clinical trials comparing combination therapy of IFNβ with statins to IFNβ alone (70), although only one reported on the incidence of neutralizing antibodies and found no difference (71). It



is important to note that the sample size was limited (*n* = 27), and statins may not be the most relevant therapeutic agents to modify the concentrations of the metabolites identified to be different in our analysis (e.g., HDL-TG). The metabolite networks revealed predictive metabolites that were highly interconnected which could be candidates for a widespread intervention, as well as unconnected metabolites which could be specifically targeted. Interactions between metabolites and patient characteristics were also identified—including baseline EDSS and acetyl acetate, smoking status and valine, and XXL-VLDL-TG with treatment type. If verified, these relationships could improve the personalization of treatment recommendations.

In conclusion, we have demonstrated the potential utility of serum metabolites and ML to predict the development of immunogenicity in MS patients. We suggest that the integration of additional molecular information (e.g., transcriptomics, genomics, proteomics) would strengthen these models, and provide novel insight into the interplay between lipids and the immunogenic response.

DATA AVAILABILITY STATEMENT

The metabolomics data presented in this study is available in Mendeley Data repository (72).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics committee of the University College London Hospitals National Health Service Trust, London, United Kingdom (18/SC/0323 and 15/SW/0109) Medical Ethics Committee of the General University Hospital in Prague (125/12, Evropský grant I.LF UK-CAGEKID) Ethikkommission der Fakultät für Medizin der Technischen Universität München, München, Germany (project no. 335/13) Ethikkommission Nordwest- und Zentralschweiz, Basel, Switzerland (project no. 305/13) Ethikkommission der Medizinischen Universität Innsbruck, Innsbruck, Austria (UN2013-0040_LEK). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

EJ, KW, MA, IP-T, and PD designed the research study. LC and AP performed ML analyses. PD and KW performed other statistical analyses. The manuscript was written by KW and EJ. AF-H coordinated anti-drug antibody testing. PN, EK, and RF provided patient serum samples and clinical assessment. All authors reviewed the manuscript and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.01527/full#supplementary-material>

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Drug Tolerant Anti-drug Antibody Assay for Infliximab Treatment in Clinical Practice Identifies Positive Cases Earlier

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A subgroup of patients treated with infliximab lose response to the treatment and one reason for this is the development of anti-drug antibodies (ADA). If used optimally, measuring drug and ADA level could lead to a more personalized and efficient treatment regime, and enable identification of ADA-positive patients before the underlying disease flares or allergic reactions occur. With the use of a drug-tolerant ADA assay which can detect ADA irrespective of drug levels in the sample, we determined the impact of ADA on treatment failure to infliximab. The aims of this study were to estimate the real-life optimal serum infliximab (sIFX) level and set a clinical threshold value for a drug-tolerant ADA assay. Trough levels of sIFX were measured with ELISA. Free ADA was measured with two drug-sensitive methods (ELISA and a bioassay) and one drug-tolerant method (Panda). Two real-life cohorts treated with infliximab were included; a cross-sectional cohort including patients with inflammatory rheumatic diseases ($n = 270$) and a prospective cohort of rheumatoid arthritis (RA) patients ($n = 73$) followed for 1 year. Normal range of sIFX was estimated from the prospective cohort and an arbitrary optimal drug level was set to be between 1 and 6 $\mu\text{g/mL}$. Using this range, optimal sIFX was found in only 60% (163/270) of the patients in the cross-sectional cohort. These patients had significantly better treatment response than those with a drug level under 1 $\mu\text{g/mL}$, who had an ADA frequency of 34% (19/56) using the drug-tolerant method. In the prospective cohort, the drug-tolerant assay could identify 34% (53/155 samples) as ADA positive in samples with sIFX level $>0.2 \mu\text{g/mL}$. ADA were seldom detected in patients with $>1 \mu\text{g/mL}$ sIFX, with three interesting exceptions. A clinically relevant ADA threshold was determined to be $>3 \text{ RECL}$ as measured with the drug-tolerant assay. In a real-life setting, there was a substantial number of patients with suboptimal drug levels and a proportion of these had ADA. Both too low and too high drug levels correlated with worse disease, but for different reasons. Adding a drug-tolerant assay enabled detection of ADA earlier and regardless of drug level at time of sampling.

Keywords: anti-drug antibody, serum infliximab, clinical threshold value, clinical effect, Panda

INTRODUCTION

Infliximab is a chimeric monoclonal antibody (mAb) blocking the effect of tumor necrosis factor alpha (TNF- α) which has been widely used since 1999 for treatment of a number of inflammatory rheumatic diseases including rheumatoid arthritis (RA). TNF- α inhibitors (TNFi) were the first monoclonal antibody therapy shown to significantly halt progression of these diseases in clinical trials (1–3), and the treatment effect is even more efficient in combination with other disease modifying anti-rheumatic drugs (DMARDs) such as methotrexate (MTX) (3). However, up to 40% of patients do not respond to TNFi treatment according to the EULAR (European League Against Rheumatism) response criteria (4–7). These patients can be categorized into those who never achieve any response (primary treatment failure), and those who lose response over time (secondary treatment failure) (8). One cause for secondary treatment failure is the development of anti-drug antibodies (ADA) (9–12). ADA results in reduced availability of the drug in the circulation (13) and therefore a lower effective dose (14–16). There is an association between non-responders and low serum trough infliximab (sIFX) levels (17) that is often due to the development of ADA (18). Therefore, it is recommended to start by first screening for the drug trough level and then usually only those with low drug level are subsequently tested for ADA.

The presence of ADA can lead to a subtherapeutic serum drug level by either neutralization of the drug, leading to hampered pharmacological activity, or through the sequestering of drug resulting in increased clearance of immune complexes (IC) via excretion through the kidneys (19, 20). ADA have also been associated with adverse effects with an increased risk of infusion-reactions, lupus, and vasculitis like events (21). Given that several studies have shown that up to 44% of patients treated with TNFi develop ADA, it is an important clinical issue to address. Routine ADA testing would allow early identification of these patients ensuring an efficient treatment regimen (3, 11, 22).

The prevalence of ADA to TNFi vary between studies, which in part can be explained by differences in concomitant medication use, timing of sampling in relation to the drug administration, treatment duration, and type of assay used for ADA detection (23–25). Standard immunoassays such as ELISA are frequently used for ADA screening. However, disadvantages of these assays include a low drug tolerance and ability to detect only free ADA. When ADA bind to the drug to form immune complexes, the antibodies become indiscernible using standard laboratory techniques, leading to a false negative result (26). One way to overcome the problem of drug interference is to use a drug tolerant, precipitation, and acid dissociation (Panda) assay. This procedure involves the addition of excess drug to the sample followed by dissociation of ADA bound to the drug before detection, making it possible to detect both free and bound ADA in samples regardless of the level of drug in the serum (27).

At times, measurement of trough TNFi level and ADA are used to monitor patients with chronic inflammatory disease when the patient has no or little clinical improvement with treatment (28). However, adjustments of dose and intervals are often made without these types of supporting data. Despite

several suggestions, there is no consensus on which ADA detection assay should be used, nor is the optimal drug trough level known. Furthermore, the outcome of ADA and drug level testing varies between different methods (25). In addition, there is a lack of knowledge about how prevalent ADA are in patients with detectable drug trough levels and at what level ADA have a clinically relevant impact (29).

MATERIALS AND METHODS

Patient Cohorts

This study included two cohorts; With patients from (1) a cross-sectional cohort from the Karolinska University Hospital, Stockholm ($n = 270$) and (2) a prospective cohort from the Sahlgrenska University Hospital, Gothenburg ($n = 73$), described in **Table 1**. In the cross-sectional study, all patients treated with infliximab in the rheumatology clinic between January 2017 to December 2017 were recruited ($n = 270$). Several samples were collected per patient at trough prior to an infusion. In the cross-sectional cohort, 43% ($n = 115$) of the included patients had RA, 44% ($n = 118$) had other type of inflammatory arthritis (spondylarthritis, ankylosing spondylitis, psoriatic arthritis, reactive arthritis, enteropathic arthritis, or undifferentiated) and 14% ($n = 37$) had other systemic inflammatory diseases. All patients (except four) in the cross-sectional cohort were switched to infliximab biosimilar InflectraTM in 2017. A total of 63% (169/270) of the patients were concomitantly treated with conventional synthetic disease-modifying anti-rheumatic drug (csDMARD) (156 with methotrexate; 6 with sulfasalazine; 4 with azathioprine; 3 with leflunomide).

In the prospective study, all RA patients initiated on infliximab from the Sahlgrenska University Hospital between 2017 and 2019 were included. Patients in the prospective cohort ($n = 73$) were included prior to initiation of infliximab treatment and followed for up to 1 year. All patients but one (previously treated with infliximab 2011–2012 and golimumab December 2016 to December 2017), were naïve to infliximab treatment at baseline. The majority of patients were concurrently treated with methotrexate, either alone ($n = 52$) or in a combination with salazopyrin ($n = 5$), plaquenil ($n = 2$) or prednisolone ($n = 9$) at the initiation of infliximab therapy. Four patients received concomitant salazopyrin only and one patient was treated with infliximab monotherapy. The patients treated at Sahlgrenska received a dosing schedule as follows; baseline, the second dose was received after 2 weeks, the third dose after 1 month, and thereafter, every 8 weeks. For this cohorts, serum samples were collected at baseline and trough prior to each infusion. The infliximab dosing regimen for this cohort was 200 mg intravenous infusion administered every 8 weeks. Patients that failed to respond were given either an increased dose and/or shortened treatment intervals or were switched to another treatment. This decision was made by the treating physician.

All patients signed informed consent to participate in this study, which was approved by Stockholm Regional Ethical Committee (2013/1034-31/3) and Gothenburg Regional Ethical Committee (1028-15, 2016-02-12).

TABLE 1 | Baseline patient characteristics in prospective and cross-sectional cohorts.

Characteristic	Cross-sectional cohort			Prospective cohort
	RA	Spondyloarthropathies	Other systemic inflammatory diseases	RA
<i>n</i> (%) ^a	115 (42.6)	118 (43.7)	37 (13.7)	73 (100)
Age (median, min-max)	65 (31–83)	51 (20–80)	45 (20–84)	52 (18–89)
Female (<i>n</i> , %)	90 (78)	40 (34)	24 (65)	58 (79)
Concomitant DMARD (<i>n</i> , %)	91 (79)	54 (46)	24 (65)	72 (98)
MTX (<i>n</i> , %)	86 (75)	49 (42)	21 (57)	68 (93)
Other DMARD (<i>n</i> , %)	5 (4)	5 (4)	3 (8)	4 (5)
Infliximab dose (median mg/IV, IQR)	210 (200–300)	250 (200–300)	260 (200–300)	200 (150–200)
Duration of treatment (median, IQR)	9 (4–18)	6 (2–11)	4 (1.5–8)	n.d.
Current smokers (<i>n</i> , %)	9 (8)	13 (11)	3 (8)	9 (12.5)
Ever smokers (<i>n</i> , %)	61 (53)	63 (53)	28 (54)	40 (56)
Never smokers (<i>n</i> , %)	53 (46)	53 (45)	17 (46)	32 (44)
Disease duration (median, IQR)	17 (11–23.5)	14 (6.75–24)	11 (4.5–20)	1.5 (0.5–10.55)
Seropositive (<i>n</i> , %)	83 (72)	n.d.	n.d.	53 (75)
Seronegative (<i>n</i> , %)	32 (28)	n.d.	n.d.	17 (24)
CRP (median mg/L, IQR)	1 (1–4)	1 (1–4)	2 (1–4.5)	4 (1–9.75)
Patient global health assessment (median, IQR)	29 (10–49)	26 (11–52)	42 (11–65)	46 (1–86)
Pain (median VAS, IQR)	24.5 (10.75–46.25)	27 (10–49)	36 (7–66)	47 (2–95)
HAQ score (median, IQR)	0.5 (0.13–1.13)	0.25 (0–1)	0.4 (0.1–1.28)	0.89 (0–2.75)
DAS28 (median, IQR)	3.13 (2.03–4.65)	n.d.	n.d.	3.6 (0.53–7.57)

RA, rheumatoid arthritis; *n*, number; IQR, interquartile range; MTX, methotrexate; DMARD, disease-modifying antirheumatic drug; CRP, C-reactive protein; RF, rheumatoid factor; seropositive is defined as RF positive, CRP positive or both; seronegative is defined as RF and CRP negative; CRP, C-reactive protein, VAS, visual analog scale; HAQ, health assessment questionnaire; DAS28, disease activity score 28; n.d., no data. ^a% of the total patients within the cohort.

Measurement of Clinical Data

Routine clinical examinations of patients were performed by treating rheumatologists at regular intervals according to local clinical practice, the Disease Activity Score in 28 joints (DAS28) was calculated and data registered in the Swedish Rheumatology Quality Register (SRQ). The first visit to evaluate the treatment response usually occurred 3–4 months after initiation of infliximab treatment. Clinical data including disease duration, rheumatoid factor (RF)/cyclic citrullinated peptide (CCP) status, smoking habits, and concomitant csDMARD treatment was retrieved from SRQ and patients' medical records. Seropositivity was defined as being CCP and/or RF positive and seronegativity was defined as being CCP and RF negative.

Assessment of Disease Activity

The composite disease activity score (DAS28) was used. The DAS28 score takes into account the number of swollen and tender in a 28-joint count, the erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP) and patient's assessment of global health on a visual analog scale (VAS-GH). Based on this score, patients were classified into the following categories: remission DAS28 <2.6, low disease activity DAS28 2.6–3.2, moderate disease activity 3.2–5.1, and high disease activity >5.1. The change in a patient's score over time, is expressed as delta (Δ) DAS28, was calculated by subtracting baseline DAS28 score from the respective final post-treatment score. Using the Δ DAS28, patients were categorized as either good, moderate, or non-responders to infliximab treatment according to the European League Against Rheumatism (EULAR) response

criteria as previously described (30). In short, good responders are defined as those with a Δ DAS28 >1.2 and a current DAS28 $\leq$ 3.2; moderate responders by a Δ DAS28 >1.2 and a current DAS28 >3.2 or a Δ DAS28 between 0.6–1.2 and a current DAS28 $\leq$ 5.1; non-responders were defined as those with a Δ DAS28 <0.6 or Δ DAS28 between 0.6–1.2 and a current DAS28 score >5.1. For the cross-sectional cohort, the DAS28 used for analyses were assessed within 3 months from the infliximab trough drug level measurement which was used for the study analyses.

ELISA for Detection of Infliximab Serum Trough Levels

Infliximab trough level was measured in patient sera using an in-house developed and validated ELISA which is used in clinical routine, as previously described (28). Briefly, microtiter plates (Nunc Maxisorp F 96, Thermo-Fisher Scientific, Roskilde, Denmark) were coated with 50 μ L per well of recombinant human TNF- α (200 ng/mL) (R&D Systems, Minneapolis, MN, USA) in 0.05 M sodium carbonate buffer pH 9.6. The plates were put on a shaker at room temperature (RT) for 2 h before being incubation overnight in +4°C. The plates were then washed three times with phosphate buffered saline (PBS) plus 0.05% pH Tween 20 and blocked with PBS + 1% bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA) and 0.05% Tween 20 (blocking buffer) for 1 h at RT. After washing, standard dilutions (0.40–100 ng/mL) of infliximab (Schering Plough, Kenilworth, NJ, USA), internal controls (defined IFX-spiked sera), and patient samples, diluted 1/500 in blocking buffer, in duplicates were added to the plate. Plates were incubated on

a shaker at RT for 1 h and washed four times before addition of alkaline phosphatase (ALP)-conjugated goat anti-human IgG (Fc-specific) (Sigma) diluted 1/10 000 in a blocking buffer. The plates were again incubated on a shaker at RT for 1 h and washed four times. The substrate (p-nitrophenyl-phosphate, 5 mg/mL in 1 M diethanolamine with 0.5 mM Mg, pH 9.8) was added and color development was monitored at 405 nm. The concentration of samples and controls was calculated from the standard curve where the lower and upper limits of quantification were 0.2 and 50 µg/mL, respectively (compensated for serum dilution 1/500).

Inhibition ELISA for Detection of Antibodies to Infliximab

ADA to infliximab was detected using an in-house developed and validated ELISA, which is based on the inhibition of labeled infliximab binding to TNF-coated ELISA plates, as previously described (28). Alkaline phosphatase (ALP) was coupled to infliximab using the Lightning-Link kit (Innova Biosciences Ltd., Cambridge, UK). The ELISA plates were coated with TNF- α , as described for the infliximab ELISA above. A standard consisting of goat anti-human IgG (Jackson Immuno Research) at a final concentration of 1 µg/mL was used. Patient sera were analyzed at final dilutions of 1:10 and 1:100. The standard defined control sera and patient samples were incubated with ALP-conjugated infliximab for 1 h at RT. After an additional wash of the TNF-coated plate, aliquots of standard, controls and patient samples were transferred to the plate in duplicates. After incubation on a shaker for 1 h at RT, the plate was washed and substrate (p-nitrophenyl-phosphate, 5 mg/mL in 1 M diethanolamine with 0.5 mM Mg, pH 9.8) was added, and color development was monitored at 405 nm. The results were transformed to percentage inhibition by normalization of the OD of the samples to that of the standard (100% inhibition) using the formula $(OD_{blank} - OD_{sample}) / (OD_{blank} - OD_{standard}) \times 100$. The lower limit of detection was set to the value plus two standard deviations obtained from measurements of normal control sera. Due to free infliximab interference with the assay, ADA could only be detected in the absence of the drug. ADA analysis was therefore limited to patient samples where serum infliximab was undetectable (<0.2 µg/mL).

ADA Detection With the Precipitation and Acid Dissociation (Panda) Method

Presence of ADA to TNF- α inhibitors was assessed using the Panda method described by Zoghbi et al. (27) on the Meso Scale Discovery® (MSD) platform. The Panda assay has demonstrated high sensitivity to detect ADA in the presence of a high concentration of drug (drug tolerant). The assay is therefore, more suitable for immunogenicity assessment of patient samples that contain detectable levels of infliximab which interfere with the detection limit of ADA in other immunoassays. Serum from 40 healthy donors (Stockholm blood center, Sweden) was collected to prepare a pool of normal healthy sera (NHS) to be used as negative control (NC) in the Panda assay. Informed written consent was given by all the donors. Human anti-IFX (clone HCA233, BIO-RAD) was prepared in NHS to be used

as a high positive control (6 µg/mL) and low positive control (1 µg/mL). The Panda assay was validated in-house before analysis of study samples. A plate specific, or floating, cut-point was applied to allow a 5% false positive rate in the screening assay. For each plate, the cut-point is calculated by multiplying the NC value of the plate with a normalization factor (NF), which is the average of all NC values obtained from six assay plates in the validation process (three operators running one plate each over two days). To resolve the problem of interference of soluble infliximab in the sera, 25 µL of patient samples were diluted 1:2 in 2% bovine serum albumin (BSA, Sigma) in PBS containing 10 µg/mL infliximab (Remicade®) in a polypropylene V-bottom plate (Thermo Scientific) in duplicate wells. ADA present in the sera were then able to bind to the excess soluble infliximab, forming immune complexes during a 1 h incubation at RT at 450 rpm. Formed immune complexes were then precipitated by addition of 50 µL 6% polyethylene glycol (PEG, Aldrich) solution to each sample (3% PEG in the plate) during overnight incubation of the plate at 4°C. The following day, the plate was centrifuged at 3,724 x g for 30 min to precipitate immune complexes into a pellet. After the supernatant was discarded, the pellets were re-suspended with a 3% PEG solution and the plate centrifuged at 3,724 g for 20 min. This step was repeated once more. After the final centrifugation step, pellets were re-suspended with 250 µL 0.25 M acetic acid, pH 3.0, to get a minimal required dilution of 1/10. Samples were thereafter transferred to a high binding carbon plate (MSD) by adding 50 µL of each sample in duplicates. The plate was thereafter incubated for 1 h at RT at 450 rpm. Following incubation, the plate was washed once with 300 µL 1x wash buffer (1xPBS, 0.1% Tween, pH 7.4) and blocked with 300 µL casein in PBS pH 7.4 (Thermo Scientific) for 1 h at RT at 450 rpm. After additional washing, 50 µL of the master mix containing 0.5 µg/mL of Sulfo-Tag conjugated infliximab (labeling ratio of 20:1 between Sulfo-Tag and IFX) (MSD GOLD™) in 2% BSA in PBS was added to the samples and incubated for 1 h at RT at 450 rpm. After the final incubation, the plate was washed once and within 5 min of adding 150 µL read buffer T (2x) (MSD) the plate was read on MESO Quickplex SQ 120 (MSD). By electrical discharge, the electrons are excited and a stable light signal is generated. This electrochemiluminescence (ECL) signal is proportional to the amount of ADA in each serum sample. A signal to background ratio was calculated by dividing the average ECL signal from an individual sample by the average ECL signal of the negative control (NHS) and expressed as relative ECL (RECL). The coefficient of variation value of $\leq 25\%$ was accepted as the maximum variation between duplicates.

Neutralizing Anti-drug Antibody Analysis

The neutralizing capacity of the ADA were measured using iLite™ infliximab NAb bioassay (Biomonitor) in 35 serum samples from 29 patients in the cross-sectional study, who previously detected ADA-positive by the ELISA method. The protocol was carried out according to the manufacturers' instructions. In short, the assay uses division-arrested TNF- α sensitive cells to measure TNF- α bioactivity. Transcription of the luciferase gene occurs when TNF- α binds to the TNF- α

receptor and the luciferase activity is inversely proportional to the amount of infliximab present in the sample. Luciferase activity was measured using GloMax Luminometer (Promega) and the antibody neutralizing activity was normalized to Renilla (31). The assay's drug tolerance is 0.65 µg/mL.

Statistical Analysis

To compare continuous variables, Mann-Whitney *U*-test for independent groups was used. All reported *p*-values were two tailed, and a *p*-value of < 0.05 was considered statistically significant. Correlation analysis was performed using Spearman's rank correlation. Receiver operating characteristic (ROC) curves were constructed to assess the cut-off of drug level- and ADA threshold values were based on EULAR response criteria (good responders vs. non-responders). The cut-off points were calculated on the basis of the best trade-off values between sensitivity and specificity. Statistical calculations were performed using Prism software (GraphPad Inc. version 8).

RESULTS

Patient Characteristics

Baseline patient characteristics of the infliximab-treated cohorts are summarized in Table 1. Patients in the prospective cohort had a median disease duration of 1.5 years (IQR 0.5–11) at baseline. In contrast, patients in the cross-sectional cohort had a wide-ranging disease and treatment duration at the time of baseline sampling. The study time points of sample collection for the prospective cohort and diagnoses for the cross-sectional cohort are illustrated in Figures S1, S2.

Serum Infliximab Levels

The sIFX values in the prospective study were found to be higher at the beginning of the treatment period when the intervals were 2 and 4 weeks between infusions. It then reached a stable range with a mean of 1.8 µg/mL [standard deviation (SD) of 2.0 µg/mL] from week 14 when infusions were 8 weeks apart (Figure 1). To determine an optimal sIFX level defined as the range of trough levels that had the sensitivity and specificity to predict good EULAR response, Receiver Operating Characteristic (ROC) curves were done for both the cross-sectional cohort and the prospective cohort (Figure 2). On the analysis, the therapeutic ranges were distributed in a U-shaped curve at the lower and upper ends. Therefore, each end were analyzed separately. Firstly, the lower end was determined on all samples with a sIFX under 3.8 µg/mL, corresponding to the mean + SD for the stable range. With an area under the curve (AUC) of 0.8, both cohorts gave a predictive optimal value of 0.95 µg/mL indicating this is the lowest sIFX level which you can expect the treatment to have good effect. For ROC analysis, the EULAR response was used as the clinical outcome and for the prospective cohort both EULAR response and remission status were used.

Using the previously suggested lower limit for optimal therapeutic effect of 1 µg/mL sIFX (32) and the data from the ROC analyses (taking into account the interassay variations), an approximate lower end of an optimal range was set to 1 µg/mL. Secondly, an upper end for the optimal range was estimated

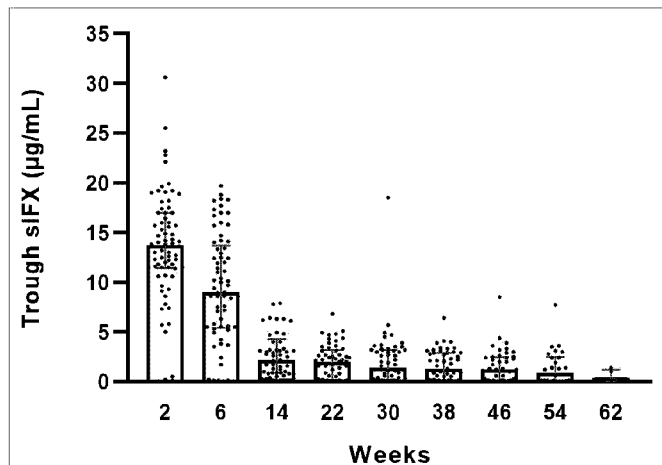


FIGURE 1 | Trough serum infliximab levels (sIFX) for all RA patients in the prospective cohort and variation over time. Total number of weeks on treatment is shown on the x-axis. The levels are presented as median (bar) and interquartile range (red). Number of individuals at each time point is illustrated in Figure S1A.

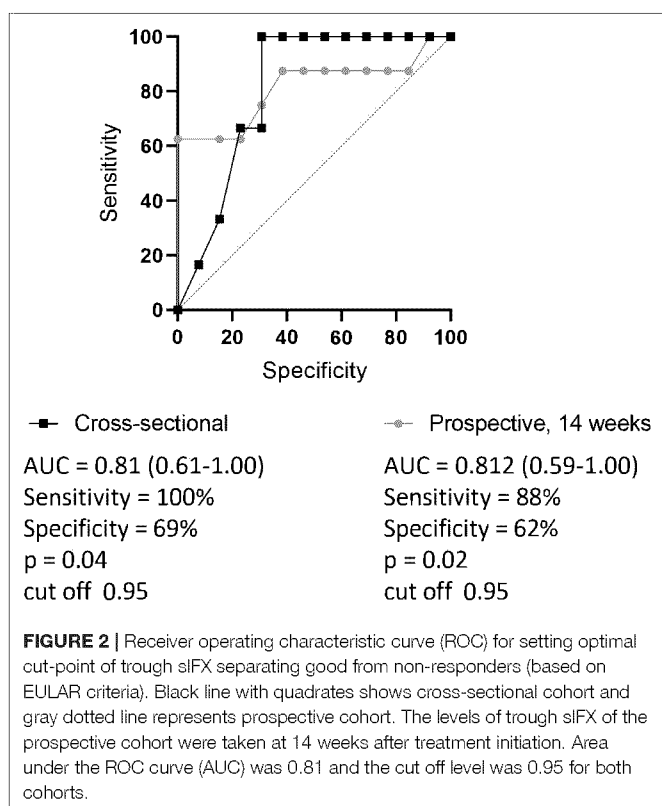
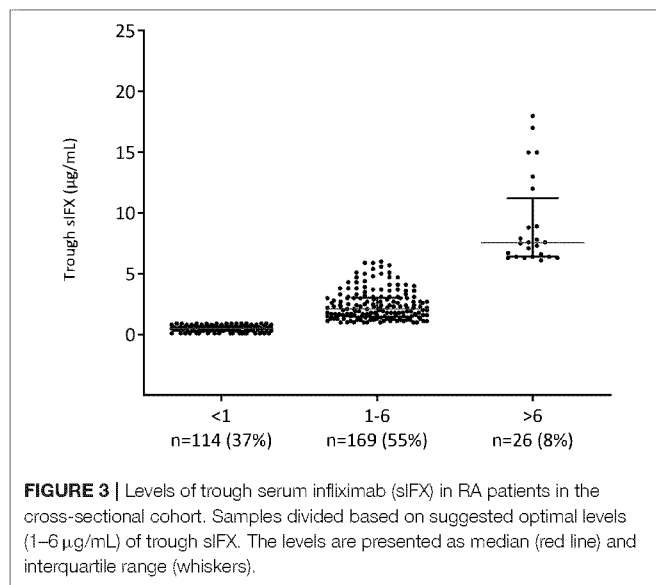


FIGURE 2 | Receiver operating characteristic curve (ROC) for setting optimal cut-point of trough sIFX separating good from non-responders (based on EULAR criteria). Black line with quadrates shows cross-sectional cohort and gray dotted line represents prospective cohort. The levels of trough sIFX of the prospective cohort were taken at 14 weeks after treatment initiation. Area under the ROC curve (AUC) was 0.81 and the cut off level was 0.95 for both cohorts.

using the mean + 2xSD of the stable sIFX period (from week 14 onward) in the prospective cohort, which was found to be 6 µg/mL sIFX in trough. This is in line with what has been shown previously for other TNFi (28, 33).

Using this range (1–6 µg/mL) as optimal, only 55% of the samples (*n* = 169) from RA patients in the cross-sectional cohort



had an optimal sIFX, 37% had lower than 1 µg/mL, and 8% had higher than 6 µg/mL (**Figure 3**). The proportions were similar for the other non-RA infliximab treated patients in this cohort, where 65% of the samples ($n = 273$) had an optimal sIFX, 24% had lower than 1 µg/mL, and 11% had sIFX higher than 6 µg/mL.

In the prospective study, only 54% of all samples ($n = 159$) collected from 65 patients from week 14 onwards had sIFX levels within the suggested optimal range, 42% of the samples had sIFX levels <1 µg/mL and 4% had a level higher than 6 µg/mL.

As a comparison, five samples which had been mistakenly collected directly after the infusion, were found to have a mean sIFX level of 46 µg/mL (median 48 µg/mL, range 42–52 µg/mL). These were not included in the analyses.

Clinical Relevance of Serum Infliximab Levels

Clinical variables were used to analyse the correlation between sIFX and treatment response in the cross-sectional cohort. For RA patients in the cross-sectional cohort, if a DAS28 score was available within 5 months of the time of sampling ($n = 34$) this was also used to evaluate the correlation. Using the DAS28, patients were then classified as previously described using the EULAR response with good, moderate or non-response categories (30). The sIFX levels were significantly lower in the non-responders group compared to the good responders (**Figure 4**). In the moderate responder group, there is one outlier case with a high sIFX level (15 µg/mL), indicating that also a high drug level might be associated with less optimal therapeutic response. The cross-sectional cohorts' sIFX levels were divided into the categories of under 1, 1–6, and above 6 µg/mL and analyzed for correlation with clinical variables, including DAS28 ($n = 34$) (**Figure 5**) and delta-DAS28 ($n = 34$) (**Figure S3**) for the RA patients. Variables on patient global assessment (PGA) (**Figure S4**), patient pain assessment (VAS) (**Figure 6**), CRP (**Figure 7**), and ESR (**Figure 8**), were analyzed for all infliximab

treated patients in the cross-sectional cohort. Clinical variables were significantly worse in patients with a sIFX trough level below 1 µg/mL compared with those with a drug level between 1 and 6 µg/mL (DAS28 ($p = 0.01$), PGA ($p = 0.01$), CRP ($p = 0.002$), and ESR ($p = 0.004$)).

Only a few patients had sIFX >6 µg/mL ($n = 8$, of which 3 were RA patients) and both CRP ($p = 0.03$) and ESR ($p = 0.007$) were significantly worse in these compared with those with a drug level between 1 and 6 µg/mL. One possible reason for a very high sIFX level could be the presence of more severe disease at treatment initiation and therefore, dosing intervals are shortened or doses increased in attempt to manage the patients' symptoms. Patients from the cross-sectional cohort with sIFX >6 µg/mL did indeed have both a higher DAS28 and a higher treatment dose (**Figure S5**).

In the prospective cohort the correlation between EULAR response and sIFX levels varied between time points, but collectively, the non-responders had a significantly lower sIFX level than the good responders (**Figure S6**).

Proportion of Patients With Free ADA

Given that the drug sensitive ELISA method only reliably detects free ADA, not bound in immune complexes, it is recommended that only samples with sIFX below the drug sensitivity of the assay (<0.2 µg/mL) are tested. Of the 73 patients in the prospective cohort, 44 (60%) had a sIFX <0.2 µg/mL at some time point allowing the sample to be tested for ADA with ELISA. Of these 44, 86% ($n = 38$), or 59% of the whole cohort, were positive for ADA. Compared to this early RA cohort, a lower proportion of the cross-sectional cohort, or 14% (37 of 270 patients) of the whole cohort were found to be ADA positive in samples with sIFX levels <0.2 µg/mL (42 of 270), probably reflecting a selection bias of patients who continue on infliximab.

ADA in Samples With Detectable sIFX Level Using Panda

With the drug-tolerant Panda method, ADA can be detected regardless of drug level, and therefore we could determine the frequency of ADA in samples with sIFX >0.2 µg/mL. A selection of samples from the cross-sectional cohort with sIFX levels ranging 0.2–7 µg/mL were analyzed with Panda (**Figure 9**). Only a 26 additional ADA positive samples were identified with this method and for the cross-sectional cohort all had a sIFX levels under 1 µg/mL. Similar results were found in the prospective cohort, with three interesting exceptions described more in detail below (**Figure 10**). A significant reverse correlation was found between ADA and sIFX levels in both cohorts ($r^2 = -0.4$, $p < 0.0001$ for cross-sectional cohort and $r^2 = -0.7$, $p < 0.0001$ for the prospective cohort).

In the prospective cohort, the kinetics of total (free and IC bound) ADA development was determined using the drug-tolerant Panda assay. ADA was found to develop early after treatment initiation and the first positive samples could already be identified prior to the 2nd infusion at week 2. The incidence of ADA increased until the 6th infusion at week 30, but cases with their first positive sample were detected up until the 9th infusion at week 54. The cumulative prevalence of ADA increased

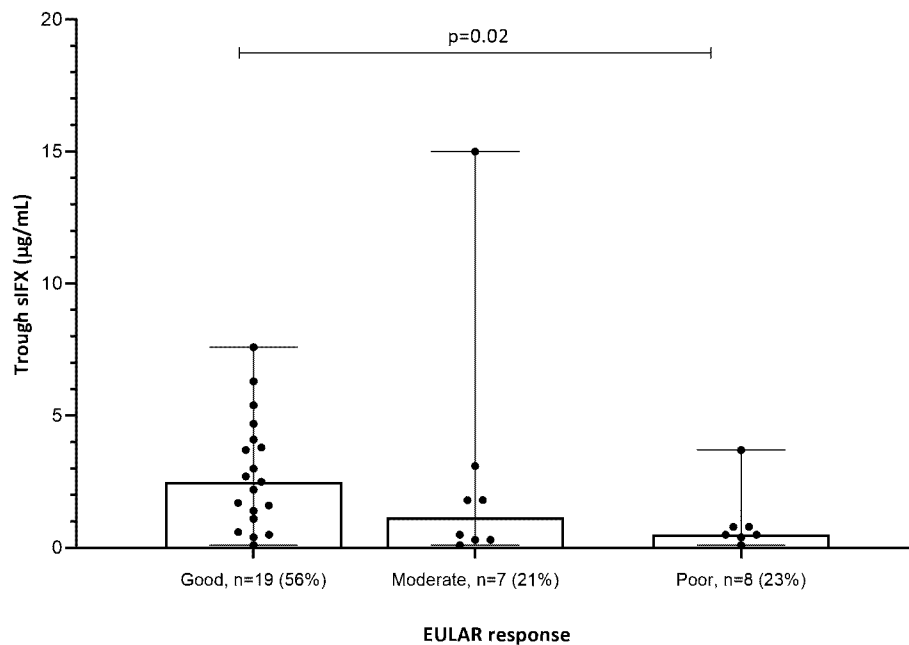


FIGURE 4 | EULAR response in relation to sIFX levels for the subgroup of RA patients in the cross-sectional cohort ($n = 34$). The sIFX levels were significantly lower in the non-responders group compared to the good responders. Percentage of patients in each EULAR group given in brackets on x axis. The levels are presented as median (bar) and interquartile range (whiskers).

up to the 6th infusion at week 30 before beginning to decrease (Figure S7).

ADA Results From ELISA Compared to Panda

Of the 108 selected prospective samples tested on both Panda and ELISA methods, there was a 72% ($n = 78$) agreement between assay results [68% ($n = 74$) confirmed positive and 4% ($n = 4$) confirmed negative]. However, there were some discrepancies between assays, with 20% ($n = 22$) positive in Panda but negative with ELISA, and 7% ($n = 8$) positive with ELISA but negative with Panda.

Of the five patients with sIFX levels $<0.2 \mu\text{g/mL}$ that were negative for ADA with ELISA, one was confirmed positive with Panda.

ADA in Complex With Drug Can Be Detected Earlier Than Free ADA

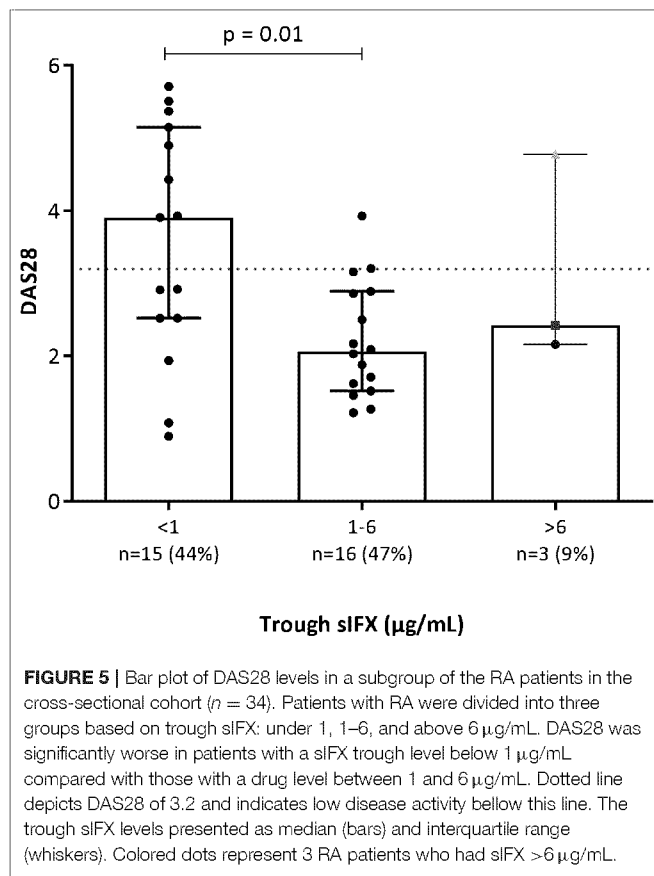
In the prospective cohort, 45 patients from whom we could retrieve samples which were taken prior of being identified as ADA positive with ELISA, were tested for ADA with Panda. Of these, 17 patients (38%) were found to be ADA positive at an earlier time point. These were on average detected 20 weeks (median 16, range 4–41 weeks) before the sIFX levels were low enough for detection of ADA with ELISA.

Three unique cases were identified which were found to be ADA positive despite very high sIFX and therefore serial samples from these cases were analyzed (Table 2). The first case was newly diagnosed and was trialled on methotrexate for 2 months without effect before initiating the first infliximab infusion. This patient

was highly ADA positive (Panda RECL = 10) already at the 2nd infusion, just 2 weeks after treatment initiation. As the sIFX was $5.8 \mu\text{g/mL}$, this patient would not have been tested for ADA with ELISA until the next infusion at week 6, when the drug level was $<0.2 \mu\text{g/mL}$. In this sample the ELISA test could then confirm ADA positivity. An infusion reaction was noted at the third infusion, which worsened despite precautions taken before the 4th infusion. The patient discontinued treatment after the 4th infusion due to infusion reactions and lack of treatment effect. The patient was switched to rituximab treatment.

The second case was also newly diagnosed and was treated with prednisolone and methotrexate for 6 months prior to their first infliximab infusion. This patient was also highly ADA positive (Panda RECL = 75) already at the 2nd infusion (week 2) and with a sIFX level of $12.2 \mu\text{g/mL}$. This patient was tested for ADA with ELISA in a subsequent sample taken at the 6th infusion, 28 weeks after treatment initiation with a serum infliximab of $0.8 \mu\text{g/mL}$. At this point, the patient was found to be ADA negative with ELISA and still positive with Panda (RECL = 22). At the 7th infusion, the sIFX level had decreased to $0.2 \mu\text{g/mL}$ and at this time point, the patient was first detected as ADA positive using the ELISA. This was 37 weeks after the first ADA positive detected using the Panda method. At this time, the patient experienced good effect of the treatment with a DAS28 of 2.8. A year later the treatment effect declined and the interval was shortened to 6 weeks without effect. The patient was then switched to etanercept.

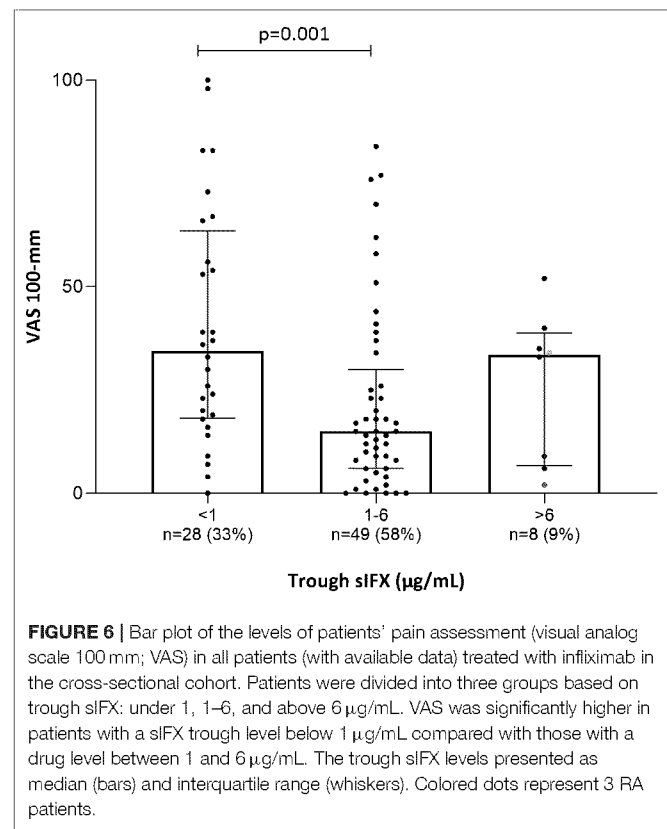
The third case was newly diagnosed with low disease activity (DAS28 at treatment initiation 3.02) and was initially managed with intra-articular cortisone injections. Methotrexate



was initially used but terminated due to a herpes zoster infection, which was managed with vaccination and anti-viral therapy. Infliximab treatment was started 2 years later, initially with good effect. This patient was ADA negative in Panda (RECL = 1) prior to the 2nd infusion, but highly positive prior to the 3rd infusion (RECL = 15) when the sIFX was still high (3.5 $\mu\text{g/mL}$). In the sample taken prior to the 4th infusion, sIFX was 0.1 $\mu\text{g/mL}$ and ADA positive with ELISA, and Panda however, the Panda positive level had dropped to 5 RECL. With the subsequent infusions the ADA increased to very high levels (RECL = 394 prior to the 8th infusion). An infusion reaction was documented after the 5th infusion and fevers after infusion 6 and 7, followed by facial skin reactions after infusion 8. The infusion reaction worsened after the 9th infusion and the patient was then shifted to adalimumab.

Clinical Threshold Value for ADA

When ADA positivity measured by Panda was stratified by patients' EULAR response categories good, moderate or non-responder, a notable difference between the proportion of ADA positive patients was observed between groups (Figure S8). However, this was less clear over time (Figure S9). Overall, a higher mean ADA value was found in the group with EULAR non-responders (15.7 RECL), whereas 79% of patients in the moderate and good EULAR response groups had a ADA result of <3 RECL. This suggests that there is a clinical threshold value for ADA of around 3 RECL, using this method. A ROC analysis using



the RECL values from prior to the 4th infusion (week 14) and the overall EULAR outcome (good and non-responders) of the patients in the prospective study, confirmed a clinical threshold value of 3.25 (AUC 0.9, $p = 0.028$) (Figure S10). When remission outcomes were also used, the clinical threshold value was 1.48 (AUC 1.0, $p = 0.01$) (Figure S10).

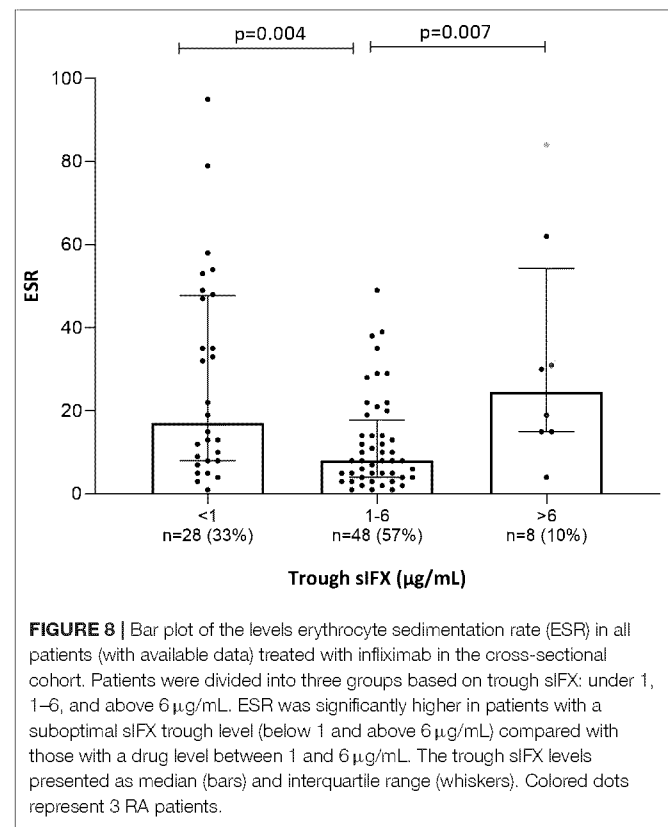
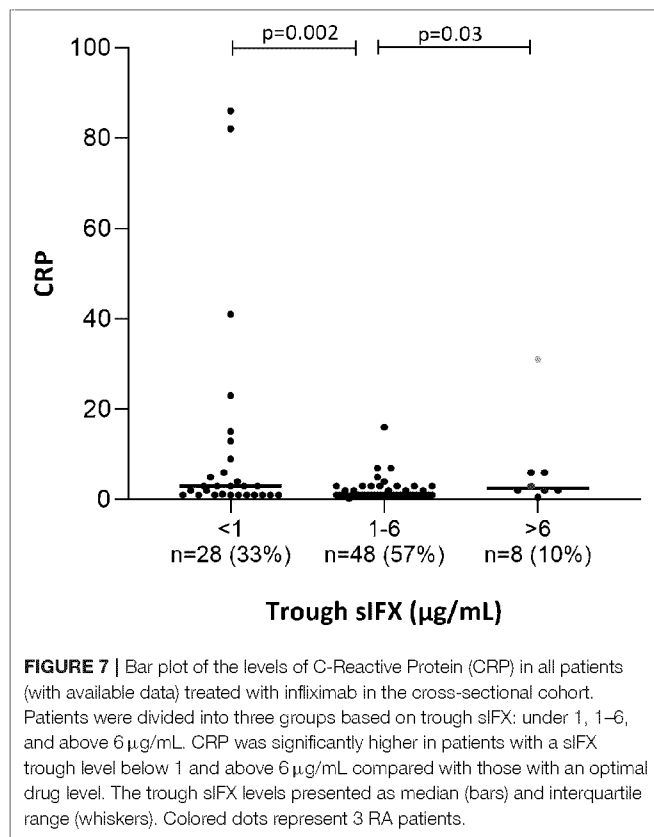
ADA Correlation to Seropositivity and to Smoking Status in RA Patients

In the prospective cohort, the level of ADA, as measured with Panda method, had a positive correlation with serological status of the patients (Figure S11). Over time, the most prominent correlation was noted prior to infusion 8 (Figure S12). This effect was due to the RF and when analyzed separately, patients who were RF positive had significantly higher ADA RECL values (Figure 11), seen also over time (Figure S13).

The smoking status of the patients was available for 54% of patients, and a highly significant correlation in ADA levels was seen between ever smokers ($n = 84$) and never smokers ($n = 63$) (p -value = 0.0001) (Figure 12), a pattern that could also be noted over time (Figure S14).

ADA to Infliximab Had Neutralizing Capacity

In the cross-sectional cohort, serum samples from 29 patients who tested positive for free ADA with the ELISA, were further tested for neutralizing capacity using the iLite bioassay. Nineteen of those patients (66%) tested positive for neutralizing ADA



against infliximab. There was a strong correlation between assays ($r = 0.9$, $p < 0.001$), particularly in those with RECL values above the clinical threshold value (RECL > 3) (Figure S15).

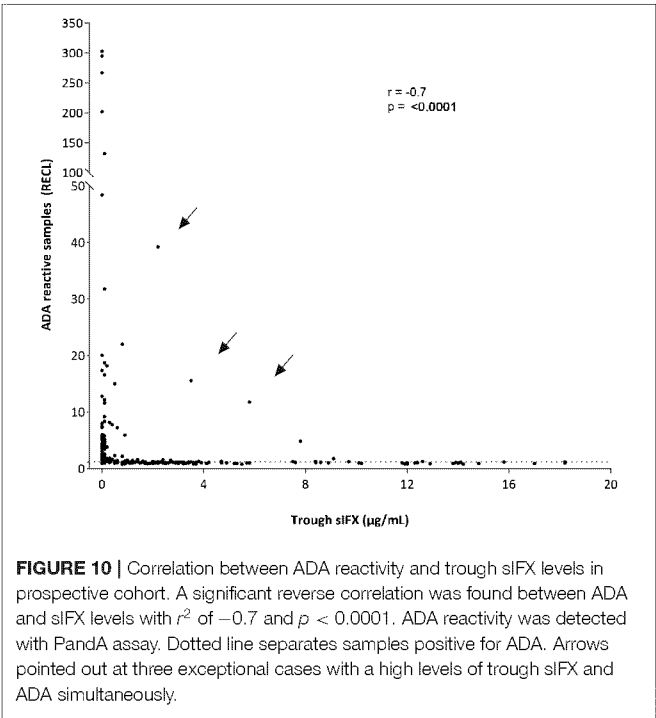
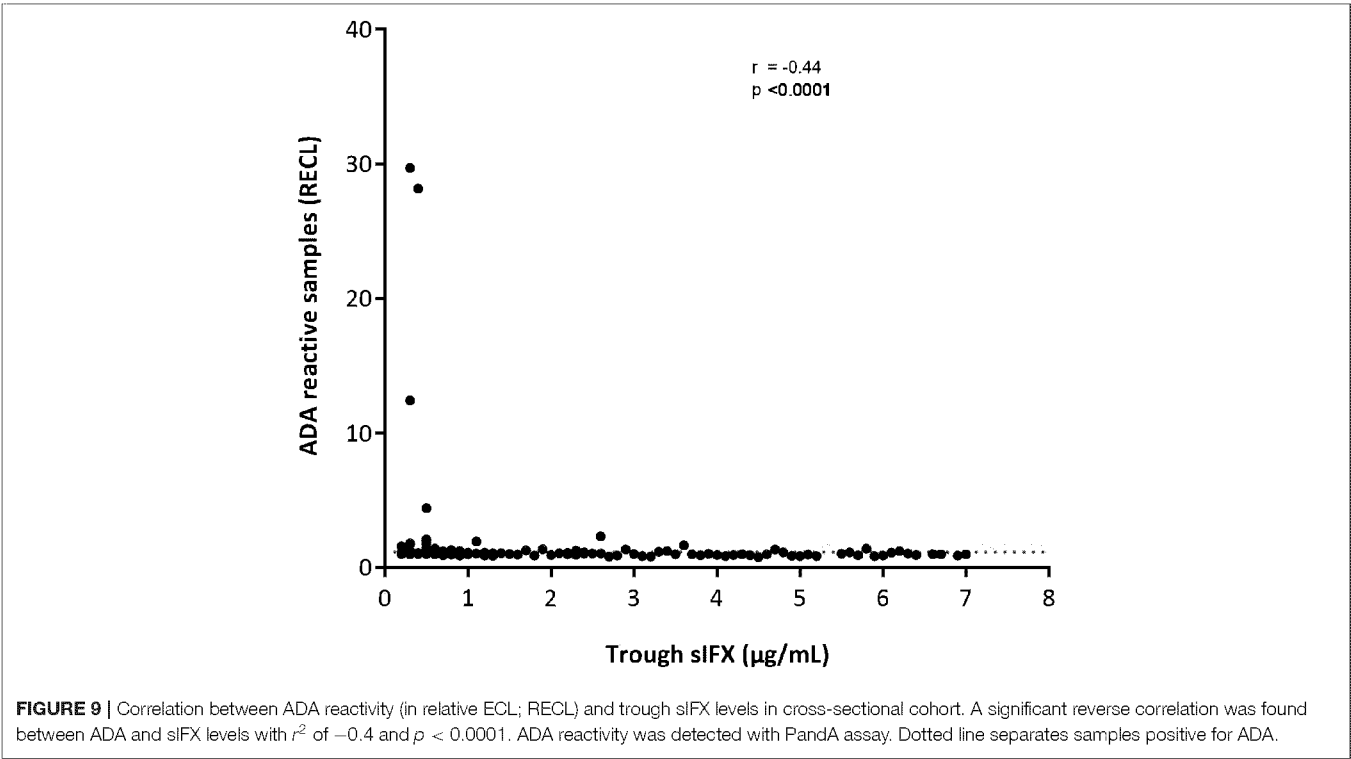
Transient and Persistent ADA

In the prospective cohort, 39 patients were identified as ADA positive with Panda method, and 29 of these had one or more samples taken after they first were determined to be positive. Therefore, they could be assigned as transient or persistent positive. The majority of these were persistent positive ($n = 22$, 56%) and had a higher peak RECL (median of 5.1, IQR 2.2–14.4) compared to the transient positive ($n = 7$, 18%) with a peak RECL value of 1.2 (IQR 1.2–1.3). Notably these groups were on either side of the suggested clinical threshold value for ADA (1.48–3.26).

Therapeutic Drug Monitoring Test Algorithm

Based on the results of this study, we suggest a treatment algorithm for interpreting the results of the sIFX and ADA tests (Figure 13). We propose as a first step, measuring the sIFX level, which allows patients to be allocated to one of four groups. **Group 1** and **Group 2** consists of patients with either undetectable infliximab levels ($<0.2 \mu\text{g/mL}$) or low sub-therapeutic sIFX trough levels ($<1 \mu\text{g/mL}$) and should therefore be tested for ADA using an ELISA or drug-tolerant assay (Panda), respectively. Not all patients with low sIFX levels

can be explained by ADA and an increased infliximab dose could therefore be considered for those found to be ADA negative at this step. For patients found to be ADA positive, other treatment options should be considered depending on the ADA level and its relationship with the clinical threshold value. If the sample is ADA positive with a RECL value >3 , the patient should be switched to another treatment due to the likely treatment inhibition and loss of effect owing to ADA. If the ADA level <3 RECL, there may still be an effect of the treatment despite the presence of ADA and it is possible the patient may only be transiently positive. In these cases, one could continue treatment, but consider repeat testing for ADA again in the coming months to monitor for ADA persistence using the quantitative Panda method. **Group 3** includes patients whose sIFX falls within the optimal sIFX range. Therefore, no action is needed if the disease is in remission or demonstrating an acceptable response to treatment. If the disease is not in remission despite sIFX being within optimal range, a drug with another mode of action might be needed. **Group 4** includes patients with a higher than normal trough sIFX level ($>6 \mu\text{g/mL}$). This might reflect a situation where the drug is not consumed as expected. Therefore, lowering the dose should be considered if the patient is responding well to infliximab. However, treatment options with another mode of action could also be considered for patients who have not responded well and have disease activity. The three exceptional cases with early high ADA titers were excluded from this algorithm and patients such as these, will only be detected



if a first tier of the ADA testing is with the PandA method prior to the second infusion. As these patients later developed infusion reactions, one might want to consider changing to an alternative treatment already at this early time point.

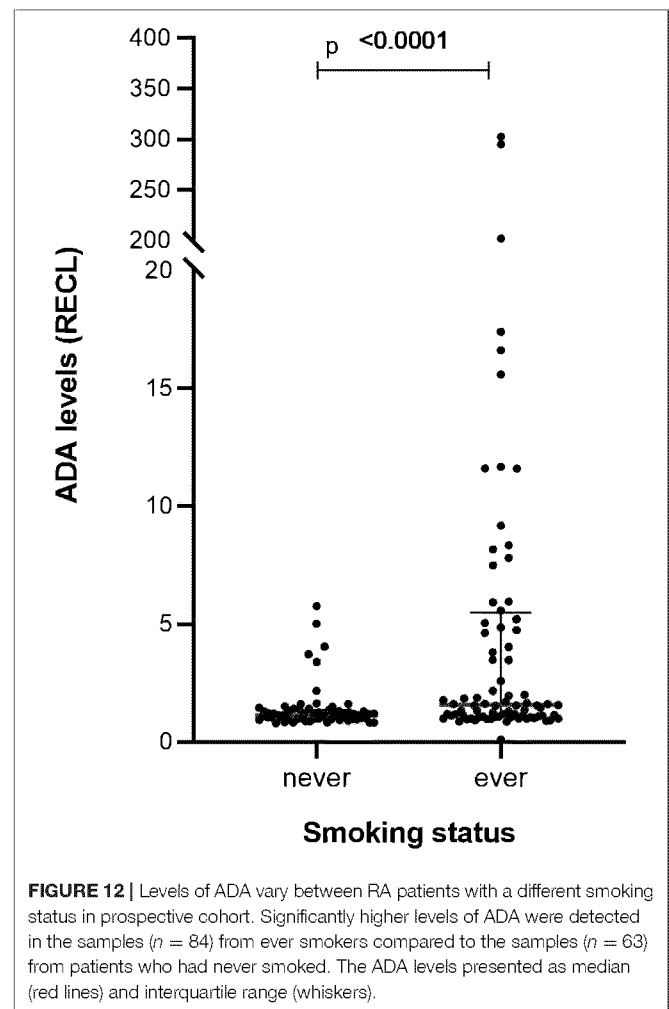
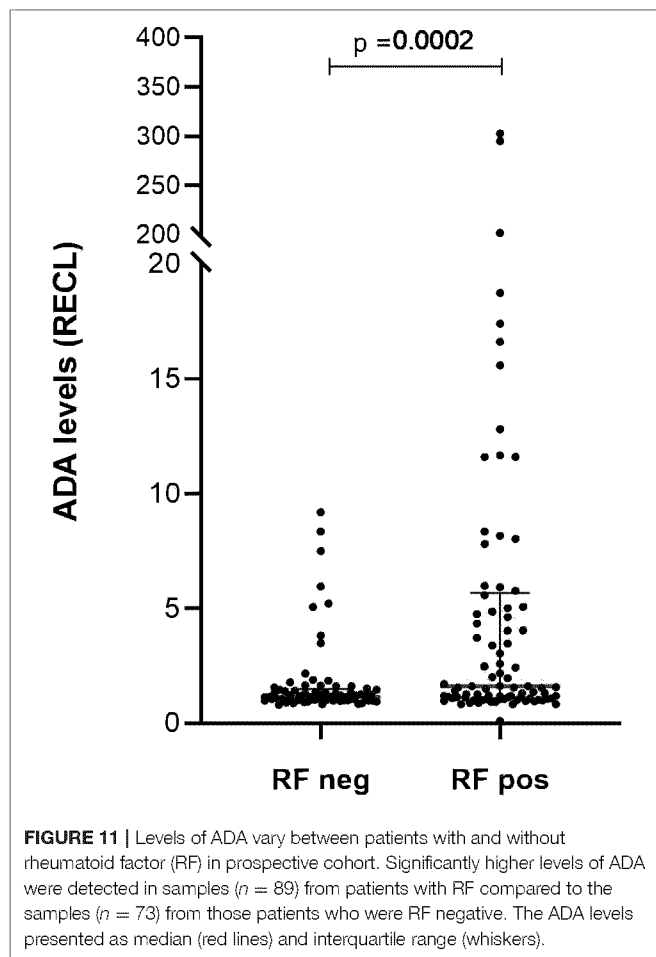
TABLE 2 | Three unique cases which were found to be ADA positive despite very high siFX.

	Infusion of Infliximab							
	2nd	3rd	4th	5th	6th	7th	8th	9th
Case #1								
PandA, RECL	10	16	31	n/a	n/a	n/a	n/a	n/a
siFX	5.8	<0.2	<0.2	n/a	n/a	n/a	n/a	n/a
ELISA	n/a	pos	pos	n/a	n/a	n/a	n/a	n/a
Case #2								
PandA, RECL	75	48	39	39	22	18	12	12
siFX	12.2	8.6	2.6	2.2	0.8	0.2	0.1	0.1
ELISA	n/a	n/a	n/a	n/a	neg	pos	pos	pos
Case #3								
PandA, RECL	1	15	5	23	267	355	394	n/a
siFX	15.7	3.5	0.05	0.0	0.0	0.0	0.0	n/a
ELISA	n/a	n/a	pos	pos	pos	pos	pos	n/a

Serial samples from these cases were analyzed. ADA was measured by Elisa and PandA assays. In all of these cases ADA have been detected with PandA already at the 2nd or 3rd infusion. Bold indicates positive ADA values.

DISCUSSION

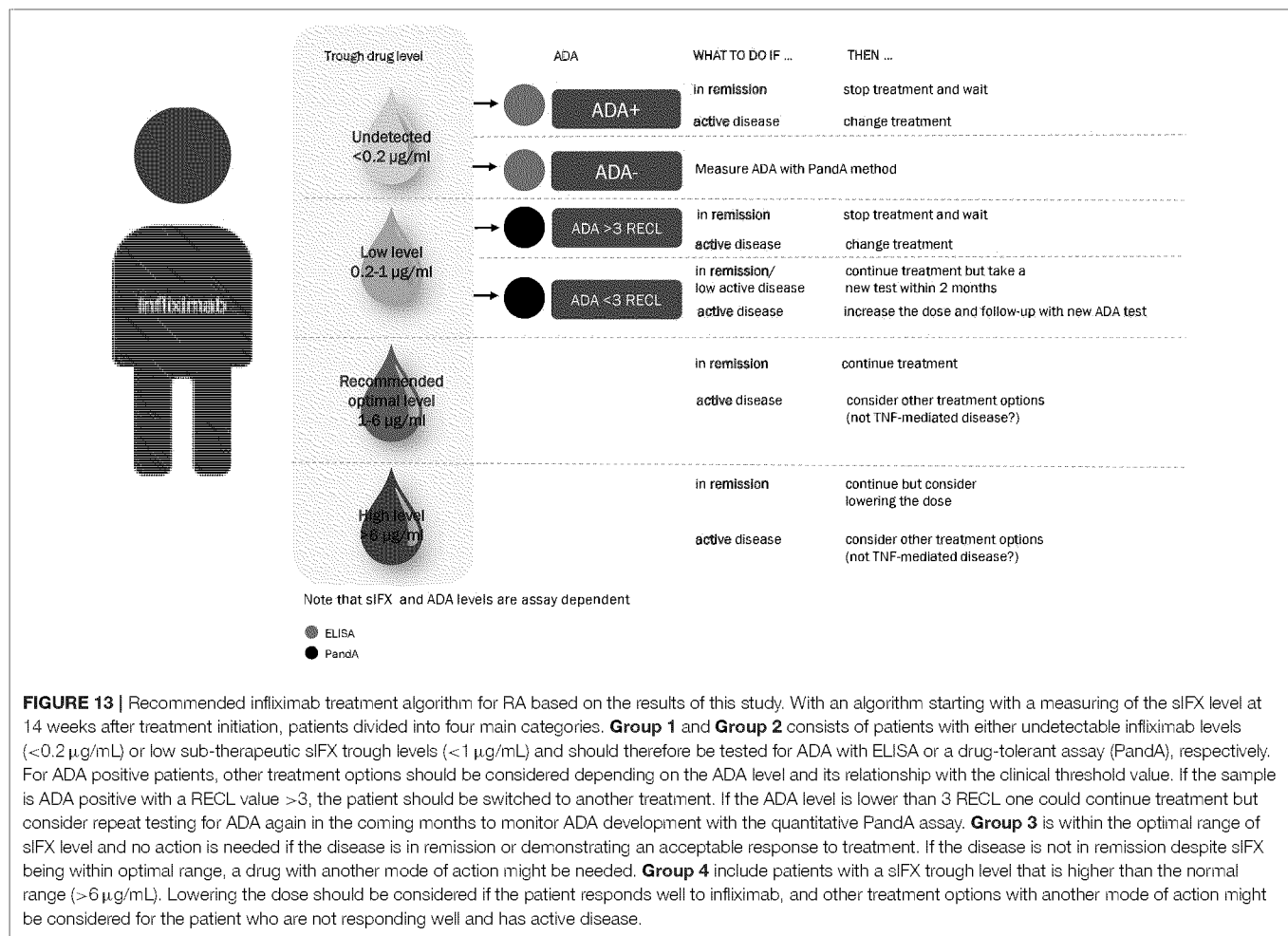
Unwanted immunogenicity is a growing challenge for management of patients treated with biopharmaceuticals. To be able to provide the highest quality of care, the consequences of ADA on safety and treatment efficacy have to be addressed. However, the benefit of integrating assessment of drug level and ADA in clinical practice has been questioned for several reasons,



including the incomprehensive conclusions of test results with very drug-sensitive ADA assays. Current recommendations advise starting with a screening of drug level and then only testing for ADA in samples with less sIFX than tolerated by the drug sensitive ADA assays (with our in-house ELISA corresponding to $<0.2 \mu\text{g/mL}$) (28, 34). This is challenged by introducing an ADA assay that is insensitive to drug level. This assay enables reliable ADA testing at any time, not only in trough, but also requires that a new drug level prompting ADA testing is established. Moreover, while there are already existing guidelines for suggested target trough concentrations for sIFX in patients with inflammatory bowel disease (35), the optimal sIFX level in rheumatic diseases is unknown. The aim of this study was to estimate an optimal sIFX range, set a recommendation for when to test for ADA using a drug-tolerant assay and to provide a clinical threshold value for this assay using two large cohorts of infliximab treated patients.

By correlating clinical data with sIFX levels and establishing a suggested optimal range, we found that a substantial proportion of patients were on a suboptimal treatment regimen in a cohort of patients that had been treated for several years. Since sIFX trough levels are highly variable between patients, the initial screening of sIFX trough level is already able to give an indication

of treatment efficacy. Collectively, our results from a range of IFX treated diseases in this cohort suggest that a trough sIFX concentration between 1 and $6 \mu\text{g/mL}$ is possibly associated with better treatment effect and therefore, a potential proxy for an optimal sIFX level. The added value of identifying patients with either too low or too high drug levels is evident, since these two groups have different reasons for their sub-optimal levels which need to be managed in different ways. As of today, although there are several recommendations, there is no consensus on the range of sIFX trough concentration at which patients achieve an optimal therapeutic response. These ranges can also vary depending on what drug, indication and assays are used and therefore, would need to be established for each method (25, 28, 36). As previous studies show, low trough levels of sIFX predict disease activity in RA and as confirmed in this study is most probably due to blocking of the therapeutic effect by ADA (37). These patients might benefit from switching to another TNFi alternative for which the patient has not developed any resistance against. This is in line with the experience with rituximab treatment, where patients developing ADA can regain treatment effect when switching to another anti-CD20 drug (38).



Patients with too high drug level were also not doing as well as those within the range of 1–6 µg/mL and therefore it is sensible to analyze these as a separate group. Using the drug tolerant Panda method, we could show that this is not ADA bound in immune complexes. Therefore, the poor treatment effect is likely attributed an initial primary treatment response, and patients have been given a higher dose in attempt to improve this. However, since the drug is not fully consumed before the next infusion, in these patients, the TNF may not be the major driver of disease severity and therefore, may benefit from a therapeutic drug with another mode of action. Similar findings are available for adalimumab treatment (36), where patients with low drug level still had good effect with another TNFi whereas those with a higher drug level did not. There are no biomarkers available that can predict what mode of action the drug should have to achieve optimal treatment response in an individual patient. Indeed, a recent study by Berkhout et al. showed that the level of circulating TNF-α during adalimumab treatment did not predict clinical response in RA and thus cannot be used as a biomarker for treatment discontinuation (39). In practice, clinicians change intervals and dose without having the drug level and ADA information, but this may be optimized with

the understanding of how to interpret the drug level and ADA data (40).

One argument against drug level and ADA testing to monitor treatment response in clinical practice is that treatment failure is evident as disease break through. However, in the cross-sectional cohort with >2 years of treatment, we observed that only two-thirds of the patients, at any investigated time points, had the suggested optimal drug concentration (1–6 µg/mL), leaving 30% of the patients with either too low or too high drug levels. For the patients with too low levels (<1 µg/mL), ADA was detected in the majority of serum samples with a drug level below 0.2 µg/mL, meaning that they have been on treatment for a long period without a biologically relevant effect of the drug. ADA was also identified in samples with measurable drug levels using the drug-tolerant assay, but rarely in those with a drug level above 1 µg/mL. Thus, by testing sIFX and ADA in clinical practice, up to one-third of the patients might receive a more optimal treatment. By using Panda for detection of ADA bound in complex with the drug, we identified more ADA positive cases. In addition, this method can be used if one needs to know the ADA status right after infusion, for example to determine if infusion reactions or serum sickness might be due

to ADA (41, 42). When comparing the ADA positivity between the two cohorts included in this study, it indeed shows that to some extent ADA positive patients are identified clinically. This is shown, as expected, by a lower frequency of ADA in the cross-sectional cohort (34% of ADA positive samples compare to 23% in the prospective, with sIFX level $>0.2 \mu\text{g/mL}$), indicating that the ADA positive patients had already been switched to another treatment due to lack of effect determined by clinical outcome. However, a substantial proportion of patients still had suboptimal sIFX level and clinically were not in remission. Thus, measuring the drug level is of clinical value since it can help with the dosing regimen. For example, large individual differences in drug concentration in patients with multiple sclerosis treated with the monoclonal antibody natalizumab has been shown by van Kempen and colleagues where the majority had high trough levels at the time of re-dosing (43). This could partly be explained by that natalizumab is administered at a fixed dose of 300 mg every 4 weeks, not accounting for body weight or pharmacokinetic differences. The authors therefore suggest that the treatment regimen should adapt to a personalized approach to allow efficient use of natalizumab (43).

We investigated the added value of using the bioassay iLite for identification of neutralizing ADA and showed that 66% of the samples were positive for neutralizing ADA. This is far less than what was reported in a study published by Schie and colleagues, which showed that the majority ($>90\%$) of the ADA to infliximab were neutralizing using a TNF competition assay (44). The difference in the number of detected neutralizing samples could be due to assay format, treatment duration at serum sampling, and the small patient cohort size. There was a good correlation between the neutralizing ADA and the Panda RECL values, showing that the level of ADA is of most importance and thus that Panda RECL values can be used as an indicator of neutralizing capacity and clinical effect. Using this approach, it is then most important to know at what level to set the clinical threshold value for ADA. Here we provide an estimate of 3 RECL with the Panda assay as the level of ADA that begins to be detrimental for the therapeutic effect.

The correlation between the Panda assay and the ELISA ADA assay was not perfect, with more discrepancies in the lower levels of ADA positivity. Moreover, the ADA-ELISA is not a quantitative assay and therefore if you want to set a clinical threshold value, then alternative more linear methods are needed. This shows the value of conducting pilot studies in real-life settings to compare assays and to determine the assay specific clinical threshold value before it is applied in the clinic. For the samples with sIFX trough level below $0.2 \mu\text{g/mL}$, the ELISA have a higher sensitivity than Panda and this is might be due to the additional washing steps required in the Panda method.

The inverse correlation between drug level and ADA was confirmed. However, five samples with a drug trough level below $0.2 \mu\text{g/mL}$ that were identified as ADA negative with ELISA were tested with Panda for potential drug/ADA immune complexes, but only one sample showed low ADA reactivity, indicating that other explanations for low drug levels need to be taken into consideration (data not shown). Since infliximab is given intravenously the compliance is controlled for and therefore,

could not be the issue here. It is possible that some patients are highly efficient in metabolizing or consuming the drug and studies of biomarkers associated with this trait might resolve this issue.

The timing of the testing is essential. From the prospective longitudinal cohort, we can conclude that the value of testing for sIFX before the initiation period of shorter intervals is over, is questionable. At week 14, the mean sIFX stabilizes, and around half of the ADA positive patients could be identified already here. However, although some of these patients were transiently positive, none of these reached the suggested clinical threshold value of 3 RECL. Some patients that later became ADA positive in ELISA could have been detected as ADA positive with Panda at earlier time points, when the sIFX levels were too high to give reliable ADA test results with ELISA. Thus, if a first-tier screening for sIFX is used, then testing at week 6, before the 3rd infusion with a drug tolerant assay, is suggested to provide the most added value to the clinical practice.

We identified three exceptional cases with high sIFX and high ADA levels for which ADA would have been detected with Panda already at the 2nd or 3rd infusion. Here, the sIFX test would not have guided the decision to test for ADA. At this time point there were no clinical parameters that would have indicated ADA positivity, but all three cases eventually had infusion reactions and secondary treatment failure. A patient with previous infliximab treatment was included in the cohort and this patient also had high ADA identified using the Panda method, already at the 2nd infusion, which would indicate that the three exceptional cases might also have had infliximab treatment before. However, according to the patients files all three were newly diagnosed and had not previously received TNFi treatment prior the start of this study.

Taken together, an infliximab treatment algorithm for RA using the assays included in this study has been suggested (Figure 13). For patients with low sub-therapeutic sIFX trough levels and no detectable ADA, a dose escalation of infliximab could be beneficial. Patients with therapeutic levels within the recommended range for the chosen endpoint but still an active disease are likely to have pharmacodynamic failure and may benefit from switching to a drug with a different mechanism of action. Samples with a serum trough drug level below $1 \mu\text{g/mL}$ (to include assay variation around $0.85 \mu\text{g/mL}$) would benefit from being screened for ADA using the drug-tolerant assay in order to discriminate whether discontinued treatment or dose escalation should be implemented. Clinical assessment scores would aid the evaluation of patients with a serum drug level above $6 \mu\text{g/mL}$ (to include assay variation around $6 \mu\text{g/mL}$) to ascertain if it could be beneficial to either lower the dose or switch to a drug with a different mechanism of action.

There are some limitations to be mentioned in this study. We have not considered other confounding factors that might increase the risk for ADA. Tatarewicz et al. found that rheumatoid factor, which are present in a majority of RA patients, can interfere with the detection of monoclonal antibodies in immunogenicity assays (45). This is particularly a problem when using a sensitive immunoassay such as the Panda. Tatarewicz and colleagues found that samples from RA patients had a higher

baseline value of ADA reactivity than healthy subjects, thus the RF could lead to a false positive signal in the immunoassay (45). Here, we used untreated RA patient serum to set a disease specific cut point, which was slightly higher than normal healthy serum. However, one can question the clinical usefulness of such practice since low positive ADA probably do not have any immediate clinical relevance. Furthermore, the goal of the treatment is to reduce inflammation and thus when this is achieved, the serum profile may be more similar to normal healthy serum.

In conclusion, this cross-sectional and prospective study examined whether measurement of sIFX trough level and ADA testing correlated with treatment response and if adding a drug tolerant assay provided additional clinically useful, accurate and timely ADA results. Decisions of either switching treatment or regulating the dose, need to be guided by evidence based optimal drug levels and a clinical threshold value for ADA. The addition of a drug tolerant assay PandA resolved cases with detectable sIFX and identified ADA positivity earlier than the drug sensitive assay. Even though test results of sIFX and ADA are both heterogeneous and dynamic and thus difficult to interpret on group level, on individual level and as a method to achieve personalized treatment, these data are valuable.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Stockholm Regional Ethical Committee (2013/1034-31/3) and Gothenburg Regional Ethical Committee (1028-15, 2016-02-12). The patients/participants provided their written informed consent to participate in this study.

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AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.01365/full#supplementary-material>

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Specificity of the T Cell Response to Protein Biopharmaceuticals

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The anti-drug antibody (ADA) response is an undesired humoral response raised against protein biopharmaceuticals (BPs) which can dramatically disturb their therapeutic properties. One particularity of the ADA response resides in the nature of the immunogens, which are usually human(ized) proteins and are therefore expected to be tolerated. CD4T cells initiate, maintain and regulate the ADA response and are therefore key players of this immune response. Over the last decade, advances have been made in characterizing the T cell responses developed by patients treated with BPs. Epitope specificity and phenotypes of BP-specific T cells have been reported and highlight the effector and regulatory roles of T cells in the ADA response. BP-specific T cell responses are assessed in healthy subjects to anticipate the immunogenicity of BP prior to their testing in clinical trials. Immunogenicity prediction, also called preclinical immunogenicity assessment, aims at identifying immunogenic BPs and immunogenic BP sequences before any BP injection in humans. All of the approaches that have been developed to date rely on the detection of BP-specific T cells in donors who have never been exposed to BPs. The number of BP-specific T cells circulating in the blood of these donors is therefore limited. T cell assays using cells collected from healthy donors might reveal the weak tolerance induced by BPs, whose endogenous form is expressed at a low level. These BPs have a complete human sequence, but the level of their endogenous form appears insufficient to promote the negative selection of autoreactive T cell clones. Multiple T cell epitopes have also been identified in therapeutic antibodies and some other BPs. The pattern of identified T cell epitopes differs across the antibodies, notwithstanding their humanized, human or chimeric nature. However, in all antibodies, the non-germline amino acid sequences mainly found in the CDRs appear to be the main driver of immunogenicity, provided they can be presented by HLA class II molecules. Considering the fact that the BP field is expanding to include new formats and gene and cell therapies, we face new challenges in understanding and mastering the immunogenicity of new biological products.

Keywords: T cell, biopharmaceuticals, therapeutic antibody, therapeutic protein, immunogenicity, epitope, T cell selection, tolerance

INTRODUCTION

Protein biopharmaceuticals (BPs) have revolutionized the treatment of many diseases and their share of the worldwide drug market continues to grow. There are three main categories of protein BPs, if we exclude recombinant vaccines and allergen-specific immunotherapy. Antibodies and molecules composed of antibody fragments are the most important category in terms of the number of BPs and market share. Hormones, growth factors and cytokines define a category of recombinant human proteins with an endogenous counterpart generally circulating at low concentration. The third category comprises replacement proteins such as clotting factors or lysosomal enzymes, which are used to restore partial or complete genetic deficiencies. All BPs exhibit marked specificity for their target and low toxicity. However, BPs may be immunogenic and promote immune responses against themselves. In particular, anti-drug antibodies (ADAs) may be produced by patients following infusion of BPs and may disturb the pharmacokinetics of BPs, neutralize their therapeutic activities or induce allergic or autoimmune symptoms, depending on the category of BPs.

Several therapeutic antibodies have been found to induce neutralizing antibodies, as illustrated by the anti-TNF α antibodies infliximab and adalimumab (1, 2). Infusion and allergic reactions have also been reported for infliximab (3). ADAs raised against the second category of BPs, which includes recombinant hormones (insulin, H2-relaxin) (4, 5), growth factors [erythropoietin (Epo), GM-CSF] (6, 7) and cytokines (IFN- β , IFN- α , IL-2) (8), might be neutralizing as well. An additional risk exists for this category, owing to the existence of endogenous forms. ADAs raised against the recombinant protein can also neutralize the protein produced by the patients and could affect critically important functions of the endogenous protein. Pure red cell aplasia (PRCA) is a rare autoimmune disease characterized by rapidly progressive severe anemia resulting from the disappearance of erythroid precursor cells in the bone marrow. PRCA can be mediated by specific ADAs induced by injections of recombinant human Epo, which has a fully human peptide sequence (6). Neutralizing antibodies are also an important issue for replacement proteins, as alternative therapies to infusion with the protein remain limited and, in some cases, may be reserved mostly for patients with severe deficiencies. Neutralizing antibodies to factor VIII (FVIII) (often called inhibitors in hemophilia papers) are found in 30% of patients with severe hemophilia A and cause severe clinical complications (9). Patients with mild or moderate severity hemophilia A have a lower incidence of inhibitors, which is in part due to residual FVIII, which actively tolerizes to the drug, but also to their lack of or lower exposure to therapeutic FVIII. Those

who have received intensive FVIII therapy have an inhibitor incidence approaching that of severe hemophilia A patients (9, 10). The incidence of inhibitors of factor IX is lower in hemophilia B (11).

All BPs have either entirely or partially humanized sequences, but humanization does not guarantee a complete lack of immune response as illustrated by the examples above. Immune tolerance is firstly a consequence of B and T cell selection during their ontogeny. B and T cell repertoires are counter-selected by self-proteins in the bone marrow (12) and the thymus (13), respectively. However, the level of endogenous counterparts of BPs, which are expected to promote tolerance, might completely differ across BP categories and patients and therefore lead to variable levels of drug tolerance. IgG1 antibodies are present in the plasma at a concentration of approximately 9 mg/mL. The sequences of the constant parts of human IgG1 antibodies are therefore present as high levels of self-proteins. In contrast, VH or VL sequences are expressed at variable and lower levels (14), while all the sequences resulting from gene rearrangement and somatic mutations are not present in the human proteome. The second category corresponds to self-proteins present at low levels and the replacement proteins forming the third category might have different levels of endogenous counterpart. In patients with complete deficiency, there is no endogenous counterpart and the replacement protein is a foreign protein for the patients. In contrast, a mutated form circulates at variable concentration (also called cross-reacting material CRM) in patients with mild or moderately severe disease (15). Completely different physiological conditions therefore shape the repertoire of BP-specific CD4 T cells across BP categories and are expected to influence the initial BP-specific T cell frequency, peptide specificity and T cell expansion upon BP infusion. As CD4 T cells participate in the initiation and control of immune responses to either foreign or self-molecules, all these parameters should impact initiation and regulation of the ADA response and therefore help to understand and anticipate ADA onset. The objective of this paper is to review recent developments in the understanding the T cell response to BPs, with a main focus on mechanisms controlling peptide specificity, T cell selection and regulation.

BASIC MECHANISMS OF THE ANTIGEN-SPECIFIC CD4 T CELL RESPONSE

CD4 T cells recognize antigens as peptides displayed by HLA class II molecules at the surface of dendritic cells (DCs). These peptides are produced by proteolysis of antigens by cathepsins in the endosomal compartments and possess appropriate amino acid sequences (also called binding motifs) (16, 17) that allow their binding to the polymorphic HLA class II molecules (18). As the polymorphic residues are mainly present in the peptide binding site of the HLA class II molecules, the bound peptides might be restricted to particular HLA class II allotypes or, in contrast, might benefit from shared binding properties across

Abbreviations: ADA, anti-drug antibody; CDR, complementarity determining region; BP, biopharmaceuticals; Epo, erythropoietin; DCs, dendritic cells; HLA, human leukocyte antigen; HC, heavy chain. HSA, human serum albumin; IFN- γ , interferon- γ ; KLH, keyhole limpet hemocyanin; LC, light chain; MAPPs, MHC-associated peptide proteomics; MS, mass spectrometry; PBMCs, peripheral blood mononuclear cells.

allotypes to bind multiple HLA class II molecules (16, 17). Some of the presented peptides are recognized by CD4 T cells (**Figure 1A**). CD4 T cells express at their surface a specific receptor (TCR), which selectively recognizes peptides anchored to the HLA molecules across the multitude of displayed peptides. This selectivity comes from the wide diversity of TCRs generated by the random and imprecise rearrangements of the V and J segments of the TCR alpha and V, D, and J segments of the TCR beta genes (22). Pioneering estimates of the diversity of the TCR repertoire suggest a lower limit estimate of 1 million different TCR beta genes (23, 24), but more recent investigations using high-throughput sequencing propose minimal estimates of 100 million unique TCR β sequences (25). This TCR repertoire is further shaped by self-peptides in the thymus (13), where many autoreactive T cells are deleted by apoptosis (**Figure 1B**). T cells leaving the thymus have not been in contact with the antigen in the periphery and are called naïve T cells. Some T cells specific for self-peptides might also be committed to thymic regulatory cells (tTregs) in the thymus (**Figure 1B**). The diversity of TCRs renders each antigen-specific naïve T clonotype very rare among the whole population of T cells, but at varying frequencies depending on the nature of the antigens (26) (**Figure 1B**). Frequencies of T cells specific for a single epitope evaluated using MHC class II tetramers (27, 28) or limiting dilution conditions vary in the range of 10^{-7} to 10^{-5} for tumor (29, 30) or foreign antigens (27, 28, 31). These frequencies are higher for full-length proteins, as exemplified by KLH or protective anthrax antigen (32, 33), but are expected to be low for recombinant forms of self-proteins owing to negative selection of T cells in the thymus (13). Upon encountering antigens in the body, specific T cell clones are activated in the lymphoid organs and expanded over the other T cells. This expansion is in the range of 10- to 1,000-fold for CD4 cells (27) (**Figure 1B**). Interestingly, the final frequencies of antigen-specific memory cells are correlated with the frequencies of precursors in the naïve repertoire, as shown in mice and humans using MHC class II tetramers (26–28). Finally, during the expansion, activated CD4 T cells transform into memory cells with helper functions. Some of the expanded T cells might also transform into induced regulatory T cells (iTreg), which are mainly characterized by their capacity to release immunosuppressive cytokines such IL-10 (**Figure 1B**).

T CELL DEPENDENCE OF THE ADA RESPONSE

Whether ADA induction is T cell-dependent is not an obvious question as pioneering work on the T cell response to FVIII using synthetic peptides did not reveal particular differences between hemophilia A patients who did or did not develop neutralizing antibodies (34–36). It thus appears important to clarify the necessary role of the T cell response in the ADA response for the three categories of BPs, as T cell dependence validates the interest of focusing on the T cell response to provide insights into immunogenicity.

From these pioneering studies, the T cell response to FVIII did not appear to be linked to the ADA response, but multiple lines of evidence later pointed to T cell dependence (34–36). Anti-FVIII antibodies are of the IgG isotype and contain somatic mutations in their variable domains indicating that they have been produced by B cells that have undergone isotype switching and affinity maturation (37), both of which are T cell-dependent. Many studies have reported the T cell response in patients with mild/moderate (38–40) or severe hemophilia A (40, 41) who developed neutralizing ADAs. Most patients with mild or moderate hemophilia A circulate a point-mutated, partially disabled FVIII protein and require only rare infusions of therapeutic FVIII, indicative of they are less likely to develop an ADA response (38, 39). However, intensive treatment to support surgery or treat major bleeds, or accumulated occasional exposures over a lifetime, increase their risk of immunogenicity substantially (10). Analysis of the epitope-specificity of the T and B cell response in these patients showed that both FVIII T cells and antibodies might be raised against the mutated epitopes, suggesting a tight regulation of the specificity of FVIII-specific T cells (38, 39, 42). An interesting observation is that low CD4 T cells resulting from HIV infection of hemophilia A patients was associated with less anti-FVIII antibodies, suggesting an important role of T cell help (43). In line with this finding in HIV infection, mouse models using FVIII-deficient mice also indicated that ADA induction against FVIII requires CD4 T cell activation (44–46).

For the second category of BPs, i.e., hormones, growth factors, and cytokines, a higher T cell response was found in ADA+ patients treated with IFN-beta as compared to ADA- patients and healthy donors (47). The T cell dependence of IFN-beta immunogenicity was strengthened by HLA association of the ADA response to IFN-beta (48, 49) and was also shown in IFN-beta transgenic mice (50). Patients treated with recombinant human erythropoietin and who developed PRCA showed a clear T cell response to recombinant human erythropoietin, in contrast to ADA- patients (51).

Finally, for the category constituted by the therapeutic antibodies, Vultaggio et al. compared the T cell response to anti-TNF α infliximab in 32 ADA+ patients, 39 ADA- patients and 10 healthy donors (52). Infliximab-specific cell proliferation was detected mainly with T cells collected from ADA+ patients, especially those who developed hypersensitivity reactions, as compared to T cells collected from ADA- patients or healthy donors. The T cell response in ADA+ patients treated with infliximab or rituximab was also observed by others (53) and the ADA response to infliximab was found to be associated with HLA-DRB1*03 (54). Isolation of human monoclonal antibodies (mAbs) raised against adalimumab (55) and natalizumab (56) confirmed the T cell dependence of their *in vivo* generation, their sequences containing multiple somatic mutations. Anti-natalizumab mAbs were isolated from donors who developed a T cell response (56). Altogether CD4 T cell response appears as a requisite to mount a ADA response for the three BP categories.

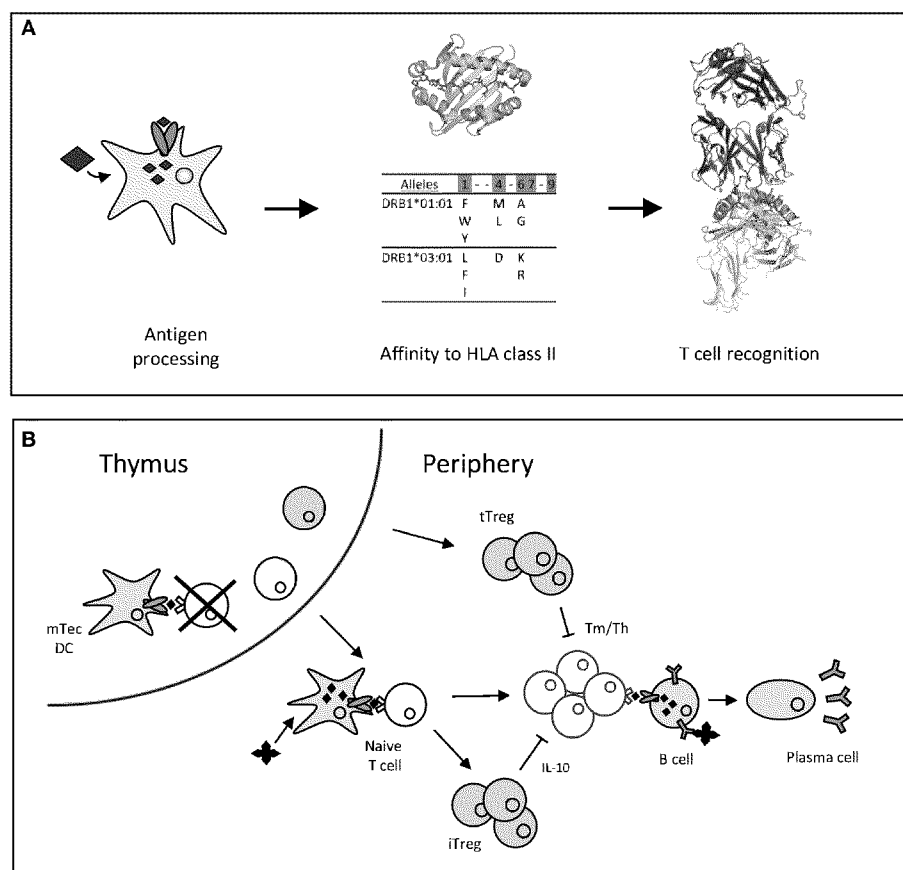


FIGURE 1 | Antigen recognition by T cells leads to T cell selection and expansion. **(A)** Peptide requirements for T cell recognition (19). A T cell epitope is processed by the dendritic cell, binds to HLA class II molecules (16, 20) and is recognized by the TCR (21). **(B)** T cells are selected positively and negatively by recognition of self-peptides in the thymus. In the periphery, the naïve T cells recognize antigenic peptides to expand and differentiate into memory or effector T cells (Tm/Teff). Thymic T cells (tTregs) are committed in the thymus to regulatory T cells, while induced regulatory T cells (iTreg), that secrete IL-10 are differentiated from the pool of naïve cells in the periphery.

T CELL RESPONSE TO BPs USING CELLS COLLECTED FROM HEALTHY DONORS WITH A VIEW TO PREDICTING IMMUNOGENICITY

A prerequisite for the generation of a CD4 T cell response to BP is the presence of T cells in the T cell repertoire that recognize epitopes within the BP. *In vitro* stimulation assays using T cells from healthy unexposed subjects are generally used to assess the potential reactivity to BP. This is in contrast to investigations of T cell responses against foreign proteins, whose T cell response is mainly investigated using donors who have already mounted an immune response to the antigens. This difference impacts both the methodologies and the outcomes of the T cell assays applied to BPs. Indeed, owing to the risk that immunogenicity issues stop the clinical development of new products, an important request from pharmaceutical companies is anticipation of these issues by selecting the least immunogenic BPs across the BP candidates at the early stages of drug development. Generally, drug selection

is driven by preclinical studies carried out in animal models. However, animal models are not considered as good models for predicting the immunogenicity of BPs in humans, the humanized proteins being recognized as non-self in animals (57). As CD4 T cells are involved in the initiation of the immune responses, T cell assays using cells collected from healthy donors have been developed to evaluate whether BPs could prime a new T cell response *in vitro* (58–61). These T cell assays evaluate whether T cells circulating in the blood of healthy donors can recognize the BPs. They are clearly different from assays that are done with cells collected from patients developing an ADA response. T cell assays using cells collected from healthy donors provide an estimate of the number of T cells prone to react to BP recognition in healthy donors, who serve as estimators of the number of T cells in the patients before BP injection. Therefore, they do not therefore directly predict immunogenicity but reveal a “potential of response,” which is one of the main factors contributing to immunogenicity (57). Multiple formats of T cell assays are used to predict BP immunogenicity. Cells introduced in the assay can be either PBMCs (PBMC assay) or

a co-culture of autologous DCs and T cells (DC:T cell assay) (58, 60, 61). Assays also differ by the number of *in vitro* stimulations with either BPs (59) or mitogenic molecules (33) and by the readout used to characterize T cell specificity (mainly CFSE, 3H-thymidine incorporation or ELISPOT). T cell assays are validated by comparing BPs known to be either immunogenic or non-immunogenic in humans (58–61), assuming that the response in *in vitro* experiments correlates with an immune response in patients. Because of the low frequency of naïve BP-specific T cells in healthy donors (26), we developed a T cell assay relying on a long-term culture phase to enrich the cell culture in specific T cells (T cell amplification assay) (59, 62). This was adapted from assays developed to identify tumor antigens (63, 64). In this assay, antibodies known to be immunogenic in multiple treated patients, such as adalimumab (2), infliximab (1, 65), and rituximab (66, 67), generate a higher T cell response (59, 68) than poorly immunogenic antibodies such as trastuzumab (69) and secukinumab (70) or fusion protein (etanercept) (71). Finally, T cell assays using healthy donors have provided most of the data on the T cell response to BPs (58–61, 68). Many T cell epitopes have also been identified using these assays, as described in the following sections.

mAbs-SPECIFIC CD4 T CELL EPITOPES AND THEIR RELATIONSHIP WITH THYMIC SELECTION

Because of HLA polymorphism, CD4 T cell epitopes of BPs are expected to vary from one donor to another as a function of their HLA allotypes, but also to be shared by multiple donors owing to common binding specificities of the HLA class II molecules (17). The location of BP-specific T cell epitopes is precious information in understanding which parts of the molecules contribute to their immunogenicity (Figure 2), but also which mechanisms take part in this response. In fact, immunological mechanisms leading to the T cell response to mAbs differ between regions mutated with respect to the germline sequences, non-mutated regions of the variable domains and constant regions (Figure 3A).

T cell epitopes have been identified in multiple therapeutic antibodies, including infliximab (53), rituximab (53), adalimumab (72), natalizumab (72), ixekizumab (73) by deriving CD4 T cell lines from cells collected from healthy donors (Figure 2). The location of CD4 T cell epitopes was found to be very specific for each therapeutic antibody (Figure 2). Two-thirds of the identified T cell epitopes of infliximab and rituximab participated to the T cell response mounted in patients, who developed an ADA response (53). In another study, the T cell response to infliximab in patients treated with infliximab appeared broader (74). To provide insights on the mechanisms leading to the T cell specificity, affinity of the mAb peptides for HLA-DR molecules, which are common worldwide was evaluated and the mAb peptides presented by HLA-DR molecules of mAb-loaded dendritic cells were identified. mAb-specific T cell epitopes are often found to bind to HLA-DR molecules with good affinity (53, 72). For example, HCDR3 of adalimumab is a hot-spot of good binders to HLA-DR

molecules (72) and contains the vast majority of identified T cell epitopes. T cell epitopes are often among the peptides identified by MHC-associated peptide proteomics (MAPPs) assay. This assay consists in loading immature monocyte-derived DCs from healthy human donors with mAb, isolating HLA-DR associated peptides from mAb-loaded DCs and identifying them by liquid chromatography-mass spectrometry (53, 72, 73, 75). Importantly, regions mutated with respect to the germline sequences and hence mainly the CDR regions appear as the main regions hosting T cell epitopes, which are therefore recognized as neopeptides (Figure 3a). Studies in mice have demonstrated that somatic hypermutations create CD4 T cell epitopes in the V regions (Figure 3a), although germline sequences do not elicit a CD4 T cell response (76). Germline sequences lead to central deletion of the specific CD4 T cell precursors in the thymus (77, 78), possibly through their presentation by thymic B cells (79) (Figure 3c). The CDR3 sequences result from the junction of V(D)J segments and TdT-catalyzed addition of nucleotides, and they therefore differ largely from germline sequences. Multiple T cell epitopes from adalimumab and ixekizumab overlap HCDR3 (Figure 3a). In CDR1 and CDR2 of the VH and VL chains of an antibody, somatic mutations are introduced during affinity maturation and seem to generate multiple epitopes of natalizumab and ixekizumab (Figure 3a). T cell clones derived from two multiple sclerosis patients who developed an ADA response specific for natalizumab were found to react with one CD4 T cell epitope overlapping the LCDR2 region, only (56). This epitope was also identified in a study performed with cells collected from healthy donors (72). Interestingly, a T cell response targeting a single epitope can suffice to mount an ADA response (56).

However, some T cells recognizing non-mutated parts of the mAb could escape thymic selection, as shown in a mouse model (78) (Figure 3b). Three T cell epitopes of adalimumab, one of natalizumab and one of ixekizumab do not contain somatic mutations with respect to the germline sequences (Figure 2) (72, 73), as evaluated by two alignment methods (80, 81). The best-fitting germline sequence of adalimumab VH is the gene segment VH3.9 (81). It is a poorly expressed germline gene segment in the human B cell repertoire (14) and hence could give rise to a partial escape of adalimumab-specific CD4 T cells from the thymus (78) (Figure 3b). However, these T cell epitopes contribute only little to the mAb-specific T cell response (Figure 2).

In addition to the T cell epitopes identified in the variable domains of therapeutic mAbs, T cell epitopes deriving from constant regions of IgG (82) and from human serum albumin (83) were found to activate tTreg cells and have been called Tregitopes. Indeed, some precursor T cells egressing from the thymus are committed to T cells with immunosuppressive function and characterized by the CD4+CD25+FoxP3+ phenotype (84). Autoreactive thymic regulatory T cells (tTregs) are positively selected on endogenous self-antigens expressed in the thymus. This selection occurs in a highly specific manner through TCR interaction of high affinity for their cognate antigen. Constant parts of immunoglobulin IgG1 and serum albumin are both highly abundant proteins and should therefore be presented as antigenic peptides by MHC II molecules

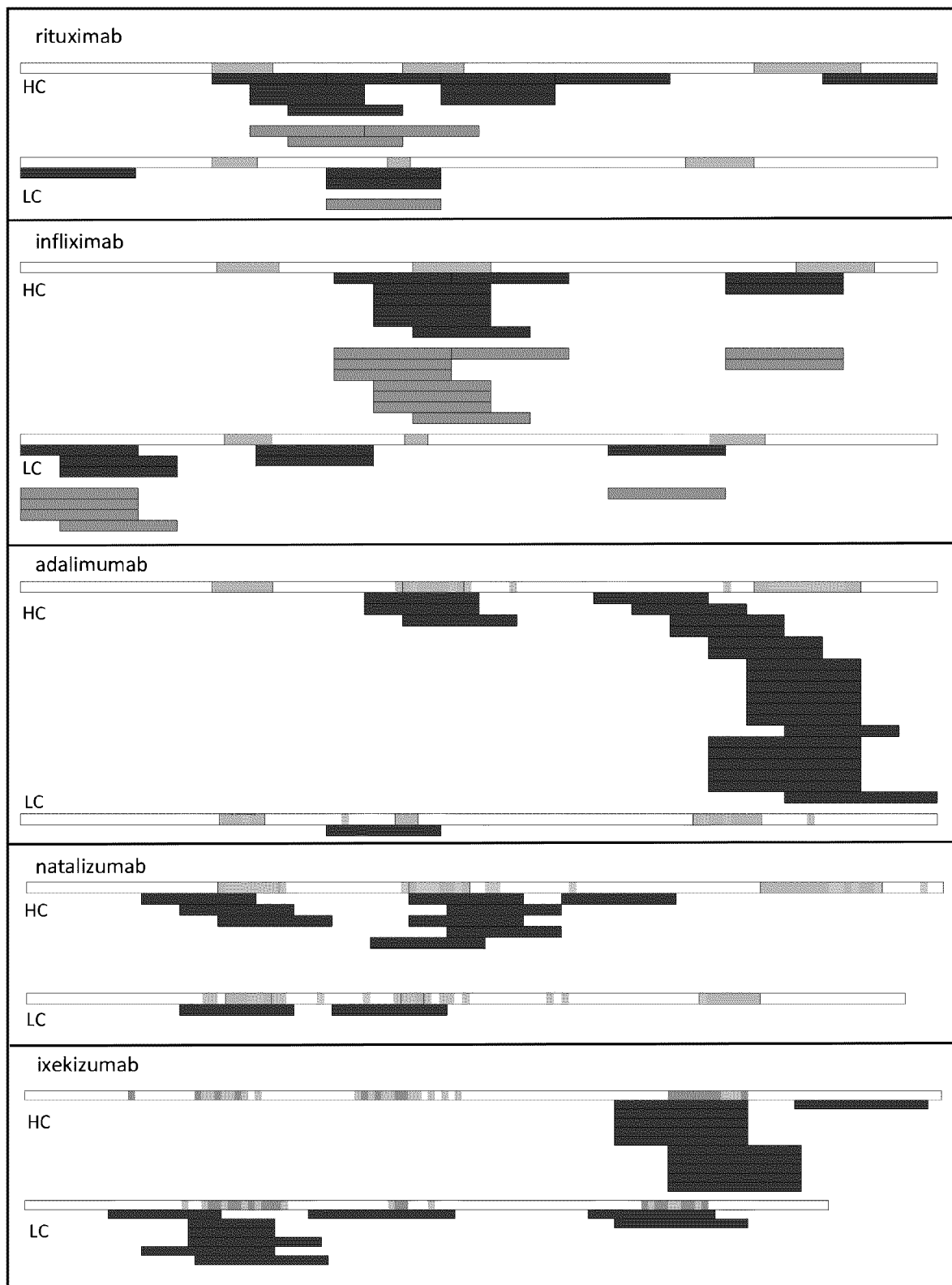


FIGURE 2 | T cell epitopes of therapeutic antibodies. T cell epitopes have been identified from T cells collected from healthy donors (red) or from patients who develop an ADA response (green) to rituximab, infliximab (53), adalimumab, natalizumab (72), and ixekizumab (73). Each bar corresponds to an individual response. CDR regions are shown in blue. Amino acids in orange correspond to mutations with respect to the best-fitting germline sequence. HC, heavy chain; LC, light chain.

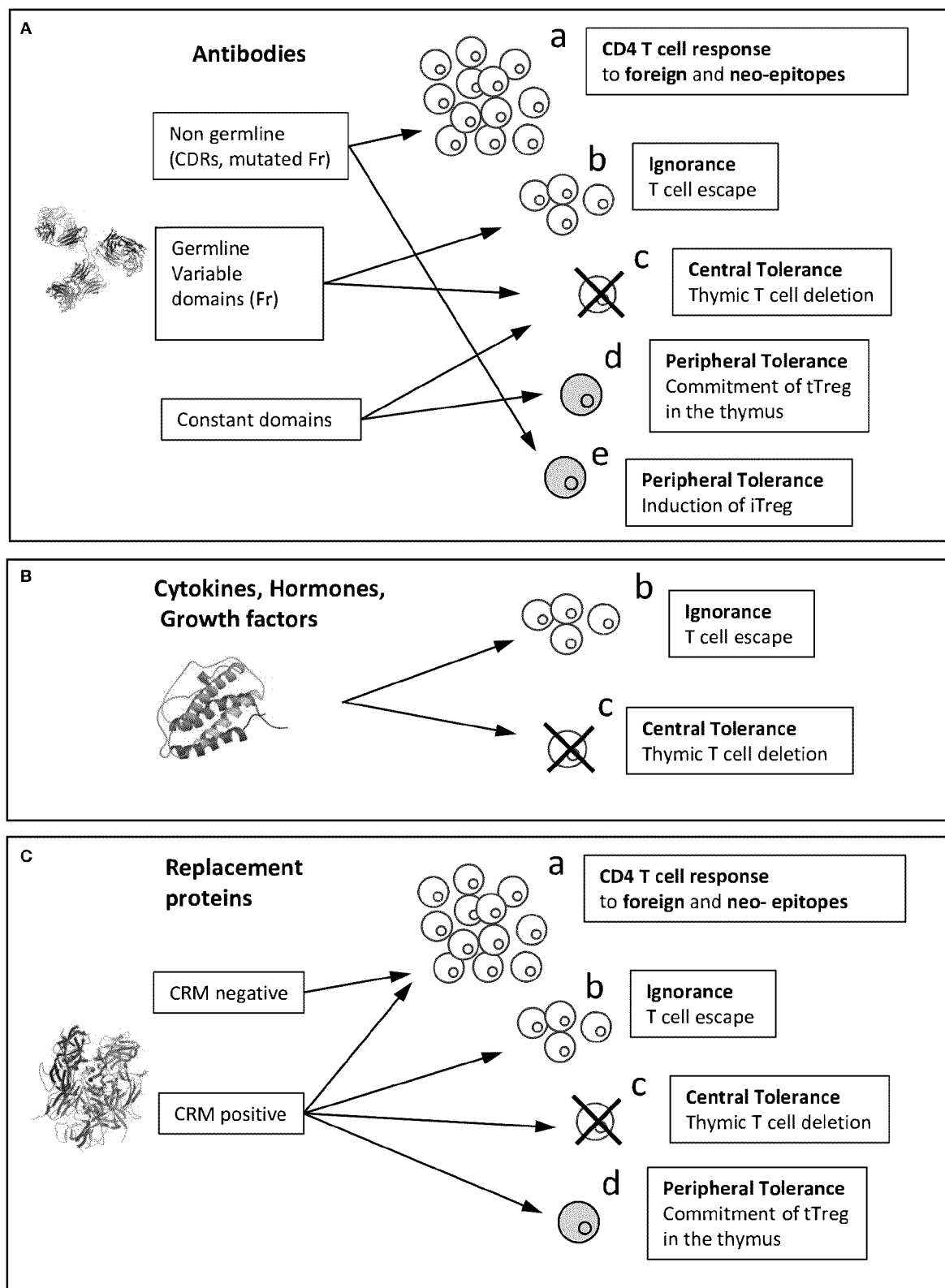


FIGURE 3 | Mechanisms of induction of the T cell response or tolerance to biopharmaceuticals. Immunological mechanisms involved in the immunogenicity of BPs are reported for the three categories of BPs: **(A)** antibody, **(B)** cytokines, hormones, growth factors, **(C)** replacement proteins. Biopharmaceuticals might be

(Continued)

FIGURE 3 | recognized by T cells as foreign in gene-deficient patients or as mutated sequences with respect to the human germline sequences (neoepitopes) (a). Non-mutated sequences might (b) stimulate T cells that have escaped thymic selection or might (c) lead to T cell tolerance by deletion of T cells in the thymus or (d) induction of thymic Tregs (tTregs). (e) Peripheral tolerance could be provided by induced Tregs (iTregs). Structure of a full-length antibody (Pdb: 21GF), human Epo (Pdb: 1BUY), and human FVIII (Pdb: 3CDZ).

expressed by medullary thymic epithelial cells (mTECs) in the thymus. Presentation of these peptides might lead to deletion of effector CD4 T cells (**Figure 3c**), which explains why CD4 T cell epitopes are not found in the constant parts of IgG, and also to selection of specific tTregs (**Figure 3d**). These Tregitopes have been shown to suppress multiple kinds of immune responses, including humoral (82, 85) and cellular (86), but were also shown to be poorly active in this regard in an alternative study (87). No BP-specific tTregs have been cloned and characterized yet and the mechanism of suppression remains to be determined. Together, the T cell repertoire specific for therapeutic mAbs appears to be shaped by creation of neoepitopes for mutated regions with respect to the germline sequences, T cell escape (also called ignorance see below) and deletion in the thymus for non-mutated regions of the variable domains and T cell deletion or tTreg generation for constant regions (**Figure 3A**).

CD4 T CELL REPERTOIRE OF HORMONES, GROWTH FACTORS, AND CYTOKINES

Mutations cannot be the only driving force of BP immunogenicity, as immunogenic hormones, growth factors and cytokines that form the second BP category are not mutated. Cytokines such as IFN- β (88), IFN- α (89), and IL-2 (90) and growth factors such as GM-CSF (7) are all known to elicit an antibody response in some patients, albeit to various degrees. *In vivo* expression of the endogenous counterpart of immunogenic BPs of the second category might not be sufficient to completely deplete the specific T cells in the thymus, leading to immunological ignorance, as named by Ohashi et al. (91) (**Figure 3b**). This immunological mechanism has been demonstrated in transgenic mice expressing a model antigen in the periphery (91). In these mice, antigen-specific T cells escape from thymic selection as the model antigen is not expressed in the thymus. T cells, however, do not react to the antigen expressed in the periphery, owing to the lack of appropriate signals of T cell activation.

Similarly, expression of the endogenous counterpart of the second BP category might be not sufficient to completely deplete the specific T cells in the thymus. T cells escape from thymic selection (**Figure 3b**), although they are specific for self-sequences and not neoepitopes (**Figure 3a**). A specific and functional T cell repertoire is therefore available to react to the corresponding BPs, but is not activated at the steady state. As an example, a high frequency of Epo-specific T cells was found in the blood of many healthy donors (62, 92). In most patients, infusion of correctly formulated recombinant Epo is well-tolerated and probably did not activate Epo-specific T cells, in the absence of co-stimulatory signals. However, altered HSA-free Epo batches might contain Epo aggregates, micelles and leachates from the

syringe stopper (93), which might favor DC maturation and antigen capture by the DCs (94) and thereby provide T cell activation signals (51). Another example of immunogenic BP of the second category is relaxin. Relaxin is a two-chain peptide hormone structurally related to insulin with anti-inflammatory activity, but its injection in humans led to the production of anti-relaxin antibodies in multiple patients (4). In agreement with its clinical immunogenicity, relaxin elicits a strong *in vitro* T cell response from cells collected from healthy donors (95).

In agreement with the concept of antigen ignorance (91) (**Figure 3b**), T cell epitopes have been identified in Epo (92), IFN- β (47), and relaxin (95), using synthetic peptides bearing unmodified human sequences. Given that the sequences of the BP are identical to those of the endogenous forms, these findings confirm that BP can be immunogenic without alteration of their sequence (**Figure 3b**), e.g., by chemical (96) or post-translational modifications to create neo-epitopes (**Figure 3a**). In fact, to our knowledge, T cell neoepitopes of the second category of BPs have not yet been identified.

One important consideration in anticipating the tolerance or ignorance of self-sequences might be direct evaluation of their expression in the thymus. Epo and IFN- β do not seem to be expressed in the thymus, in agreement with their capacity to mount a T cell response (47, 51, 62, 92). In contrast, the thymic expression of relaxin has been reported (95), but it is unknown whether relaxin is expressed by mTECs to promote T cell selection. To our knowledge, insulin is the only hormone known to induce central tolerance, as depletion of insulin expression in mTECs has been shown to induce spontaneous anti-insulin autoimmunity in mice (97) (**Figure 3d**). In the case of insulin, a particularity is, that in some cases, insulin is given to diabetic patients with autoantibodies against self-proteins and peptides including insulin itself (98, 99). Hence autoantibodies to insulin may be preexisting, i.e., present before the first injection of insulin. Multiple studies have identified proinsulin and not only insulin as the main target of autoreactive CD4 T cells, many clones being specific for the C-peptide human proinsulin (100–102). Mechanisms of insulin immunogenicity are therefore not representative of the other immunogenic hormones, but suggest that expression of self-antigens in mTEC might be investigated to define the immunogenicity risk of therapeutic proteins. In summary T cells specific for cetagory including hormones, growth factors and cytokines are therefore either produced by ignorance or deleted by central tolerance (**Figure 3B**).

CD4 T CELL EPITOPES OF REPLACEMENT PROTEINS, ILLUSTRATED BY FVIII

The third category of BPs comprises replacement proteins such as clotting factors or lysosomal enzymes. To our knowledge, there

are no T cell epitopes identified in lysosomal enzymes such as α -glycosidase (Pompe disease), α -galactosidase (Fabry disease), or in factor IX (hemophilia B) in humans, but only in mice (103). In contrast, multiple studies have sought to identify CD4 T cell epitopes of FVIII from severe hemophilia A patients (41, 104), mild/moderate hemophilia A patients (38, 39, 105, 106) and FVIII-deficient humanized mice (46).

T cell reactivity against FVIII appears very complex and depends on the residual amount of endogenous FVIII. The grade of hemophilia A from severe to mild is related to the remaining level of FVIII function. Deficiencies range from complete lack of circulating FVIII, which results in the most severe hemophilia A cases, to an altered function due to missense mutations, small insertions or deletions. ADA incidence varies from 30% in severe hemophilia A patients to 5–20% in mild/moderate hemophilia A patients and may be linked to the remaining amount of FVIII (CRM+) and may not be related to its functionality (107). Hemophilia B patients have a reduced risk of neutralizing antibodies, probably because patients produce a circulating and dysfunctional factor IX (CRM+) (11).

In severe hemophilia A patients, partial or complete lack of FVIII (CRM-) renders the infused FVIII a foreign molecule (Figure 3a). In line with the large size of FVIII, multiple potential epitopes have been found to be displayed by HLA class II molecules (108, 109) and are across all FVIII domains (108–110). The number of presented peptides is diminished by the association of FVIII with von Willebrand factor (110), which limits the uptake of FVIII by DCs (111). In FVIII-deficient HLA-DR15-transgenic mice, which are designed to mimic severe hemophilia A, the T cell response is supported by 8 dominant epitopes, which exhibit a good affinity for different HLA-DR molecules. T cell epitopes identified in FVIII (36, 38, 39, 106) are some of the presented peptides (108) but all the presented FVIII peptides did not elicit a T cell response as already observed for therapeutic mAbs (53, 72). The T cell response in severe hemophilia A patients may appear to focus on only one high-avidity epitope, when assessed with HLA-Class II tetramers (41), which is at variance with studies using T cell proliferation assays suggesting a much broader T cell response to FVIII (34).

In mild/moderate hemophilia A patients, the remaining material of FVIII circulating in the blood might exert selective pressure on the CD4 T cells in the thymus (Figure 3c). This is supported by observation patients with mild/moderate hemophilia that carry a mutation. CD4 T cell clones isolated from these patients target the mutated epitope (i.e., a neoepitope) (Figure 3a), suggesting that T cells reactive to the non-mutated variant were negatively selected. Both the epitope with the R2150H mutation and the non-mutated counterpart bind with high affinity to multiple HLA-DR molecules, the mutation supposedly being in tight contact with the TCR (38). The epitope with the A2201P mutation (39, 105) was the same as that found in severe hemophilia A patients (41). In contrast to the mutated regions, the remaining parts of FVIII did not give rise to specific T cells in the mild/moderate hemophilia A patients and appeared to be tolerated (Figure 3c).

Strikingly, induction of central tolerance (Figure 3c) by endogenous FVIII does not seem to be important in healthy

donors. Indeed, a large repertoire of FVIII-specific CD4 T cells is found in the blood of healthy donors (112), suggesting that these cells escape negative thymic selection (Figure 3b). Half of the FVIII-specific T cells were naïve or memory T cells and the frequency of memory cells remained low as compared to memory cells specific for foreign antigens (112).

This repertoire of FVIII-reactive T cells might also trigger the naturally-occurring anti-FVIII Abs found in many healthy subjects and maybe even for the onset of acquired hemophilia (113). Owing to the higher incidence of ADAs in severe hemophilia A patients as compared to mild/moderate hemophilia A patients, the different level of FVIII deficiency might enlarge the FVIII-specific CD4 T cell repertoire from the already large repertoire found in healthy donors, by expanding T cells of higher avidity for their cognate antigen, a concept demonstrated for a tumor antigen in mice (114). Alternatively, the loss of FVIII expression in hemophilia A patients could be accompanied by a deficiency of thymic regulatory T cells (tTregs). Indeed, absence of cognate antigen has been shown to lead to a severe loss of tTreg positive selection (115). Deficiency in expression of FVIII in hemophilia A patients may therefore impact FVIII-specific tTreg selection (Figure 3d). But to date, the role of tTreg cells in the FVIII T cell response has only been assessed by removing CD4+ CD25+ T cells from T cell proliferation assays (105) or by adoptive transfer of tTregs into mice challenged with FVIII plasmid (116). No FVIII peptides have yet been characterized for tTreg recognition. Further studies using samples from mild/moderate or severe hemophilia A patients or healthy donors are required to provide a more complete picture of the FVIII T cell epitopes and how they entail or prevent ADA onset. Nevertheless, how the T cell repertoire is shaped in CRM- and CRM+ patients appears to involve multiple immunological mechanisms.

ROLE OF SUBSETS OF BP-SPECIFIC CD4 T CELLS IN THE INDUCTION AND REGULATION OF THE ADA RESPONSE

Different subsets of T cells participate in the initiation and regulation of many adaptive immune responses, their frequencies, phenotypes, onset and persistence might be a source of understanding, as already observed for vaccines (117) and allergen immunotherapies (118, 119). To date, the phenotypes of T cells specific for BPs in treated patients have been investigated in few studies, mainly in the context of mAbs (52, 53, 120). Overall, the data suggest that the ADA response might be controlled by the balance between antibody-inducing and antibody-suppressing cytokines. Although ADA follow-up is requested by the authorities during clinical trials, characterization of the T cell response is not mandatory, but recommended. Ethical issues might also limit the collection of blood samples, especially as in most genetic deficiencies, including hemophilia and lysosomal diseases, the patients might be very young. As mentioned above, effector T cells specific for infliximab (52, 53, 120), rituximab (53), and natalizumab

(56) collected from patients who developed an ADA response were revealed by proliferation assays or secretion assays of inflammatory cytokines. Individual patterns of the cytokines including IFN- γ , IL-13 (120), IL-5, and IL-17 (53) were found across the patients. IL-13 appeared to be associated with detection of specific IgE (52). T cell clones specific for infliximab were found to be of TH1, TH2, or TH0 cells (120) and should provide help to the B cells to produce ADA. By contrast, many of the T cell clones (120) or bulk T cells (53, 120) also secreted IL-10, an immunosuppressive cytokine, which is known to suppress T cell proliferation and IgG secretion (121) (Figure 3e). IL-10-secreting T cell clones specific for infliximab inhibited the proliferation of co-cultured effector T clones also specific for infliximab in an IL-10-specific manner (120). Interestingly, longitudinal analysis of the infliximab-specific T cell response highlighted the upregulation of IL-10 throughout the treatment, while IFN- γ was mainly expressed at the first infusion. Patients who developed ADAs produced little IL-10 at the beginning of the treatment and exhibited low IL-10/IFN- γ ratios (74). IL-10 was therefore found to be a cornerstone of ADA regulation. IL-10-producing cells are induced by infusion with the therapeutic antibody (Figure 3e) and differ from tTreg cells (Figure 3d), which are directly committed to regulatory T cells in the thymus (84). Besides IL-10 production (121), induced Treg cells (iTregs) (Figure 3e) might acquire a Foxp3+ CD25+ CD4+ phenotype (120) similar to that of tTregs (Figure 3d), but they share the same CD4 T cell epitopes with effector CD4 T cells, as they are produced from the same precursor cells, as shown for allergen immunotherapy (122). In studies of the T cell response to infliximab (52, 120), almost all patients generated an IL-10 response, including patients who did not generate ADA (ADA-). It is generally thought that ADA- patients did not develop any immune response against the BP. As shown in this study, they might develop a suppressive T cell response, which hampers the ADA response. Isolated immunomonitoring of the ADA response, while neglecting the T cell response, may therefore underestimate the number of patients who generate an immune response to infliximab (1, 65). Further investigations are required to generalize these observations to other anti-TNF therapeutic antibodies or other BPs. In conclusion, detailed analyses of the T cell response to BPs are insufficient in number, but the existing studies are important in deciphering a part of the mechanisms of immunogenicity in patients treated with BPs.

PERSPECTIVES

In the last decade, our understanding of the T cell response to BPs has benefited from findings from various immunology-related fields including vaccinology and cancer immunology. T cell assays relying on the generation of T cell lines have been adapted from experiments done to identify tumor T cell epitopes (63). Prediction of T cell epitopes is done using algorithms trained with foreign sequences, especially from bacterial and viral antigens (123). The next decade may benefit from methods

developed to investigate the T cell response in healthy donors and in patients, from the identification of their own T cell epitopes, and from pioneering work on T cells in patients treated with BPs. It is important to extend studies to many more BPs, as the paucity of clinical data is hampering progress. Little attention has also been paid to understanding how the T cell response is triggered. A soluble molecule generally leads to immune tolerance rather than immune response (124) and needs adjuvant activity to switch on the response (125). It is unclear how BPs acquire this ability to prime the T cell response. Identified hints are the capacity to aggregate (126), which may occur *in vivo* (127), to contain trace levels of impurities (128), to form immune complexes (129), and to interact with immune cells (130). Moreover, patient- treatment- or disease-specific factors may also have to be considered. An important opportunity provided by T cell epitope identification is to de-immunize BPs by removing T cell epitopes from their sequences (131–133). The challenge of de-immunization is to take into account the wide diversity of HLA in humans and to maintain BP functionality. Multiple molecules have already been engineered including enzymes (132), FVIII (134), and mAbs (56, 133), but to our knowledge none has yet been challenged in a clinical setting. Finally, multiple other BPs different from proteins, such as advanced therapeutic medicinal products, are being actively developed and studied in terms of immunogenicity issues. Pre-existing immunity to recombinant adeno-associated virus vectors or lentiviral vectors compromises the efficacy of gene therapy, while *de novo* antibody and cytotoxic CD8 T cell responses could eliminate cells transfected with the therapeutic products (135). Pre-existing immunity has also been demonstrated for the bacterial CAS9 protein and could dramatically limit genome editing tools such as CRISPR/Cas9 technology (136). All these new therapeutic approaches might benefit from already off-the-shelf technologies to investigate the T cell response and from recent advances in single-cell analysis (137) and next-generation sequencing (138).

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Benefits of Prophylactic Short-Course Immune Tolerance Induction in Patients With Infantile Pompe Disease: Demonstration of Long-Term Safety and Efficacy in an Expanded Cohort

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Immune tolerance induction (ITI) with a short-course of rituximab, methotrexate, and/or IVIG in the enzyme replacement therapy (ERT)-naïve setting has prolonged survival and improved clinical outcomes in patients with infantile Pompe disease (IPD) lacking endogenous acid- α glucosidase (GAA), known as cross-reactive immunologic material (CRIM)-negative. In the context of cancer therapy, rituximab administration results in sustained B-cell depletion in 83% of patients for up to 26–39 weeks with B-cell reconstitution beginning at approximately 26 weeks post-treatment. The impact of rituximab on serum immunoglobulin levels is not well studied, available data suggest that rituximab can cause persistently low immunoglobulin levels and adversely impact vaccine responses. Data on a cohort of IPD patients who received a short-course of ITI with rituximab, methotrexate, and IVIG in the ERT-naïve setting and had ≥ 6 months of follow-up were retrospectively studied. B-cell quantitation, ANC, AST, ALT, immunization history, and vaccine titers after B-cell reconstitution were reviewed. Data were collected for 34 IPD patients (25 CRIM-negative and 9 CRIM-positive) with a median age at ERT initiation of 3.5 months (0.1–11.0 months). B-cell reconstitution, as measured by normalization of CD19%, was seen in all patients ($n = 33$) at a median time of 17 weeks range (11–55 weeks) post-rituximab. All maintained normal CD19% with the longest follow-up being 248 weeks post-rituximab. 30/34 (88%) maintained negative/low anti-rhGAA antibody titers, even with complete B-cell reconstitution. Infections during immunosuppression were reported in five CRIM-negative IPD patients, all resolved satisfactorily on antibiotics. There were no serious sequelae or deaths. Of the 31 evaluable patients, 27 were up to date on age-appropriate immunizations. Vaccine titers were available for 12 patients after B-cell reconstitution and adequate humoral response was observed in all except an

inadequate response to the Pneumococcal vaccine ($n = 2$). These data show the benefits of short-course prophylactic ITI in IPD both in terms of safety and efficacy. Data presented here are from the youngest cohort of patients treated with rituximab and expands the evidence of its safety in the pediatric population.

Keywords: anti-drug antibodies, enzyme replacement therapy, immune tolerance induction, anti-rhGAA IgG antibody, alglucosidase alfa, immunogenicity

INTRODUCTION

Lysosomal storage disorders (LSDs) are a group of inherited metabolic disorders caused by disease-associated variants in genes encoding catabolic enzymes active in the lysosome. The deficiency or complete absence of endogenous enzyme leads to a build-up of undegraded macromolecules in lysosomes affecting various target tissues depending on the specific enzyme deficiency (1–3). Although there is no cure for LSDs, the development of enzyme replacement therapy (ERT) aimed at replacing the deficient lysosomal enzymes and reducing the toxic substrate accumulation has greatly improved the course for several LSDs including Gaucher disease, Fabry disease, Pompe disease, Mucopolysaccharidoses (MPS) I, II, IVA, VI, and VII, and Wolman disease, with additional therapeutic proteins in development (4, 5). Despite the success of ERTs in improving outcomes for patients with LSDs, the development of anti-drug antibodies (ADA) against the therapeutic protein remains a challenge that impacts both the safety and efficacy of the treatment (6).

In 2006, the FDA approved alglucosidase alfa (recombinant human acid alfa-glucosidase, rhGAA) for treatment of Pompe disease, an autosomal recessive LSD caused by disease-associated variants in the GAA gene resulting in deficiency of acid-alpha glucosidase (GAA), predominantly affecting skeletal, smooth, and cardiac muscle (7, 8). Pompe disease has a phenotypic spectrum ranging from classic infantile Pompe disease (IPD) to late-onset Pompe disease. Classic IPD is the most severe end of the disease spectrum, with patients presenting with severe cardiomyopathy in the first few days to weeks of life and rarely surviving beyond 2 years of age without treatment (9). The availability of ERT with alglucosidase alfa has changed the natural course of Pompe disease, significantly prolonging survival and improving long-term clinical outcomes. Despite the considerable benefits of ERT, the overall response is heterogeneous and impacted by multiple factors including age at ERT, the extent of underlying pathology, the dose of ERT, and development of anti-drug antibodies. Additionally, there are other limitations of ERT including clearance by non-muscle tissue, limited cellular uptake in muscles, inability to cross blood-brain barrier, and variability of skeletal muscle response (10, 11). Published literature has demonstrated that long-term IPD survivors often have residual physical impairments including muscle weakness, hypernasal speech, dysphagia with a risk of aspiration, ptosis, and risk of arrhythmias (12).

The negative impact of IgG ADA to alglucosidase alfa in patients with IPD has been well established since the first clinical

trial (13–15). In two alglucosidase alfa clinical trials, 89% (35/39) of patients (NCT00125879, $n = 16/18$ and NCT00053573, $n = 19/21$) with IPD developed ADA to alglucosidase alfa and a subset developed high and sustained IgG antibody titers (HSAT) causing suboptimal treatment response resulting in clinical deterioration and death despite treatment with ERT (9, 14). Of critical importance, the development of ADA to ERT is strongly influenced by the patient's genetic variants which determine whether any GAA protein is generated, even if non-functional, as the production of a non-functional enzyme may still tolerize the immune system to some extent. Patients with two null variants, produce no GAA, resulting in the immune system recognizing rhGAA as foreign (16). IPD patients with two null GAA variants are considered cross-reactive immunologic material (CRIM)-negative and are at the highest risk of developing significant ADA to ERT (16, 17). A previous study assessing the impact of CRIM status on treatment outcomes in Pompe disease showed that CRIM-negative patients who received ERT monotherapy were either deceased or ventilator-dependent by age of 27.1 months due to the development of ADA (16). Immune tolerance induction (ITI) in the ERT-naïve setting has been established as a strategy to diminish the development and minimize the impact of ADA on treatment response to ERT and has become the standard of care for CRIM-negative IPD patients (18–23). Although endogenous GAA detected in CRIM-positive IPD patients can tolerize them to ERT, up to 32% of CRIM-positive IPD patients also develop deleterious ADA to ERT and the clinical course is indistinguishable from that of CRIM-negative IPD patients (24). Some CRIM-positive IPD patients at high risk of developing deleterious ADA can be identified based on previously reported GAA variants in IPD patients who developed HSAT or based on development of HSAT by an older sibling. We thus have instituted an immunomodulation approach at ERT initiation based on an algorithm of early immune response data in this subset of CRIM-positive IPD patients considered as high risk (22–24).

Different approaches to overcoming the challenges of ADA to ERT have been tried in patients with Pompe disease with varying degrees of success (25). These immunomodulation approaches, initiated with ERT, are intended to target B and T cells to induce long-term immune tolerance and improve treatment response to ERT (21, 23, 26, 27). While approaches have differed in terms of drug combinations, all approaches included rituximab, an anti-CD20 monoclonal antibody, which targets antibody-producing B cells. We previously reported success in inducing immune tolerance with a short-course of rituximab, low-dose of methotrexate, with/without IVIG in an international cohort

of 19 CRIM-negative IPD patients (23). Combination therapy targeting different cells of the immune system (B and T cells) to prevent the cascade for antibody development was the rationale for using rituximab and methotrexate. Rituximab is a chimeric monoclonal antibody that has been approved for multiple types of malignancy and autoimmune diseases. Methotrexate, an inhibitor of dihydrofolate reductase, impacts rapidly dividing T and B cells (28, 29). Experience from rheumatologic disorders has shown that the addition of methotrexate with other biological therapy has prevented the development of ADA against therapeutic proteins (30). We added IVIG at a dose of 500 mg/kg to provide passive immunity until B cell reconstitution would assure production of antibodies to pathogens. The duration of B cell depletion varied based upon patient age, treatment indication, additional immunosuppressive medication administration, and duration of treatment. An example of this is that children typically achieve B cell reconstitution within 1 year of rituximab discontinuation, while adults can take up to 2 years (31). Long-term follow-up studies show that there is skewing of B cell populations to naïve phenotypes resulting in prolonged low immunoglobulin levels and impaired responses to vaccines (32–37). Published literature of rituximab use in autoimmunity and malignancy has shown that though rare, infections can occur when B cells are undetectable (36, 38, 39).

The short 5-week course of ITI was able to tolerize IPD patients to ERT, as evidenced by negative/low anti-rhGAA IgG antibody titers even after B cell reconstitution and ability to receive age-appropriate routine vaccination. The data suggested that combination therapy was safely tolerated and was successful in inducing immune tolerance in most IPD patients. However, the long-term safety of this approach was not evaluated and published data on the safety of immunomodulation with rituximab, especially in pediatric populations, is limited. At this time, there are limited data in the literature on the long-term safety of rituximab, especially in patients younger than a year of age. Considering that the majority of patients with infantile Pompe disease are initiated on treatment within weeks of birth, it is important to understand the long-term safety of treatment in such a young and medically fragile population. The purpose of this study was to determine if patients with IPD who received rituximab experienced long-term impairment of the immune system, as described in the literature for its use in diverse disease settings. In the current study, we present the long-term safety and efficacy of short-course immune tolerance induction (ITI) in a relatively large cohort of CRIM-negative and CRIM-positive IPD patients by evaluating anti-rhGAA IgG antibody titers, absolute neutrophil count (ANC), aspartate aminotransferase (AST), alanine aminotransferase (ALT), B cell and T cell quantitation, vaccination history and titers against vaccines, left ventricular mass index (LVMI), and overall and ventilator-free survival.

MATERIALS AND METHODS

Patients and Inclusion Criteria

Inclusion criteria for the present study were based on the following; (1) a confirmed diagnosis of IPD with two disease-associated GAA variants and low GAA enzyme activity (8), (2) a

history of ITI with rituximab, methotrexate, with or without IVIG in ERT-naïve setting, and (3) at least 6 months of follow-up data since initiation of ITI. A retrospective chart review of qualifying patients with IPD was conducted. Data on patients included in the previous publication were reviewed and further longitudinal data since the last publication on patients who met the inclusion criteria were included for the analysis (23).

Ethics Approval

All patients were enrolled in a Duke institutional review board (IRB)-approved study protocol (Pro00001562; Determination of Cross-Reactive Immunological Material [CRIM] Status and Longitudinal Follow-up of Individuals with Pompe disease; LDN6709 Site 206; ClinicalTrials.gov NCT01665326). One CRIM-negative IPD patient included in this study had IRB/ethics committee approval from a local institution. All other patients were enrolled in the Duke IRB-approved study through written informed consent from a parent or legal guardian (15, 22, 40–42).

Immune Tolerance Induction (ITI)

The 5-week short-course immune tolerance induction approach included four doses of weekly rituximab (375 mg/m², intravenously), and three cycles of low-dose methotrexate (0.4 mg/kg; three doses per cycle with first three ERT infusions, subcutaneously or orally), as described previously (Supplementary Figure 1) (22). To provide passive immunity during B cell suppression, monthly IVIG at 500 mg/kg was added to the combination therapy during the time of B cell suppression. To avoid any significant interference of IVIG with antibody-dependent cellular cytotoxicity mediated B cell depletion, the first dose of IVIG was administered 24–48 h after the first dose of rituximab. There was no recommended supplier for rituximab, methotrexate, and IVIG in ITI protocol that was shared with treating physicians. To assess the safety of ITI, patients were monitored for incidence of infection around the time of ITI, decrease in ANC, and increase in AST, and/or ALT levels. An ANC of <750 cells/mm³ or AST and/or ALT >3 times their respective baseline values were considered as adverse events. Routine vaccinations, except for the flu shot were withheld while patients were B cell suppressed. Vaccinations were resumed following evidence of B cell reconstitution, defined as normalization of CD19% (40).

Patients were classified into three groups based on anti-rhGAA IgG antibody titers; (1) HSAT, defined as titers of ≥51,200 on two or more occasions at or beyond 6 months on ERT (15), (2) sustained intermediate titer (SIT), defined as titers of ≥12,800 and <51,200 on ERT (Lumizyme™ prescribing information) (15, 41), and (3) low titer (LT), defined as titers of ≤6,400. The cutoffs of 51,200 and 12,800 were utilized based on the findings from previous publications and Lumizyme™ prescribing information (15, 41). Previous studies have demonstrated that CRIM-positive and CRIM-negative IPD patients who developed anti-rhGAA IgG titers of ≥12,800 had poor clinical outcomes (15, 17, 42). Additionally, the Lumizyme prescribing information states that patients developing sustained anti-αglucosidase alfa antibody titers of ≥12,800 may have a poorer clinical response to treatment, or may lose motor function

as antibody titers increase. Patients with antibody titers $\geq 12,800$ at Week 12 of treatment had an average increase in α -glucosidase alfa clearance of 50% from Week 1 to 12. In our study with ITI, patients were considered immune tolerant if they met the following criteria: (1) were seronegative (did not develop anti-rhGAA IgG antibodies) or maintained anti-rhGAA IgG titers of $\leq 6,400$ throughout ERT, and (2) were able to receive age-appropriate routine vaccines.

Data Collection

Clinical data including GAA variants, CRIM status, age at diagnosis, age at ERT initiation, dose of ERT, longitudinal anti-rhGAA IgG antibody titers, LVMI, motor status, feeding status, and pulmonary status were extracted from medical records provided by the principal care provider of the patient. CRIM status was determined by western blot analysis in skin or blood at Duke GSD/LSD Enzymology Laboratory and confirmed by GAA variants or was predicted based on GAA variants as previously described (43). Anti-rhGAA IgG antibody titers were determined by Sanofi Genzyme (Framingham, MA, USA) by enzyme-linked immunosorbent assay and confirmed using radioimmunoprecipitation as previously described (8). Since the decrease in ANC and elevations in AST and ALT have been noted with treatment with rituximab and methotrexate, we evaluated these values in patients on the ITI protocol. ANC, ALT, and AST levels were monitored bi-weekly during ITI followed by monthly monitoring until return to baseline levels. Flow cytometry was performed in CLIA certified laboratories to define the following cell populations: CD19, CD3, CD3CD4, CD3CD8. Lymphocyte quantitation including CD19% was evaluated to monitor B cell suppression and B cell reconstitution. Lymphocyte quantitation was performed every 4 weeks until B cell recovery, then every 3–6 months. B cell depletion was defined as detection of CD19% below 1%. B cell reconstitution was measured in terms of normalization of CD19% to the normal range for the age as previously described (40). Additionally, CD3, CD4, and CD8% were evaluated to monitor T cell response. Titers against Diphtheria, Tetanus, Pneumococcal, Measles, Mumps, and Rubella (MMR) were assessed after vaccination and humoral response to vaccines were categorized as adequate (immune) or inadequate (not immune). Humoral response to Tetanus, Diphtheria, and MMR were determined to be adequate based on the respective CLIA certified laboratory reference ranges. The response to the Pneumococcal vaccine was determined to be adequate if $>50\%$ of serotypes had an antibody concentration of >1.3 micrograms per milliliter, as all patients were <6 years old (44). B cell reconstitution, vaccination status at baseline and after ITI, and titers against routine vaccines were collected to assess if administration of rituximab resulted in long-term immunodeficiency. Data collection continued until October 2019 or until at least 6 months had passed since the initiation of ERT and ITI, at which time the database was locked for analysis.

Statistics

Overall survival and ventilator-free survival for patients who received ITI were analyzed using the Kaplan-Meier method with two-tailed *P*-values generated using the log-rank test and

compared to a historical cohort of CRIM-negative IPD patients who received ERT monotherapy (15). Age at ERT, age at diagnosis, longitudinal anti-rhGAA IgG antibody titers, and LVMI were compared using Wilcoxon/Kruskal-Wallis rank-sum test. Analyses were performed with JMP Pro 14.0. Descriptive data are presented as medians.

RESULTS

Patients and Treatment Details

From our international cohort of infantile Pompe disease patients (IPD), 34 patients (25 CRIM-negative and 9 CRIM-positive) who met all inclusion criteria and had received a short-course of ITI with rituximab, methotrexate, and/or IVIG in ERT-naïve setting were identified. ADDIN EN.CITE (23) Of the 25 CRIM-negative patients, 17 patients (CN1 to CN17) were included in our previous publication and met the inclusion criteria for the current study. Three CRIM-negative IPD patients (CN1, CN2, and CN6) received ITI with rituximab and methotrexate and did not receive IVIG, as per the local treating physician's decision.

Patient demographics, age at diagnosis, age at ERT initiation, GAA variants, dose of ERT, current age, age at death, and CRIM status are shown in **Table 1**. The median age at diagnosis was 2.4 months (range, 0.0–5.9 months) and 4.2 months (range, 0.0–10.9 months) for CRIM-negative and CRIM-positive IPD groups, respectively (**Table 2**). The median age at ERT and ITI initiation was 3.1 months (range, 0.1–6.7 months) and 4.8 months (range, 0.1–11.0 months) for CRIM-negative and CRIM-positive groups, respectively (**Table 2**). There were no statistically significant differences in age at diagnosis ($p = 0.3189$) or age at ERT initiation ($p = 0.2828$) between CRIM-negative and CRIM-positive IPD patients. At the time of database lock, 27 IPD patients were alive (18 CRIM-negative and 9 CRIM-positive) and 7 CRIM-negative IPD patients were deceased (**Table 1**). No statistically significant differences were observed in age at diagnosis ($p = 0.2584$) or age at ERT initiation ($p = 0.2246$) between living and deceased IPD patients.

Anti-rhGAA IgG Antibody Titers (Table 3)

Of the 34 IPD patients, 30 patients [88%; 21 CRIM-negative (84%) and 9 CRIM-positive (100%)] were immune tolerant with the longest follow-up of 348 weeks following ITI. Of these 30 IPD patients, sixteen (47%; 11 CRIM-negative and 5 CRIM-positive) remained seronegative (did not develop detectable anti-rhGAA IgG antibodies) and 14 (41%; 10 CRIM-negative and 4 CRIM-positive) developed low antibody titers (defined as titers of $\leq 6,400$) throughout the course of ERT. Of the four CRIM-negative IPD patients who failed to tolerate, two (6%) (CN10 and CN12) developed sustained intermediate titers (titers of $\geq 12,800$ and $<51,200$) and two (6%) (CN13 and CN21) developed high and sustained antibody titers (titers of $\geq 51,200$ on two or more occasions). None of the CRIM-positive IPD patients who received immunomodulation developed SIT or HSAT. There was no recognizable difference in baseline characteristics between CRIM-negative IPD patients who developed HSAT/SIT and those who maintained low/negative anti-rhGAA IgG antibody titers (**Table 1**). The median peak anti-rhGAA IgG antibody titers

TABLE 1 | Demographics and treatment history.

Patient/Gender	GAA disease-associated variants		CRIM status	Age at diagnosis (months)	Age at ERT initiation (months)	Current age (months)	ERT dose at the time of initiation
	Allele 1	Allele 2					
ALIVE PATIENTS							
CN1/F	c.341insT	c.341insT	Negative	1.9	3.8	148.8	20 mg/kg EOW*
CN3/F	c.2608C>T	c.2608C>T	Negative	2.5	3.0	112.0	20 mg/kg EOW*
CN4/M	c.546+2T>C	c.546+2T>C	Negative	3.5	4.6	111.2	20 mg/kg EOW
CN5/F	c.236_246del	c.236_246del	Negative	2.0	2.5	108.4	20 mg/kg EOW*
CN10/M	c.2560C>T	c.1292_1295dupTGCA	Negative	2.4	2.6	92.3	20 mg/kg Weekly*
CN11/F	c.2560C>T	c.2560C>T	Negative	0.3	1.3	88.8	20 mg/kg EOW*
CN12/F	c.258dupC	c.2227C>T	Negative	2.6	3.1	71.1	20 mg/kg EOW*
CN13/M	c.1754+2T>A	c.1822C>T	Negative	0.9	1.8	87.5	20 mg/kg EOW*
CN14/F	c.2237G>A	c.437delT	Negative	5.9	6.6	71.5	20 mg/kg EOW
CN16/F	c.2560C>T	c.2560C>T	Negative	Prenatal	0.1	50.5	20 mg/kg EOW*
CN17/M	c.2560C>T	c.525delT	Negative	3.3	3.6	43.1	20 mg/kg Weekly*
CN18/F	c.1195-18_2190-20del	c.1195-18_2190-20del	Negative	3.9	4.4	53.7	20 mg/kg EOW*
CN19/M	c.1827C>G	c.2662G>T	Negative	0.3	0.8	41.5	20 mg/kg EOW*
CN20/M	c.525delT	c.1694_1697delTCTC	Negative	0.9	1.0	20.2	20 mg/kg EOW*
CN22/M	c.1548G>A	c.2560C>T	Negative	4.9	5.4	41.8	20 mg/kg EOW
CN23/M	c.525delT	c.2560C>T	Negative	0.7	1.4	39.0	20 mg/kg EOW*
CN24/M	c.1051delG	c.1579delA	Negative	0.4	0.5	27.0	20 mg/kg EOW*
CN25/M	c.525delT	c.2560C>T	Negative	Prenatal	0.1	8.1	40 mg/kg EOW
CP1/M	c.1912G>T	c.2481+102_2646+31del	Positive	4.5	4.8	70.6	20 mg/kg EOW*
CP2/M	c.2457_246delCTG	c.2560C>T	Positive	3.8	4.0	70.9	20 mg/kg EOW*
CP3/F	c.1844G>A	c.1844G>A	Positive	10.9	11.0	109.5	20 mg/kg EOW
CP4/M	c.2105G>T	c.2512C>T	Positive	Prenatal	0.7	73.1	20 mg/kg EOW
CP5/F	c.525delT	c.2481+110_2646+39del	Positive	6.0	7.3	87.8	20 mg/kg EOW
CP6/F	c.1841C>A	c.2481+102_2646+31del	Positive	4.2	5.1	46.5	40 mg/kg EOW*
CP7/M	c.1118T>G	c.1118T>G	Positive	Prenatal	0.1	21.2	20 mg/kg Weekly*
CP8/M	c.716delT	c.871C>T	Positive	6.0	6.4	42.9	20 mg/kg EOW*
CP9/F	c.1843G>A	c.1933G>C	Positive	0.1	0.9	36.9	20 mg/kg EOW
DECEASED PATIENTS							
CN2/M	c.1548G>A	c.525delT	Negative	2.4	3.6	56.9	20 mg/kg EOW
CN6/F	c.525delT	c.2560C>T	Negative	0.3	0.4	63.2	20 mg/kg Weekly
CN7/F	c.2560C>T	c.2560C>T	Negative	3.0	3.4	25.4	20 mg/kg EOW
CN8/F	c.525_526delTG	c.525_526delTG	Negative	5.5	6.7	30.0	20 mg/kg EOW
CN9/F	c.2560C>T	c.2560C>T	Negative	3.2	3.9	15.0	20 mg/kg EOW
CN15/F	c.2560C>T	c.2560C>T	Negative	5.1	6.6	15.5	20 mg/kg EOW*
CN21/F	c.2238G>A	c.2560C>T	Negative	5.9	6.3	25.1	40 mg/kg EOW*

GAA, gene encoding acid α -glucosidase; CRIM, Cross-reactive immunologic material; ERT, enzyme replacement therapy; EOW, every other week.

*Patient was initiated on or subsequently received ERT at a higher dose than the recommended dose of 20 mg/kg EOW.

were 200 (range, 0–51,200) and 0 (range, 0–200) for ITI treated CRIM-negative and CRIM-positive IPD groups, respectively (Table 2). The median peak anti-rhGAA IgG antibody titers were 0 (range, 0–6,400) for tolerized ($n = 21$) and 38,400 (range, 25,600–51,200) for nontolerized CRIM-negative IPD patients ($n = 4$). The median anti-rhGAA IgG antibody titers at the final assessment were 100 (range, 0–51,200) for ITI treated CRIM-negative IPD and 0 (range, 0–100) for ITI treated CRIM-positive IPD at the median timepoint following ERT initiation

of 108 weeks (range, 19–351 weeks) and 104 weeks (range, 35–272 weeks) for ITI treated CRIM-negative and CRIM-positive groups, respectively (Figure 1). The median anti-rhGAA IgG antibody titers at the final assessment were 0 ($n = 21$; range, 0–6,400) and 19,200 ($n = 4$; range, 3,200–51,200) for tolerized and nontolerized CRIM-negative IPD patients, respectively. Overall, 88% of IPD patients who received immunomodulation in the ERT-naïve setting either remained seronegative or maintained low anti-rhGAA IgG antibody titers (Table 3).

TABLE 2 | Summary of age at diagnosis, age at ERT initiation, anti-rhGAA IgG antibody titer, B cell, and LVMI.

	<i>N</i>	Median	Range
Age at diagnosis			
CRIM-negative	25	2.4 months	0.0–5.9 months
• Alive CRIM-negative	18	2.0 months	0.0–5.9 months
• Deceased CRIM-negative	7	3.2 months	0.3–5.9 months
CRIM-positive (all alive)	9	4.2 months	0.0–10.9 months
Alive (CRIM-negative and CRIM-positive)	27	2.4 months	0.0–10.9 months
Age at ERT initiation			
CRIM-negative	25	3.1 months	0.1–6.7 months
• Alive CRIM-negative	18	2.5 months	0.1–6.6 months
• Deceased CRIM-negative	7	3.9 months	0.4–6.7 months
CRIM-positive	9	4.8 months	0.1–11.0 months
Alive (CRIM-negative and CRIM-positive)	27	3.0 months	0.1–11.0 months
Peak anti-rhGAA IgG antibody titer			
CRIM-negative	25	200	0–51,200
• CRIM-negative tolerized	21	0	0–6,400
• CRIM-negative nontolerized	4	38,400	25,600–51,200
CRIM-positive (all tolerized)	9	0	0–200
Anti-rhGAA IgG antibody titer at last assessment (time since ERT initiation)			
CRIM-negative	25	100 (108 weeks)	0–51,200 (19–351 weeks)
• CRIM-negative tolerized	21	0 (103 weeks)	0–6,400 (19–351 weeks)
• CRIM-negative nontolerized	4	19,200 (228 weeks)	3,200–51,200 (72–343 weeks)
CRIM-positive (all tolerized)	9	0 (104 weeks)	0–100 (35–272 weeks)
B cell response			
Time to B cell depletion	31	3 weeks	1–5 weeks
Time to B cell reconstitution	33	17 weeks	11–54 weeks
LVMI at baseline			
CRIM-negative	20	178.2 g/m ²	55.5–448.9 g/m ²
CRIM-positive	8	221.0 g/m ²	93.98–628.6 g/m ²
LVMI at most recent follow-up (time since ERT initiation)			
CRIM-negative	24	62.9 g/m ² (84 weeks)	46.0–257.0 g/m ² (9–437 weeks)
CRIM-positive	8	69.7 g/m ² (73 weeks)	61.0–174.6 g/m ² (23–102 weeks)

N, number; CRIM, cross-reactive immunologic material; ERT, enzyme replacement therapy; LVMI, left ventricular mass index.

Safety Measures

ANC, ALT, and AST Data

In the first 10 weeks on ERT and ITI, AST and ALT data were available for 28 IPD patients. Only one IPD patient (CN14) had an increase in AST >3 times baseline value and subsequently decreased to baseline levels 6 weeks following the last dose of rituximab. None of the other IPD patients exhibited such an increase in AST or ALT during immunomodulation. AST decreased to baseline levels 6 weeks following the last dose of rituximab. Moreover, since methotrexate and rituximab can

induce neutropenia, ANC data for the first 10 weeks on ITI were analyzed and were available in 21 IPD patients. Eight patients (6 CRIM-negative and two CRIM-positive; patients: CN10, CN11, CN12, CN13, CN16, CN24, CP6, and CP7) developed ANCs of <750 cells/mm³ following immunomodulation. Neutropenia was transient and ANC level returned to normal in all patients within 23 weeks following cessation of immunomodulation.

Infections During Immunomodulation

Detailed information on the presence or absence of infections during ITI was available for 23 IPD patients (Table 3): five patients (CN3, CN4, CN12, CN14, and CN18) experienced infections while immune suppressed (23). Of these five IPD patients, details on four IPD patients have been reported previously (23). Central line infections and bacteremia were observed in Patient CN3 (*Enterococcus faecalis*, *Pseudomonas fluorescens/putida*, and *Enterococcus raffinosus*) and Patient CN12 (*Klebsiella pneumoniae*) requiring central line removal and antibiotic treatment. Patient CN4 had a respiratory syncytial virus infection and Patient CN14 suffered an episode of aspiration pneumonia and enterovirus/rhinovirus infection during immunomodulation. Patient CN18 experienced rhinorrhea, ear infection, and *Escherichia coli* urinary tract infection 4 weeks following completion of rituximab administration. Infections were managed with antibiotics without interrupting ERT infusions or ITI therapy. Overall, ITI was safely tolerated without any life-threatening infections.

B Cell Reconstitution

Longitudinal follow-up of CD19% was available for 33 IPD patients (except patient CN6), with the longest follow-up of 248 weeks following the last dose of rituximab (Table 3). T cell percentages (CD3+, CD4+, CD8+) were within normal ranges for age making CD19% an appropriate measure for B cell reconstitution (Supplementary Figure 2). Complete B cell reconstitution, defined as normalization of CD19% for age, was seen in all 33 IPD patients. The median time to B cell depletion was 3 weeks ($n = 31$; range, 1–5 weeks) following initiation of ERT + ITI and the median time to complete B cell reconstitution was 17 weeks ($n = 33$; range, 11–54 weeks) following the last dose of rituximab. Following B cell reconstitution, all patients continued to maintain normal B cell counts, as measured by CD19%, with a median follow-up of 43 weeks ($n = 33$; range, 11–248 week). B cell reconstitution within 3 months following the last dose of rituximab was observed in three IPD patients (CN5, CN24, and CP9) and three patients (CN7, CN8, and CP3) experienced B cell recovery later than 9 months following the last dose of rituximab.

Vaccination and Titers Against Vaccines

Data on routine vaccination prior to initiation of ITI were available on 19 IPD patients (13 CRIM-negative and 6 CRIM-positive) (Table 3). Of these 19 patients, 13 patients received age-appropriate vaccination prior to immunomodulation. Vaccination details post immunomodulation were available on 31 IPD patients (24 CRIM-negative and 7 CRIM-positive) and all except four CRIM-negative patients (CN15, CN20, CN22,

TABLE 3 | B cell reconstitution, infections, vaccination, anti-rhGAA IgG antibody titers, and ITI protocol deviations in IPD patients treated with immunomodulation.

ID	Rounds of ITI	B Cell recovery (weeks post-RTX)	Weeks post-RTX at last CD19% follow-up	Infections	Vaccination prior to ERT	Vaccination up to date for age	Anti-rhGAA IgG antibody titers			ITI protocol deviations
							Peak titers (weeks on ERT)	Last titers (weeks on ERT)	Immune tolerant (Yes/No)	
ALIVE PATIENTS										
CN1	1	Yes (17)	17	No	NA	Yes	1,600 (38)	200 (103)	Yes	Did not receive IVIG
CN3	1	Yes (19)	43	Enterococcus faecalis, Pseudomonas fluorescens/putida, Enterococcus raffinosus	NA	Yes	0	0 (281)	Yes	IVIG: 1 dose during ITI + 2 doses after ITI
CN4	1	Yes (17)	21	RSV infection	NA	Yes	0	0 (284)	Yes	IVIG started at Week 4 on ERT
CN5	1	Yes (11)	73	No	NA	Yes	0	0 (269)	Yes	None
CN10	2	Yes (19)	195	No	NA	Yes	25,600 (198)	25,600 (198)	No	Maintenance rituximab every 2 to 3 months following ERT+ITI for 32 months
CN11	1	Yes (32)	156	No	NA	Yes	200 (69)	0 (351)	Yes	3rd cycle of MTX was administered with 4th ERT infusion instead of 3rd ERT infusion
CN12	1	Yes (25)	33	Klebsiella pneumoniae	NA	Yes	25,600 (94)	3,200 (258)	No	None
CN13	>2	Yes (17)	208	No	Yes	Yes	51,200 (71)	12,800 (343)	No	Multiple cycle of ITI
CN14	1	Yes (25)	82	Aspiration pneumonia, enterovirus/rhinovirus	Yes	Yes	200 (81)	200 (81)	Yes	None
CN16	1	Yes (27)	91	No	No	Yes	0	0 (174)	Yes	None
CN17	1	Yes (13)	29	No	NA	Yes	6,400 (54)	3,200 (135)	Yes	None
CN18	1	Yes (13)	45	URI rhinorrhea; UTI E. Coli; Ear infection	Yes	Yes	200 (34)	100 (161)	Yes	None
CN19	1	Yes (17)	159	NA	Yes	Yes	0	0 (112)	Yes	None
CN20	1	Yes (13)	13	NA	Yes	No	0	0 (19)	Yes	None
CN22	1	Yes (16)	29	No	No	No	200 (108)	200 (108)	Yes	None
CN23	1	Yes (20)	102	No	Yes	Yes	0	0 (144)	Yes	None
CN24	1	Yes (12)	36	No	NA	NA	0	0 (31)	Yes	None
CN25	1	Yes (19)	19	No	No	No	0	0 (31)	Yes	None
CP1	1	Yes (18)	21	NA	Yes	NA	0	0 (35)	Yes	None
CP2	1	Yes (13)	248	NA	Yes	Yes	0	0 (171)	Yes	None
CP3	1	Yes (43)	75	NA	NA	NA	100 (4)	100 (78)	Yes	None
CP4	1	Yes (19)	19	NA	No	Yes	200 (22)	0 (272)	Yes	None
CP5	1	Yes (13)	53	NA	Yes	Yes	0	0 (39)	Yes	None
CP6	1	Yes (14)	164	NA	NA	Yes	0	0 (117)	Yes	2nd cycle of MTX was withheld. Three cycles of MTX administered with 1st, 3rd, and 4th ERT infusions.
CP7	1	Yes (15)	37	No	No	Yes	100 (66)	100 (66)	Yes	None
CP8	1	Yes (17)	141	NA	NA	Yes	0	0 (105)	Yes	None
CP9	1	Yes (12)	28	NA	Yes	Yes	200 (26)	100 (104)	Yes	None

(Continued)

TABLE 3 | Continued

ID	Rounds of ITI	B Cell recovery (weeks post-RTX)	Weeks post-RTX at last CD19% follow-up	Infections	Vaccination prior to ERT	Vaccination up to date for age	Anti-rhGAA IgG antibody titers			ITI protocol deviations
							Peak titers (wks on ERT)	Last titers (wks on ERT)	Immune tolerant (Yes/No)	
DECEASED PATIENTS										
CN2	1	Yes (23)	51	No	NA	Yes	0	0 (67)	Yes	Two doses of MTX with 3rd ERT infusion and additional cycles of MTX administered with 4th, 5th, and 6th ERT infusions. Did not receive IVIG.
CN6	1	NA	NA	No	No	Yes	0	0 (70)	Yes	
CN7	2	Yes (55)	59	No	Yes	Yes	6,400 (31)	3,200 (91)	Yes	
CN8	2	Yes (43)	43	No	NA	Yes	6,400 (29)	6,400 (46)	Yes	
CN9	1	Yes (22)	35	No	NA	Yes	1,600 (38)	800 (45)	Yes	
CN15	1	Yes (22)	31	No	Yes	No	200 (3)	200 (32)	Yes	None
CN21	1	Yes (13)	17	NA	Yes	Yes	51,200 (65)	51,200 (72)	No	None

RTX, rituximab; ERT, enzyme replacement therapy; NA, not available; wks, weeks; ITI, immune tolerance induction; MTX, methotrexate; IVIG, intravenous immunoglobulin.

and CN25) were up to date on their routine vaccination. Patient CN22 did not receive any vaccinations as per the decision of the parents/legal guardians. Patient CN15 was deceased, due to disease progression, prior to completion of routine immunization. Immunization was not yet resumed in Patients CN20 and CN25 at the time of database lock. After B cell reconstitution, titers against routine vaccines were performed in 12 patients (8 CRIM-negative and 4 CRIM-positive) and were categorized as adequate or inadequate based on the reference antibody values (Table 4). Two CRIM-positive IPD patients (CP2 and CP8) demonstrated an inadequate response to certain serotypes of the Pneumococcal vaccine. These two CRIM-positive IPD patients (CP2 and CP8) had an adequate humoral response to other vaccines where titers against the vaccine were performed. Four IPD patients (CN18, CN19, CN21, and CP2) had received age-appropriate vaccination prior to ITI and had titers against vaccines available (Tables 3, 4). All four IPD patients showed adequate humoral responses, although, it was not possible to determine if the response to the pre-ITI vaccine was maintained or lost, as all four patients received revaccination following complete B cell reconstitution after cessation of immunomodulation.

Left Ventricular Mass Index (LVMI)

LVMI at baseline was available for 28 IPD patients (20 CRIM-negative and 8 CRIM-positive) and at follow-up for 32 IPD patients (24 CRIM-negative and 8 CRIM-positive) (Table 5). Median LVMI at baseline was 178.2 g/m^2 ($n = 20$; range, $55.5\text{--}448.9 \text{ g/m}^2$) and 221.0 g/m^2 ($n = 8$; range, $93.98\text{--}628.6 \text{ g/m}^2$) for CRIM-negative and CRIM-positive IPD groups, respectively, with the upper limit of normal LVMI at 64 g/m^2 . Median LVMI at the most recent follow-up of CRIM-negative IPD patients was 62.9 g/m^2 ($n = 24$; range, $46.0\text{--}257.0 \text{ g/m}^2$) at a median time since ERT initiation of 84 weeks (range, 9–437 weeks). Median LVMI at the most recent follow-up of CRIM-positive IPD patients was 69.7 g/m^2 ($n = 8$; range, $61.0\text{--}174.6 \text{ g/m}^2$) at a median time since ERT initiation of 73 weeks (range, 23–102 weeks). It is important to note that all patients experienced decreases in their LVMI with 17 IPD patients (13 CRIM-negative and 4 CRIM-positive) having LVMI within the normal range (below 64 g/m^2) at the most recent follow-up. In contrast, CRIM-negative IPD patients from the original alglucosidase alfa clinical trials, who were not tolerized, but treated with ERT monotherapy, had progressive increases in their LVMI beyond the first 6 months on ERT (8, 9, 45, 46).

Overall and Invasive Ventilator-Free Survival (Figure 2)

Of the 34 IPD patients, 27 (18 CRIM-negative and all 9 CRIM-positive) were alive and seven were deceased (all CRIM-negative; CN2, CN6, CN7, CN8, CN9, CN15, and CN21) at the time of database lock. Among the living IPD patients, the median current age is 70.6 months (range, 8.1–148.8 months). Of the seven deceased CRIM-negative patients, the median age of death was 25.4 months (range, 15.0–63.2 months). The cause of death for all seven was cardiorespiratory failure due to disease progression and was unrelated to ITI. However, there were no

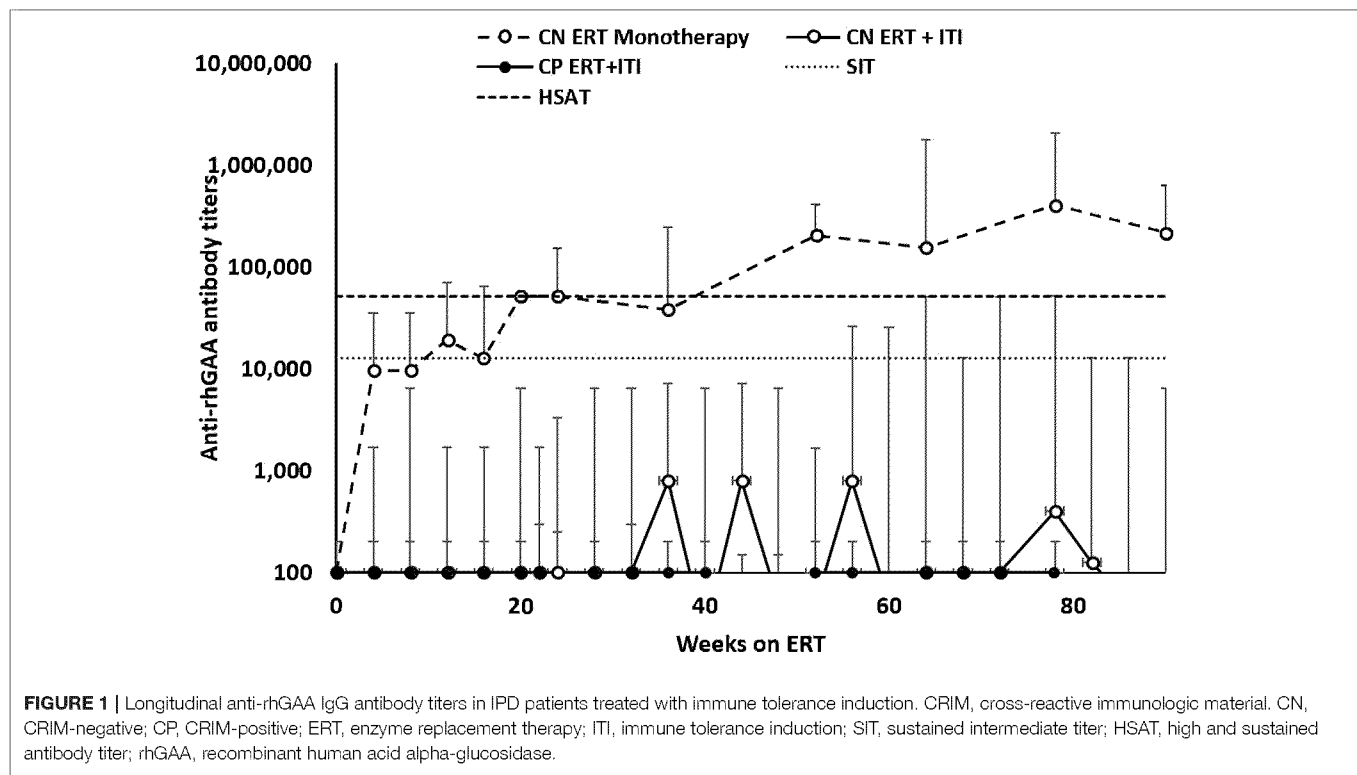


TABLE 4 | Humoral response to routine vaccinations.

Patient	Tetanus	Diphtheria	Measles	Mump	Rubella	Pneumococcal
CN3	Adequate	ND	Adequate	Adequate	Adequate	Adequate
CN11	Adequate	Adequate	Adequate	Adequate	Adequate	ND
CN12	Adequate	Adequate	Adequate	Adequate	Adequate	Adequate
CN16	Adequate	Adequate	ND	ND	ND	ND
CN17	Adequate	Adequate	Adequate	Adequate	Adequate	Adequate
CN18	Adequate	Adequate	Adequate	Adequate	ND	ND
CN19	Adequate	Adequate	ND	Adequate	Adequate	ND
CN21	Adequate	Adequate	ND	ND	ND	ND
CP2	Adequate	ND	Adequate	Adequate	Adequate	Inadequate
CP6	Adequate	Adequate	ND	ND	Adequate	ND
CP8	Adequate	Adequate	ND	ND	Adequate	Inadequate
CP9	ND	ND	ND	ND	ND	Adequate

ND, Not done.

statistically significant differences in either age at diagnosis ($p = 0.0896$) or age at ERT initiation ($p = 0.0693$) between living and deceased CRIM-negative IPD patients (Table 1). Mortality in this population better reflects the extent of pathology prior to treatment initiation.

Median age at ERT initiation was 3.0 months (range, 0.1–11.0 months) and 3.9 months (range, 0.4–6.7 months) and median age at diagnosis was 2.4 months (range, 0.0–10.9 months) and 3.2 months (range, 0.3–5.9 months) for living and deceased groups, respectively. Among living CRIM-negative IPD patients ($n = 18$) median age at diagnosis (2.0 months; range, 0.0–5.9

months) and median age at ERT initiation (2.5 months; range, 0.1–6.6 months) were earlier compared to the median age at diagnosis (3.2 months; range, 0.3–5.9 months) and median age at ERT initiation (3.9 months; range 0.4–6.7 months) in deceased CRIM-negative IPD patients ($n = 7$). Although lacking statistical significance, even a relatively short delay in ERT may impact extent of clinical benefits and lead to permanent muscle loss.

As previously reported, all CRIM-negative IPD patients from original clinical trials, who received ERT monotherapy, were either deceased or invasive ventilator-dependent by 27.1 months of age (17). In the current cohort of CRIM-negative IPD

TABLE 5 | Efficacy of ERT + ITL.

ID	LVMI		Motor Status		Ventilation Status		Feeding Status	
	Baseline	Final assessment (weeks on ERT)	Baseline	Final assessment (weeks on ERT)	Baseline	Final assessment (weeks on ERT)	Baseline	Final assessment (weeks on ERT)
ALIVE PATIENTS								
CN1	NA	NA	Hypotonia	Ambulatory (378)	No support	No support (378)	Oral	Oral (378)
CN3	160.3	57.8 (437)	Head lag, severe hypotonia, and motor delay	Ambulatory; wheelchair as needed, mostly for transportation (450)	Oxygen	No support (450)	NG tube	Oral (450)
CN4	445.8	68 (274)	Head lag, and antigravity movements (arms > legs)	Can move arms against gravity (286)	Oxygen and BiPAP at night	Invasively ventilated (271)	NG tube	G Tube (271)
CN5	277	80 (334)	Severe hypotonia, floppy baby, and no head or neck control	Ambulatory (76)	Oxygen	No support (76)	NG tube	Oral (76)
CN10	NA	58.4 (227)	Hypotonia	Ambulatory (199)	Invasively ventilated	No support (199)	TP	Oral (199)
CN11	140.6	53.7 (252)	Motor status and milestones appropriate for the age	Ambulatory (182)	No support	No support (213)	Oral	CPAP with nasal mask (199)
CN12	156.7	53.82 (208)	Hypotonia	Ambulatory (273)	No support	Vest/cough assist (273)	Oral	Oral (273)
CN13	NA	53.5 (217)	Hypotonia	Severely hypotonic, unable to hold head up, rollover, or sit unassisted. Can move both arms weakly (350)	No support	Invasively ventilated (350)	Oral	G Tube (350)
CN14	176	48 (105)	Hypotonia	NA	BiPAP	BiPAP (92)	G tube	G tube (92)
CN16	65.4	58.8 (48)	Head lag, and hypotonia	Normal developmental milestones (58)	CPAP for a week	No support (37)	Oral	Oral (37)
CN17	433.1	49.9 (124)	Normal symmetric bulk and appeared to have normal tone	Stands with support and braces, sits unassisted rollside to side, and can lift head up. Crawls and pushes to quadruped and creeps on hands and knees. (130)	No support	No support (130)	Oral	G Tube, eating puree orally (130)
CN18	448.9	62.7 (185)	Delayed motor milestones	Ambulatory (195)	Nasal O2	Recommended CPAP at night (195)	Oral and NG tube	Oral (195)
CN19	NA	63.02 (163)	NA	Ambulatory (162)	NA	No support (162)	NA	Oral (162)
CN20	180.4	80.4 (23)	Hypotonia	Generalized hypotonia (50)	No support	Overnight BiPAP (59)	NG tube	G tube (59)
CN22	211.9	192.5 (26)	Mild hypotonia and delayed head control at 3 months but rolling	Ambulatory. Walks, runs, jumps, feeds self, plays with siblings, dresses self, and walks upstairs (136)	No support	No support (136)	NG tube	NG tube and oral (136)
CN23	156.2	60.2 (141)	Mild hypotonia	Ambulatory; meeting developmental milestones (101)	No support	No support (101)	Oral	Oral (101)
CN24	158	46 (47)	Delayed milestones	Ambulatory. Walking, running, and jumping (88)	No support	No support (156)	Oral	Oral (156)
CN25	55.5	62.3 (9)	Age appropriate gross motor skill development	Meeting developmental milestones (27)	No support	No support (27)	Oral	Oral (27)

(Continued)

TABLE 5 | Continued

ID	LVMI		Motor Status		Ventilation Status		Feeding Status	
	Baseline	Final assessment (weeks on ERT)	Baseline	Final assessment (weeks on ERT)	Baseline	Final assessment (weeks on ERT)	Baseline	Final assessment (weeks on ERT)
CP1	423.6	75.1 (31)	Mild axial hypotonia with head lag	Ambulatory with moderate hypotonia. Severe delay in gross motor development (196)	No support	No support (220)	Oral, NG Tube at night	Oral (220)
OP2	186	61 (81)	Good muscle strength and tone. Minimal head lag, lifts head up	Not ambulatory. Does not move legs in supine, requires assistance for head control in supported sitting (274)	No support	Invasively ventilated (274)	NG Tube	G Tube (274)
CP3	628.6	174.6 (76)	Head lag.	Ambulatory. Mild proximal weakness (100)	No support	No support (411)	Oral	Oral (411)
OP4	251.7	75.6 (23)	NA	Ambulatory. Uses a wheelchair for transportation (297)	No support	Oxygen at night (268)	Oral	Oral (297)
CP5	248	105.3 (98)	NA	NA	NA	NA	NA	NA
CP6	NA	NA	General hypotonia	Ambulatory. Low muscle tone, global muscle weakness, and delayed motor skills (167)	High flow nasal cannula	No support (167)	NG Tube	Oral (167)
OP7	93.98	64.2 (37)	Normal muscle tone	Ambulatory; Low tone (72)	No support	No support (72)	Oral	Oral (72)
OP8	194	62 (70)	Rolling supine to left and right, and side lying	Ambulatory (137)	No support	No support (137)	Oral	Oral (137)
CP9	122	63 (102)	Slightly decreased tone. Able to control head without support	Ambulatory. Able to get up and down from the floor and steps with assistance (130)	No support	No support (135)	Oral	Oral (135)
DECEASED PATIENTS								
CN2	NA	257 (63)	Hypotonia	Prop-sits unassisted, rolls from supine to side lying, and bears weight through lower extremities in supported standing (80)	Transient ventilation for 3 days	No support (67)	Oral	Oral (80)
CN6	409.6	92.3 (53)	Axial hypotonia, withdraws extremities to stimulation, weak grasp	Sits with support and minimal capacity for weight-bearing on lower extremities (53)	Invasively ventilated	Invasively ventilated (off ventilator 10–12 hours a day) (58)	G tube	G tube (58)
CN7	317.2	144.9 (54)	Head lag and unable to sit or rollover	Standing with support (46)	Invasively ventilated	Oxygen and BiPAP at night (46)	NG Tube	G Tube (46)
CN8	347.1	107.9 (36)	Severe hypotonia	Able to move arms against gravity but near-complete lower extremity immobility (50)	Invasively ventilated	BiPAP at night (50)	NG Tube	G Tube (50)
CN9	220	83 (39)	Unable to independently hold head or sit unsupported	Not able to hold head or sit unsupported (46)	No support	Invasively ventilated (46)	NG Tube	GJ Tube (46)
CN15	127.5	118.3 (28)	Hypotonia	Not ambulatory (38)	Invasively ventilated	Invasively ventilated (38)	NG tube	G tube (38)
CN21	160.1	76 (15)	NA	Not ambulatory, severely limited motor skills (56)	Invasively ventilated	Invasively ventilated (56)	NG Tube	G Tube (56)

LVMI, left ventricular mass index; ERT, enzyme replacement therapy; ITI, immune tolerance induction; NA, not available; NG, nasogastric tube; TP, transpyloric.

Two CRIM-positive IPD patients had inadequate humoral response to Pneumococcal vaccine while demonstrating adequate response to other age-appropriate vaccines. It is not clear if the lack of response to Pneumococcal vaccine in these two patients was due to immunosuppression or normal variability in the efficacy of vaccine. As per the CDC, the efficacy of PCV13 is 45.6–75.0% and PPSV23 is 60–70%.

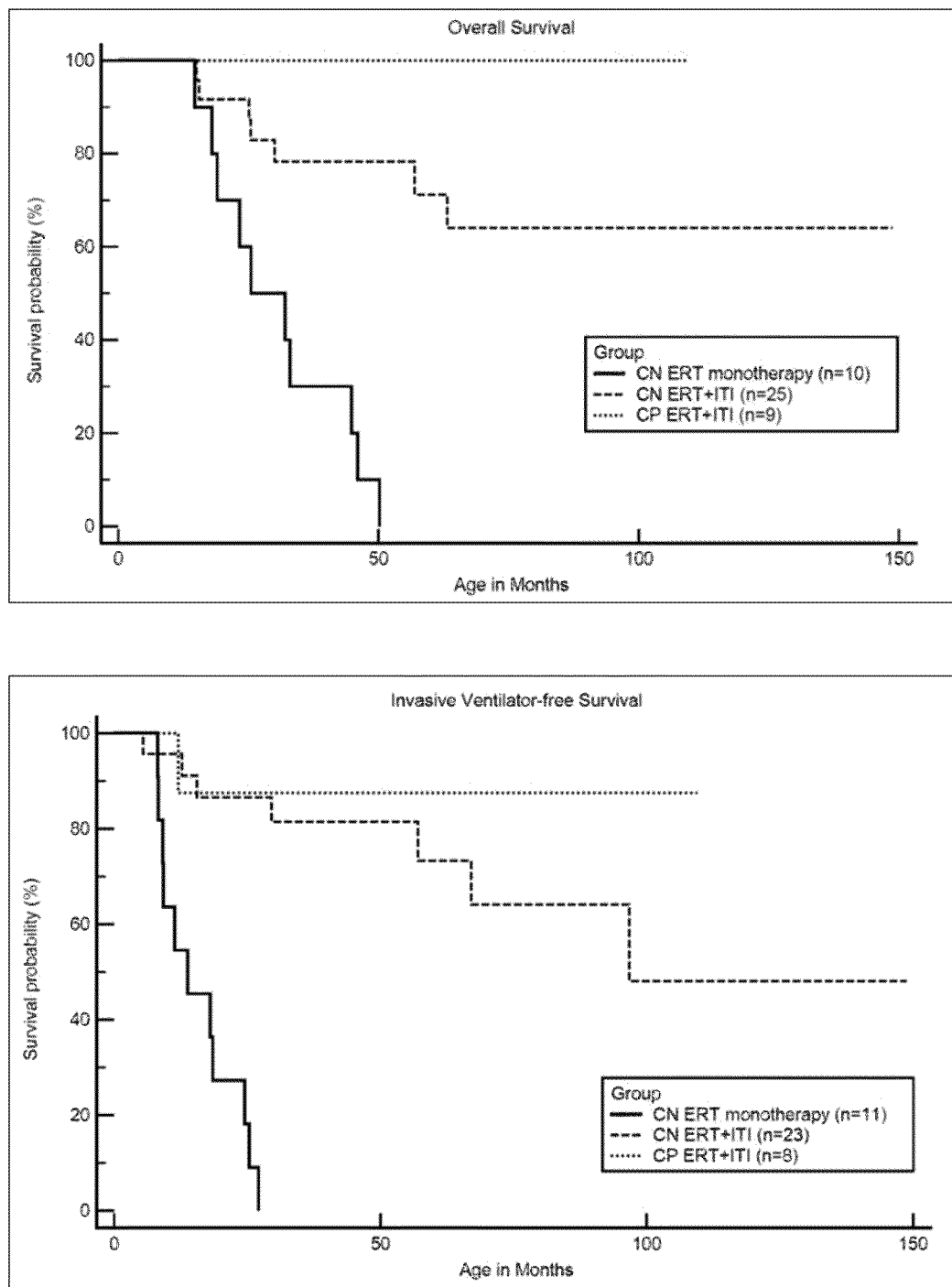


FIGURE 2 | Kaplan-Meier survival analysis: overall and invasive ventilator-free survival. CN, CRIM-negative; CP, CRIM-positive; ERT, enzyme replacement therapy; ITI, immune tolerance induction.

patients, 16 CRIM-negative IPD patients (64%) were living without the need for invasive ventilation with the age of the oldest survivor being 148.8 months (range, 8.1–148.8 months) (Figure 2). Invasive ventilator-free survival was significantly ($p = 0.0010$) improved in CRIM-negative IPD patients who received ERT with ITI compared to ERT monotherapy.

In the current analysis, at baseline, eleven CRIM-negative (CN1, CN9, CN11, CN12, CN13, CN17, CN20, CN22, CN23, CN24, and CN25) and seven CRIM-positive (CP1, CP2, CP3, CP4, CP7, CP8, and CP9) IPD patients did not require any respiratory support whereas six CRIM-negative IPD patients (CN2, CN6, CN7, CN8, CN15, and CN21) were invasively

ventilated (Table 5). Of these six invasively ventilated IPD patients, three (CN7, CN8, and CN10) were able to come off invasive ventilation with patient CN10 requiring no respiratory support at the most recent follow-up. At the most recent follow-up, 13 CRIM-negative (CN1, CN2, CN3, CN5, CN10, CN11, CN16, CN17, CN19, CN22, CN23, CN24, and CN25) and 6 CRIM-positive (CP1, CP3, CP6, CP7, CP8, and CP9) IPD patients did not require respiratory assistance whereas six CRIM-negative (CN4, CN6, CN9, CN13, CN15, CN21) and one CRIM-positive (CP2) IPD patients were invasively ventilated.

DISCUSSION

The negative impact of high and sustained anti-rhGAA IgG antibody titers to treatment response has been evident since the first clinical trial of alglucosidase alfa (46). Although the published literature has supported that abrogation of the immune response to ERT improves the efficacy of ERT in patients with Pompe disease (22, 23, 47), the long-term safety with the use of rituximab in such a young and medically fragile population has been an outstanding concern, thus prompting this study. This is the largest cohort of CRIM-negative and CRIM-positive IPD patients, to our knowledge, treated with ITI in the ERT-naïve setting with the longest follow-up of 148 months on ERT. It is also the youngest cohort of patients that have received rituximab for any indication; 16 patients were initiated on rituximab ages ≤ 3 months. In the published literature, the experience on safety of rituximab has been reported on patients aged 4 months to 18 years (33–35, 38, 48–53).

We found important clinical improvements from the initiation of a short course of ITI with rituximab, methotrexate, and IVIG concomitant with ERT. These improvements included reduced need for mechanical ventilation, LVMI, improved motor ability and longer overall survival. Importantly, three CRIM-negative IPD patients, who were invasively ventilated at baseline, no longer required invasive ventilation at the most recent follow-up, demonstrating a significant reversal in the disease course. This was an important finding as IPD patients are rarely able to come off ventilatory support once invasively ventilated. This further demonstrates the benefits of initiation of a short course of ITI with rituximab, methotrexate, and IVIG concomitant with ERT.

Immunomodulation was largely successful in reducing the development of anti-rhGAA IgG antibody titers. Thirty patients with IPD (88%), who received prophylactic ITI, either did not develop ($n = 16$) or maintained low anti-rhGAA IgG antibody titers ($n = 14$). Four CRIM-negative IPD patients developed anti-rhGAA IgG antibody titers in the SIT or HSAT ranges; one of the SIT patient's rhGAA IgG antibody titers subsequently decreased to 3,200 at the final assessment. There were no recognizable differences in baseline characteristics between IPD patients who maintained low antibody titers and IPD patients who developed SIT or HSAT. There is no apparent explanation as to why these four IPD patients did not respond to ITI similarly to other patients in the current cohort. One hypothesis for the lack of response is resistance to rituximab.

Rituximab resistance is known to be a common occurrence in naïve patients; however, its mechanism is incompletely understood. The potential mechanism of rituximab resistance is Fc receptor genetic variants affecting the affinity of effector cells for rituximab, complement depletion, and loss or decreased expression of CD20 on target antigen (54). Another possible reason for the development of high sustained anti-rhGAA IgG antibody titers in a few cases (CN10, CN12, CN13, and CN21) is incomplete B cell depletion. Rituximab has important limitations in that it doesn't deplete plasma cells, as they do not express CD20. Additionally, murine models have shown that 5% of B cells in lymph nodes survive CD20 depletion strategies (55). Although another recognized challenge with the use of rituximab is infusion-related reactions; with an incidence of infusion-related reactions of 25% of NHL patients and 25% of CLL patients interestingly, we did not observe any infusion-related reactions to rituximab in the current cohort of IPD patients.

Major concerns with the use of rituximab in patients with neoplastic and autoimmune disorders consist of significant delays in B cell recovery, skewing of B cell subpopulation to immature phenotype, and inability to mount a protective humoral response to vaccines (32). The addition of methotrexate to rituximab in the immunosuppressive regimen IPD patients receive only heightens the potential concerns. Much of the published data on rituximab originated from the treatment of adult populations where the extent of symptomatic hypogammaglobulinemia with an average of 4–6% of patients on rituximab requiring IVIG for symptomatic hypogammaglobulinemia. Persistent hypogammaglobulinemia was seen in up to 40% of patients on rituximab (usually those on long courses of rituximab to treat lymphoma), vaccine response was sometimes altered, and B cell reconstitution could take up to 24 months (36, 37). Small studies in pediatric populations have been more optimistic showing normalization of B cells by 1 year in nearly all patients, regardless of indication or duration of rituximab, and hypogammaglobulinemia rates at a maximum of 22% (33, 34, 38, 52). Our cohort of IPD patients is the largest group of patients under 1 year of age evaluated for rituximab impact. Most notably, all of the IPD patients experienced complete B cell reconstitution after discontinuation of rituximab with a relatively fast median time to reconstitution (median = 17 weeks) supporting the contention that the younger the patient the more rapid the reconstitution after immunomodulation with rituximab.

Methotrexate is an inhibitor of dihydrofolate reductase, an enzyme necessary for the synthesis of purine nucleotides and thymidylate. It predominantly affects rapidly dividing cells, such as lymphocytes, by interfering with DNA synthesis and repair. In addition to impacting B and T cells, methotrexate can help to prevent the development of ADA to rituximab. Methotrexate's ability to prevent ADA development stems from different lines of evidence—A murine study showed that methotrexate's interaction with BAFF (B cell activating factor of the TNF family), a driver of B cell activation, is important in the prevention of ADA development (28, 29). In rheumatic disease, concomitant therapy with a biologic and methotrexate prevented the development of antibodies against the biologic (30). Available

data on methotrexate's impact on B cells indicate that it does not impact the overall CD19% but rather impacts B cell subsets and immunoglobulin levels (56–58). Our tracking of B cell depletion is limited by inconsistent collection of absolute counts with consistent reporting of CD19%. Future analysis will be necessary to assess B cell subsets and immunoglobulin levels during immunomodulation and immune reconstitution.

Although data were available for a small number of patients (titers available for 12 patients), patients generally had protective vaccine titers to polysaccharide (T cell-independent) antigen and conjugated (T cell-dependent) antigen. All IPD patients demonstrated an adequate humoral response against tetanus and diphtheria vaccines, and all except two CRIM-positive IPD patients (CP2 and CP8) had adequate pneumococcal titers. It is not clear if the lack of response to polysaccharide vaccines in these two patients was due to immunosuppression or normal variability in response to pneumococcal vaccines.

The short course of prophylactic ITI was safely tolerated without any major adverse events. Although infections were reported in five CRIM-negative patients which required treatment with antibiotics and central line removal in two patients, no interruption in ERT or immunomodulation was required in any of the patients. At the most recent follow-up, seven IPD patients were deceased at a median age of 25.4 months (range, 15.0–63.2 months). The cause of death was cardiorespiratory failure due to disease progression and was unrelated to immunomodulation but likely pertained to the extent of disease progression prior to treatment. Overall survival was significantly ($p = 0.0001$) improved in CRIM-negative IPD patients who received ITI with ERT compared to CRIM-negative IPD patients on ERT monotherapy.

Various immunomodulation strategies using rituximab in patients with Pompe disease have been reported in the literature. The combination of rituximab, methotrexate, with or without IVIG initiated along with the first ERT infusion, as shown in the current study, has proven to be the most successful strategy in inducing immune tolerance in patients with IPD (25). The immunomodulation strategy reported by Elder et al. used a combination of rituximab, mycophenolate/sirolimus, and IVIG in five IPD patients (four CRIM-negative and one CRIM-positive) (27). This protocol required long-term immune suppression and more significantly delayed ERT initiation by at least 3 weeks which can be very detrimental in a rapidly progressive irreversible muscle disease (59). Poelman et al. utilized a combination of rituximab, methotrexate, and IVIG similar to our ITI protocol, however, with a different dosing schedule of methotrexate, in three IPD patients (one CRIM-negative and two CRIM-positive) (26). All three patients developed anti-rhGAA IgG antibodies with two developing HSAT. Although B cell reconstitution was observed in all three patients, B cell reconstitution also resulted in an increase in rhGAA IgG antibody titers. In contrast, our immunomodulation approach was able to tolerate 84% of CRIM-negative and 100% of CRIM-positive IPD patients as evidenced by the maintenance of low or complete absence of anti-rhGAA IgG antibody titers even well after B cell recovery (22, 23).

To our knowledge, this the largest cohort of patients with IPD treated with ITI in ERT-naïve settings and the largest cohort of

pediatric patients under a year of age evaluated for the safety of rituximab. Overall, this short course of immune modulation in the ERT-naïve setting significantly increased the likelihood of achieving long-term immune tolerance to ERT and did not lead to any long-term sequelae. Patients who received ITI were able to receive routine vaccinations and demonstrated adequate humoral immune responses. The data suggest that short-course prophylactic immunomodulation with rituximab, methotrexate, and IVIG initiated in the ERT-naïve setting is safe and efficacious in achieving long-term immune tolerance to ERT. The addition of this ITI regimen to ERT is life-saving and our data show that the benefits of adding immune modulation (ITI regimen) outweigh the risks in this setting.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/Supplementary Material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Duke Health Institutional Review Board. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

Conception and study design and development of methodology were performed by AD and PK. Analysis and interpretation of data were performed by AD, CB, JS, and PK. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.01727/full#supplementary-material>

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The Molecular Mechanisms That Underlie the Immune Biology of Anti-drug Antibody Formation Following Treatment With Monoclonal Antibodies

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Monoclonal antibodies (mAbs) are a crucial asset for human health and modern medicine, however, the repeated administration of mAbs can be highly immunogenic. Drug immunogenicity manifests in the generation of anti-drug antibodies (ADAs), and some mAbs show immunogenicity in up to 70% of patients. ADAs can alter a drug's pharmacokinetic and pharmacodynamic properties, reducing drug efficacy. In more severe cases, ADAs can neutralize the drug's therapeutic effects or cause severe adverse events to the patient. While some contributing factors to ADA formation are known, the molecular mechanisms of how therapeutic mAbs elicit ADAs are not completely clear. Accurate ADA detection is necessary to provide clinicians with sufficient information for patient monitoring and clinical intervention. However, ADA assays present unique challenges because both the analyte and antigen are antibodies, so most assays are cumbersome, costly, time consuming, and lack standardization. This review will discuss aspects related to ADA formation following mAb drug administration. First, we will provide an overview of the prevalence of ADA formation and the available diagnostic tools for their detection. Next, we will review studies that support possible molecular mechanisms causing the formation of ADA. Finally, we will summarize recent approaches used to decrease the propensity of mAbs to induce ADAs.

Keywords: monoclonal antibodies, anti-drug antibodies, immune response, immunogenicity, neutralizing antibodies

INTRODUCTION

In the last three decades, the pharmaceutical industry experienced a massive shift toward the use of protein drugs, often referred to as “biologics.” Biologics offer higher specificity and better characterized mechanisms of action compared to small molecule drugs, and their use has revolutionized the treatment of a wide range of diseases and disorders. In general, monoclonal antibodies (mAbs) are the most widely used class of biologics (1).

Monoclonal antibodies account for a growing number of blockbuster drugs with their US sales reaching over \$24 billion (2), and will maintain a dominant position in the pharmaceutical market that exceeds \$125 billion by the end of 2020 (3).

To date, over 73 mAbs have been approved by the United States Food and Drug Administration (FDA) and the European Medicines Agency (EMA). Hundreds more mAbs are in different stages of clinical development. mAbs are used for various clinical indications including cancer, chronic autoimmune and inflammatory diseases, allergies, infections, transplantations, and cardiovascular diseases (4).

The mechanism of action (MOA) of mAbs can vary across different use cases. For example, the anti-CD20 rituximab induces cell death by binding to surface receptors, resulting in a signaling cascade that leads to apoptosis (5). Other mAbs, including the anti-HER-2 trastuzumab, block receptor-ligand interactions to achieve a desired effect, either by blocking the receptor domain to inhibit an activation signal by removing a soluble ligand entirely from circulation (6). mAbs can also induce fragment crystallizable (Fc)-dependent effector functions such as antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), which are important for the anti-CD20 drug obinutuzumab that is used for the treatment of lymphoproliferative disorders (7). Other mAbs target specific proteins involved in pathogenesis of disease, such as anti-TNF α mAbs infliximab and adalimumab that are used to treat inflammatory bowel disease (IBD) and rheumatoid arthritis (RA) (8). Other mAbs in this category are omalizumab, an anti-IgE mAb that is used to treat patients with allergic asthma (9), palivizumab which targets an epitope in the A antigenic site of the F protein of the respiratory syncytial virus (RSV) (10), and bezlotoxumab which binds and neutralizes *Clostridium difficile* toxin B (11). Some mAbs, such as cetuximab and panitumumab (12), target the epidermal growth factor receptor (EGFR) which is overexpressed in a number of cancers. In recent years, checkpoint inhibitor mAbs were also developed to manipulate anti-tumor T-cell responses, like the anti-PD-1 nivolumab that is used to treat melanoma and non-small cell lung cancer (13).

The tremendous progress in mAb discovery began in 1975, when Köhler and Milstein reported *in vitro* screening and production of murine mAbs from hybridomas (14). In the late 1980s, murine mAbs were in rapid clinical development, but had significant drawbacks as they were often induced allergic reactions and the formation of human anti-mouse antibodies (HAMA). Examples include T101 used to treat chronic lymphocytic leukemia (CLL) and cutaneous T cell lymphoma (CTCL), and 9.2.27 to treat melanoma (15). Additionally, murine mAbs exhibited a relatively short half-life in humans, possibly due to low affinity toward the human neonatal Fc receptor (FcRn) (16), and were relatively poor recruiters of effector functions, crucial for some mAb efficacy (17).

To overcome the immunogenicity and reduced effector function of murine mAbs, chimeric antibodies (mouse-human) were next developed by fusing the antigen-specific variable domain of a murine mAb with the constant domains of a human mAb. This resulted in chimeric mAbs of approximately 65% human origin by amino acid content (18). Human gene

sequences were mostly taken from the κ light chain and the IgG1 heavy chain, as IgG1 has the highest efficiency in activating complement and cytotoxic effector cells, and the κ light chain is more common in human serum antibodies (19, 20). The development of chimeric mAbs indeed reduced immunogenicity and increased efficacy. For example, metastatic colorectal carcinoma patients who received the chimeric mAb 17-1A did not show any toxic or allergic reactions, and the chimeric antibody was significantly less immunogenic than its parental murine antibody (21).

Chimeric mAbs exhibited an extended half-life and reduced immunogenicity, but they still presented a considerably high propensity for ADA induction (22). Aiming to further reduce mAb immunogenicity, humanized mAbs were developed by grafting the murine complementarity determining regions (CDR) onto framework regions (FR) of the human mAb heavy and light chain variable domains (V_H and V_L , respectively), for mAbs that are approximately 95% human (23). mAb humanization often significantly reduces immunogenicity and ADA formation (24).

Technological advances of phage display technology (25, 26) based on human single chain Fv (scFv) libraries (27) next enabled the discovery of antibodies comprised entirely of human genes. These human mAbs were additionally aided by the more recent development of transgenic mouse strains expressing human antibody variable domains (28–30).

While both humanized and fully human mAbs reduce immunogenic potential and show properties similar to human endogenous IgGs, they fail to completely eliminate mAb immunogenicity and ADA formation (31). **Table 1** summarizes mAbs that are currently approved in the US and EU, along with their reported immunogenicity rates.

In the past decade, next-generation sequencing (NGS) technologies enabled a rapid increase in the capacity to sequence human and animal genomes (32). Like many other areas of modern biology, NGS is now frequently used in basic and applied immunology. NGS is often applied for sequencing the V_H and V_L antibody domains (33–36), as well as T-cell receptors (37, 38) and antibody derivative [e.g., scFv, F(ab)] libraries screened using display systems (39–41). NGS analysis of B cells can elucidate the features of antibody immune responses at a molecular level, and has been further exploited for advanced mAb discovery and engineering (42–44).

In addition to NGS of bulk populations, single-cell sequencing comprises an important group of technologies for antibody discovery, as single cell data is necessary to reveal the native V_H and V_L pairing. Previous studies were able to obtain V_H and V_L chain pairing from isolated plasmablasts (PB) in immunized mice (34, 45, 46) and antigen-specific PB from tetanus-vaccinated human patients (33).

A recently introduced technology combines proteomic analyses of antibodies in blood or secretions with NGS analysis of antibody-encoding B cells. Proteomics thus provides invaluable information about the molecular, monoclonal properties of human serum antibodies in health and disease (46–48). All of the above recently developed technologies have expedited mAb discovery and revolutionized our understanding about the nature

TABLE 1 | Approved mAb and their reported ADA rates.

International non-proprietary name	Brand name	Target	Format	Indication first approved or reviewed	First EU/US approval year	%ADA	%ntADA	References
Adalimumab	Humira	TNFA	Human IgG1	Rheumatoid arthritis	2003/2002	28%	Not reported	(139, 140, 141)
Alemtuzumab	Lemtrada; MabCampath, Campath-1H	CD52	Humanized IgG1	Multiple sclerosis; chronic myeloid leukemia#	2013; 2001#/2014;2001#	67.1–75.4	Not reported	(102, 103)
Alirocumab	Praluent	PCSK9	Human IgG1	High cholesterol	2015/2015	5.1%	1.30%	(142)
Atezolizumab	Tecentriq	PD-L1	Humanized IgG1	Bladder cancer	2017/2016	30–48%	Not reported	https://www.accessdata.fda.gov/drugsatfda_docs/label/2018/761034s010lbl.pdf
Avelumab	Bavencio	PD-L1	Human IgG1	Merkel cell carcinoma	2017/2017	4.10%	Not reported	https://www.accessdata.fda.gov/drugsatfda_docs/label/2018/761069s002lbl.pdf
Basiliximab	Simulect	IL-2R	Chimeric IgG1	Prevention of kidney transplant rejection	1998/1998	1.17%	Not reported	https://www.accessdata.fda.gov/drugsatfda_docs/label/2003/basnov010203LB.htm
Belimumab	Benlysta	BLyS	Human IgG1	Systemic lupus erythematosus	2011/2011	0–4.8%	Not reported	(74)
Benralizumab	Fasenra	IL-5R α	Humanized IgG1	Asthma	2018/2017	15.62%	Not reported	(143)
Bevacizumab	Avastin	VEGF	Humanized IgG1	Colorectal cancer	2005/2004	0%	0%	(144)
Bezlotoxumab	Zinplava	<i>Clostridium difficile</i> enterotoxin B	Human IgG1	Prevention of <i>Clostridium difficile</i> infection recurrence	2017/2016	0%	0%	https://www.accessdata.fda.gov/drugsatfda_docs/nda/2016/761046Orig1s000ClinPharmR.pdf
Brodalumab	Siliq, LUMICEF	IL-17R	Human IgG2	Plaque psoriasis	2017/2017	2.70%	0%	(145)
Burosumab	Crysvita	FGF23	Human IgG1	X-linked hypophosphatemia	2018/2018	0%	0%	https://www.ultragenyx.com/file.cfm/29/docs/Crysvita_Full_Prescribing_Information.pdf
Canakinumab	Ilaris	IL-1 β	Human IgG1	Muckle-Wells syndrome	2009/2009	<1%	0%	https://www.accessdata.fda.gov/drugsatfda_docs/nda/2016/125319Orig1s085,086,087MedR.pdf
Cemiplimab	Libtayo	PD-1	Human mAb	Cutaneous squamous cell carcinoma	2019/2018	1.30%	Not reported	https://www.accessdata.fda.gov/drugsatfda_docs/label/2018/761097s000lbl.pdf
Cetuximab	Erbix	EGFR	Chimeric IgG1	Colorectal cancer	2004/2004	22.36%	Not reported	(90)
Crizanlizumab	Adakveo	CD62 (aka P-selectin)	Humanized IgG2	Sickle cell disease	In review/2019	0–1.6%	0%	https://www.accessdata.fda.gov/drugsatfda_docs/label/2019/761128s000lbl.pdf
Daratumumab	Darzalex	CD38	Human IgG1	Multiple myeloma	2016/2015	0.70%	Not reported	https://www.ema.europa.eu/en/documents/variation-report/darzalex-h-c-4077-ii-0002-epar-assessment-report-variation_en.pdf

(Continued)

TABLE 1 | Continued

International non-proprietary name	Brand name	Target	Format	Indication first approved or reviewed	First EU/US approval year	%ADA	%ntADA	References
Denosumab	Prolia	RANK-L	Human IgG2	Bone Loss	2010/2010	0%	0%	(146)
Dinutuximab	Unituxin	GD2	Chimeric IgG1	Neuroblastoma	2015/2015	28%	Not reported	(147)
Durvalumab	IMFINZI	PD-L1	Human IgG1	Bladder cancer	2018/2017	2.90%	Not reported	https://www.accessdata.fda.gov/drugsatfda_docs/label/2018/761069s002lbl.pdf
Eculizumab	Soliris	C5	Humanized IgG2/4	Paroxysmal nocturnal hemoglobinuria	2007/2007	0%	0%	(148)
Elotuzumab	Empliciti	SLAMF7	Humanized IgG1	Multiple myeloma	2016/2015	33.30%	Not reported	(149)
Emapalumab, emapalumab-lzsg	Gamifant	IFN γ	Human IgG1	Primary hemophagocytic lymphohistiocytosis	In review/2018	5%	1.60%	https://www.accessdata.fda.gov/drugsatfda_docs/nda/2018/761107Orig1s000MultidisciplineR.pdf
Erenumab	Aimovig	CGRP receptor	Human IgG2	Migraine prevention	2018/2018	8.90%	0%	https://www.accessdata.fda.gov/drugsatfda_docs/nda/2018/761077Orig1s000SumR.pdf
Evolocumab	Repatha	PCSK9	Human IgG2	High cholesterol	2015/2015	0.16%	0%	(150)
Evolocumab	Dupixent	IL-4R α	Human IgG4	Atopic dermatitis	2017/2017	2–6%	4–9%	https://www.accessdata.fda.gov/drugsatfda_docs/label/2018/761055s007lbl.pdf
Fremanezumab	Ajovy	CGRP	Humanized IgG2	Migraine prevention	2019/2018	0.4–1.6%	0.06–0.9%	https://www.accessdata.fda.gov/drugsatfda_docs/label/2018/761089s000lbl.pdf
Galcanezumab	Emgality	CGRP	Humanized IgG4	Migraine prevention	2018/2018	12.50%	Most ADA were ntADA	https://www.accessdata.fda.gov/drugsatfda_docs/nda/2018/761063Orig1s000ClinPharmR.pdf
Golimumab	Simponi	TNF α	Human IgG1	Rheumatoid and psoriatic arthritis, ankylosing spondylitis	2009/2009	31.70%	Not reported	(151)
Guselkumab	TREMFYA	IL-23 p19	Human IgG1	Plaque psoriasis	2017/2017	5.50%	0.40%	https://www.accessdata.fda.gov/drugsatfda_docs/nda/2017/761061Orig1s000MultidisciplineR.pdf
Ibalizumab, ibalizumab-uiyk	Trogarzo	CD4	Humanized IgG4	HIV infection	2019/2018	0.83%	0.83%	https://www.accessdata.fda.gov/drugsatfda_docs/nda/2018/761065Orig1s000ClinPharmR.pdf
Infliximab	Remicade	TNF	Chimeric IgG1	Crohn's disease	1999/1998	66.70%	Not reported	(139, 152)
Ipilimumab	Yervoy	CTLA-4	Human IgG1	Metastatic melanoma	2011/2011	26%, 1.1–5.4%	Not reported, 0%	(153), United States Product Information 2018
Ixekizumab	Taltz	IL-17a	Humanized IgG4	Psoriasis	2016/2016	9%	Not reported	(154)
Lanadelumab	Takhzyro	Plasma kallikrein	Human IgG1	Hereditary angioedema attacks	2018/2018	12%	Not reported	https://www.accessdata.fda.gov/drugsatfda_docs/label/2018/761090s000lbl.pdf

(Continued)

TABLE 1 | Continued

International non-proprietary name	Brand name	Target	Format	Indication first approved or reviewed	First EU/US approval year	%ADA	%ntADA	References
Mepolizumab	Nucala	IL-5	Humanized IgG1	Severe eosinophilic asthma	2015/2015	3%	<1%	(155)
Mogamulizumab	Poteligeo	CCR4	Humanized IgG1	Mycosis fungoides or Sézary syndrome	2018/2018	3.90%	0%	https://www.accessdata.fda.gov/drugsatfda_docs/nda/2018/761051Orig1s000MultidisciplineR.pdf
Natalizumab	Tysabri	$\alpha 4$ integrin	Humanized IgG4	Multiple sclerosis	2006/2004	8–9%	Not reported	(156)
Necitumumab	Portrazza	EGFR	Human IgG1	Non-small cell lung cancer	2015/2015	4.10%	1.40%	https://www.accessdata.fda.gov/drugsatfda_docs/label/2015/125547s000lbl.pdf
Nivolumab	Opdivo	PD1	Human IgG4	Melanoma, non-small cell lung cancer	2015/2014	12.7%, 4.1–37.8%	0.8%, 0–4.6%	(157) https://www.accessdata.fda.gov/drugsatfda_docs/label/2019/125554s070lbl.pdf
Obiltoximab	Anthim	<i>B. anthracis</i> PA	Chimeric IgG1	Prevention of inhalational anthrax	In review/2016	0%	0%	(158)
Obinutuzumab	Gazyva, Gazyvaro	CD20	Humanized IgG1	Chronic lymphocytic leukemia	2014/2013	7%	Not reported	https://www.accessdata.fda.gov/drugsatfda_docs/label/2017/125486s017s018lbl.pdf
Ocrelizumab	OCREVUS	CD20	Humanized IgG1	Multiple sclerosis	2018/2017	0.9%, 0.2–0.5%	0.15%, 0–0.2%	https://www.accessdata.fda.gov/drugsatfda_docs/nda/2017/761053Orig1s000ClinPharmR.pdf , (159)
Ofatumumab	Arzerra	CD20	Human IgG1	Chronic lymphocytic leukemia	2010/2009	<1%	Not reported	https://www.accessdata.fda.gov/drugsatfda_docs/label/2016/125326s062lbl.pdf
Olaratumab	Lartruvo	PDGFR α	Human IgG1	Soft tissue sarcoma	2016/2016	3.50%	3.50%	https://www.accessdata.fda.gov/drugsatfda_docs/nda/2016/761038Orig1s000MultiDisciplineR.pdf
Omalizumab	Xolair	IgE	Humanized IgG1	Asthma	2005/2003	0%	0%	(160)
Palivizumab	Synagis	RSV	Humanized IgG1	Prevention of respiratory syncytial virus infection	1999/1998	1.80%	0%	(161)
Panitumumab	Vectibix	EGFR	Human IgG2	Colorectal cancer	2007/2006	4.60%	1.60%	https://www.accessdata.fda.gov/drugsatfda_docs/label/2009/125147s080lbl.pdf
Pembrolizumab	Keytruda	PD1	Humanized IgG4	Melanoma	2015/2014	1.80%	0.50%	(162)
Pertuzumab	Perjeta	HER2	humanized IgG1	Breast Cancer	2013/2012	0.60%	Not reported	(163)

(Continued)

TABLE 1 | Continued

International non-proprietary name	Brand name	Target	Format	Indication first approved or reviewed	First EU/US approval year	%ADA	%ntADA	References
Ramucirumab	Cyramza	VEGFR2	Human IgG1	Gastric cancer	2014/2014	3.80%	0.18%	https://www.accessdata.fda.gov/drugsatfda_docs/nda/2014/125477Orig1s000MedR.pdf
Ravulizumab (ALXN1210)	Ultomiris	C5	Humanized IgG2/4	Paroxysmal nocturnal hemoglobinuria	2019/2018	>0.5%	0%	https://www.accessdata.fda.gov/drugsatfda_docs/nda/2018/761108Orig1s000MultidisciplineR.pdf
Raxibacumab	(Pending)	<i>B. anthracis</i> PA	Human IgG1	Anthrax infection	NA/2012	0%	0%	https://www.accessdata.fda.gov/drugsatfda_docs/label/2012/125349s000lbl.pdf
Reslizumab	Cinqaero, Cinqair	IL-5	Humanized IgG4	Asthma	2016/2016	4.8–5.4%, 5%	Not reported, 0%	(164, 165)
Risankizumab	Skyrizi	IL-23 p19	Humanized IgG1	Plaque psoriasis	2019/2019	24%	14%	https://www.accessdata.fda.gov/drugsatfda_docs/nda/2019/761105Orig1s000MultidisciplineR.pdf
Rituximab	MabThera, Rituxan	CD20	Chimeric IgG1	Non-Hodgkin lymphoma	1998/1997	26–37%, 12.5%	Not reported	(73, 144)
Romosozumab	Evenity	Sclerostin	Humanized IgG2	Osteoporosis in postmenopausal women at increased risk of fracture	NA/2019	18.10%	4.60%	https://www.accessdata.fda.gov/drugsatfda_docs/nda/2019/761062Orig1s000MultidisciplineR.pdf
Sarilumab	Kevzara	IL-6R	Human IgG1	Rheumatoid arthritis	2017/2017	14–19.3%	1.8–3.3%	https://www.accessdata.fda.gov/drugsatfda_docs/nda/2017/761037Orig1s000ChemR.pdf
Secukinumab	Cosentyx	IL-17a	Human IgG1	Psoriasis	2015/2015	0.41%	0.20%	(166)
Siltuximab	Sylvant	IL-6	Chimeric IgG1	Castleman disease	2014/2014	0.20%	0%	(167)
Tildrakizumab	Ilumya	IL-23 p19	Humanized IgG1	Plaque psoriasis	2018/2018	6.8–8.8%, 4.1–8.2%	2.7–3.34%, 0.6–3.2%	https://www.accessdata.fda.gov/drugsatfda_docs/nda/2018/761067Orig1s000MultidisciplineR.pdf , (168)
Tocilizumab	RoActemra, Actemra	IL-6R	Humanized IgG1	Rheumatoid arthritis	2009/2010	5	Not reported	(169)
Trastuzumab	Herceptin	HER2	Humanized IgG1	Breast cancer	2000/1998	16.30%	Not reported	(144)
Ustekinumab	Stelara	IL-12/23	Human IgG1	Psoriasis	2009/2009	6.50%	Not reported	(170)
Vedolizumab	Entyvio	$\alpha 4\beta 7$ integrin	humanized IgG1	Ulcerative colitis, Crohn's disease	2014/2014	17%	Not reported	(171)

of the immune responses, including in the formation of ADAs following immunization and administration of mAbs.

Monoclonal antibodies immunogenicity is mainly manifested in ADA generation (49). The formation of ADAs alters a drug's bioavailability and pharmacokinetic and pharmacodynamic properties, and most often reduces drug efficacy (50, 51). ADAs have a significant impact on mAb drug safety, as they can lead to serious adverse immune reactions in the clinic (52). Patients with ADAs can be stratified by their effect on the clinical treatment course. Patients are designated as having primary loss of response (LOR) when the administered mAb fails to show any efficacy within several weeks following treatment initiation, or secondary LOR when patients show significant side effects or the drug loses effectiveness over time despite an initial therapeutic response (53–55).

For multiple decades, many studies focused on possible mechanisms that govern ADA formation, development of improved assays for ADA detection, and advancement of tools for immunogenicity and prediction of ADA formation. This review provides an overview on these topics, underlining the challenges and potential solutions for this important research field. While this review focuses on ADA as an important outcome of mAb immunogenicity, there are other immunogenicity outcomes such as allergic reactions, cytopenia, and anaphylaxis that are widely reviewed elsewhere (56).

THE MOLECULAR MECHANISMS THAT LEAD TO ADA FORMATION

Anti-drug antibodies can be generated by a T-cell dependent or independent B cell activation pathway. In the T-cell dependent pathway, mAbs act as antigens and are internalized by antigen presenting cells (APCs), processed, and presented to T cells via the cognate interaction between the MHC class II molecules and T-cell receptor. Depending on the cytokine milieu during this interaction, several different immune responses can occur (57). In the T-cell dependent pathway, ADAs are generated when a T helper cell (Th) differentiates into a Th1 or Th2 phenotype and, following their cognate interactions with B cells, induces the proliferation of plasma cells (PC) that secrete ADAs. Previous studies showed that a Th2 response mostly induce ADA production of the IgG4 isotype, in comparison to the Th1 response, that in the case of anti-factor VIII elicits the generation of IgG1 and IgG2 ADA (58, 59).

For example, infliximab-specific Th2 cells can be detected in circulation after infliximab infusion, and these cells were correlated with the presence of infliximab-specific ADA (60). Interestingly, this cellular response was observed mostly in patients with hypersensitivity reactions, rather than in the LOR group. In another study, T cell epitopes of infliximab and rituximab were identified by isolating antibody-specific T cells after repeated rounds of antibody-loaded dendritic cells (DCs) in co-culture (61). These T cells were specific to peptides derived from V_H and V_L and encompassed CDRs and FRs, reflecting the immunogenicity of the chimeric part of these antibodies. Importantly, these peptides were

also eluted from antibody-loaded DCs, highlighting the importance of MHC Class II antigen presentation in the ADA formation process.

In contrast, for the T cell independent pathway mAbs with multiple epitopes can crosslink B cell receptors (BCRs) and stimulate B cells to differentiate into PC to produce ADAs (62–66). It was previously demonstrated that impurities and aggregates of the mAbs may increase the number of adjacent epitopes on the mAb, potentially steering the immune response toward a T-cell independent pathway by B cell crosslinking (67–70).

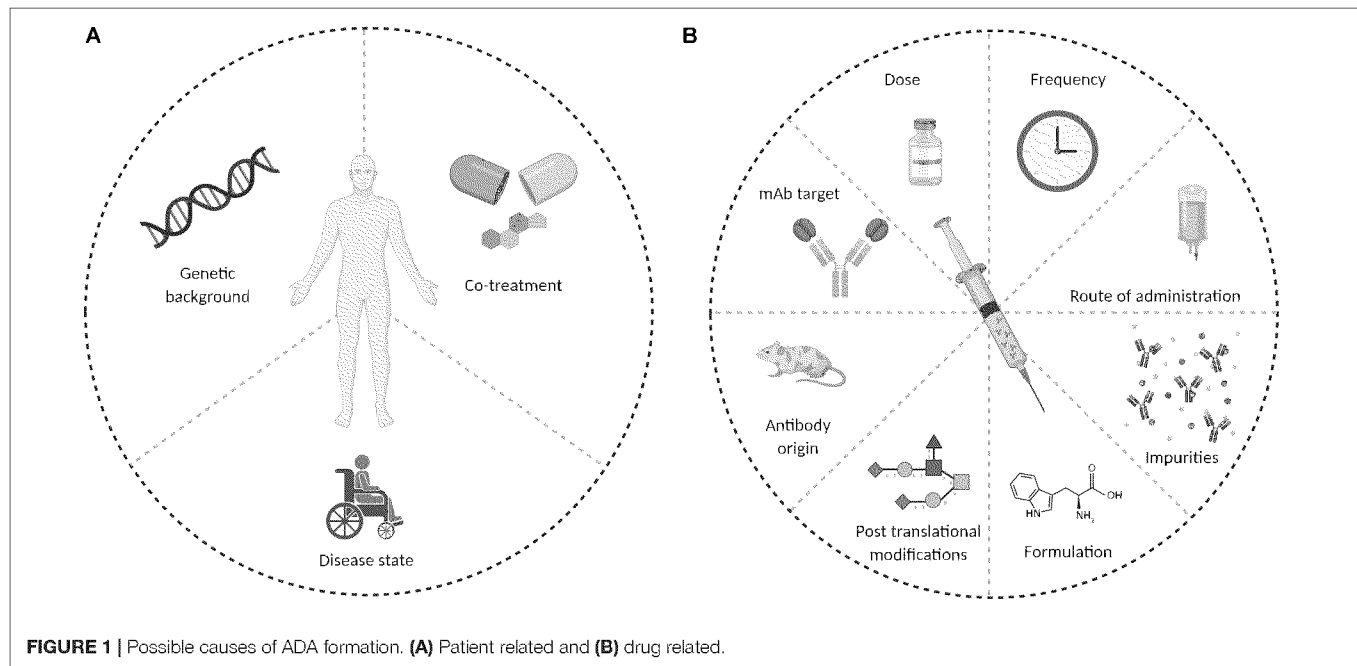
DRUG AND PATIENT CHARACTERISTICS CONTRIBUTING TO ADA FORMATION

Anti-drug antibodies formation depends on the interplay between several factors, which can be patient-related or drug-related. Possible causes for ADA formation are summarized in Figure 1.

Patient-Related Factors

The study of why and how ADAs are generated is complicated by the fact that some patients develop ADAs and some, with the same clinical indication and receiving the same therapeutic mAb, do not. The extent of immunogenicity thus differs among patients receiving the same mAb, which could be related to the immune pathways underlying the pathogenesis of the disease (71). For example, RA patients have a higher likelihood of developing ADAs toward a mAb drug than spondyloarthritis patients (57). When examining a specific disease or immune target, different mAbs may have a varying effect on the induction of ADAs. RA patients develop higher ADA levels when treated with two different mAbs (72). In multiple sclerosis (MS) patients, treatment with rituximab (chimeric anti-CD20 mAb) generated an unwanted immune response in up to 37% of patients (73). On the contrary, belimumab (a fully human anti B-cell activating factor (BAFF) mAb), which is used to treat systemic lupus erythematosus (SLE) patients, showed low rates induction ADA (74). Of note, in autoimmune diseases the hyperactivation of both the innate and adaptive immune responses may further complicate the study of mAb immunogenicity (57, 75). On the other hand, when administering mAbs to cancer patients, ADA formation often depends on the stage of the cancer. ADA levels tend to be higher in early stages of the disease than in later stages (76).

Much of the variability in the propensity of administered mAb to induce ADA formation may result from different immune contexts; Principally, disease status and HLA alleles, which could promote or inhibit an ADA response. The idea that ADA formation is often derived from a T-dependent response has recently led to studies focusing on how ADA formation correlates with HLA polymorphism in the population. Although limited by sample size, Benucci et al. showed that patients with the HLA-DR β -11, HLA-DQ-03, and HLA-DQ-05 alleles were at a higher risk to develop ADA responses after treatment with an anti-TNF mAb (5 different mAbs were included in this study)



(77). Another report revealed that a G1m1 allotype in the IgG1 created a protease cleavage site in the CH3 domain of the antibody Fc and enabled presentation of a CH3_{15–29} peptide epitope (78). The CH3_{15–29} peptide epitope was tolerated in patients with a G1m1 allotype. However, donors homozygous for nG1m1 did not natively display the G1m1 MHC-II peptide and developed T cell CD4⁺ responses against antibody therapeutics containing the G1m1 allotype sequence; these ADA were also correlated with HLA-DRB1*07 allele. Some therapeutic mAbs (including trastuzumab) do not harbor this allotype, which could partially explain differences in immunogenicity across different mAb drugs (78, 79). This allotype difference could impact future development of antibody products, since ~40% of the Caucasian population is homozygous for nG1m1, and thus may be at a greater risk for ADA generation (80). In two recent studies, ADA formation against infliximab and adalimumab was correlated with the HLA-DQA1*05A > G genotype in IBD patients (81, 82). One detailed recent study examined the immune response to natalizumab, a humanized monoclonal IgG4 antibody to $\alpha 4$ integrins that is used to treat patients with MS, and that induces ADA formation in ~6% of the patients. The immune response was found to be polyclonal and targeted different epitopes of the natalizumab idiotype, with a single immunodominant T cell epitope spanning the FR2-CDR2 region of the V_L (83). Generation of a T cell-dependent ADA response is also a multifactorial process, depending not only on the existence of a potential MHC-II peptide epitope in the mAb, but also on the ability of that epitope to be processed, presented and recognized by T cells. The influence of HLA allotypes on the probability of ADA responses should be considered during the design of immunogenicity studies and clinical trials for mAb development. Conclusions from studies that rely on smaller cohorts might not have general applicability for ADA predictions if the study

population has substantially different MHC-II gene backgrounds from a larger treatment population.

Drug-Related Factors

The molecular mechanisms that lead to induction of ADAs were initially related to the murine origin of the first mAbs, which were recognized as “non-self” by the human immune system. Unfortunately, even the use of complete human antibody genes has not completely eliminated immunogenicity and the associated induction of ADA (84). Fully human mAbs contain new epitopes in the CDRs that can steer the immune response through an idiotype/anti-idiotype interaction (85, 86). As discussed above, mAb-derived peptides presented by MHC-II are necessary for T cell-dependent ADA formation. Efforts to remove T cell epitopes during mAb engineering are used consistently, but the high genetic variability of human populations greatly complicates efforts to remove all MHC-II-binding peptides from human mAbs (87, 88).

Changes in Fc glycosylation may also affect ADA induction. The removal of N-linked glycosylation of the Fc was shown to reduce immunogenicity (89). Fully human mAbs lacking Fc functions were also shown to be immunogenic and have direct effects on the ability to recruit macrophages and activate complement. For example, galactose- α -1,3-galactose, which is a foreign glycan not found in humans, is present on the antigen-binding (Fab) portion of the cetuximab V_H (a chimeric mAb used in cancer therapy targeting the EGF receptor). This glycan was shown to induce ADA formation of the IgE isotype, and was responsible for anaphylactic reactions in patients (90, 91). On the other hand, immunogenicity is sometimes linked to impurities in the formulation process, and not necessarily due to glycosylation differences. A review of the differences between 18 biosimilars and mAbs originators concluded that the differences between

them are mainly in glycosylation patterns, and do not impact immunogenicity (92).

Other drug related factors that play a role in mAb immunogenicity are “danger signals” that are released by tissues undergoing stress, damage or abnormal death. The danger model was first suggested in 1994, where it was first postulated that the immune system responds to substances that cause damage, rather than to those that are simply foreign (93, 94). In the case of therapeutic antibodies, process related impurities (such as aggregates and residual DNA or proteins from the mAb expression system) can influence immunogenicity (95).

The mAb target may also have high importance for the MOA of ADA formation. We recently found that repeated administration of infliximab (a TNF α antagonist) results in a vaccine-like response, where ADA formation is governed by the extrafollicular T cell-independent immune response (96). The administration of infliximab blocks TNF α and shifts the immune response toward the marginal zone (MZ) instead of the germinal center (GC), as observed in TNF α knockout mice (97). Another possible explanation is that a strong T cell-independent immune response in the MZ may be induced by a drug/ADA/TNF α immunocomplex (IC). As a trimer, TNF α may form “super complexes” upon engagement with TNF α antagonistic antibodies (98–100).

Another example of mAb target importance is alemtuzumab, a mAb specific to the CD52 lymphocyte cell surface glycoprotein. Alemtuzumab is used to treat MS (101) and induces ADAs in about 85% of patients, of which around 92% develop neutralizing ADAs (102). Alemtuzumab's high frequency of ADA induction may be related to CD52 expression patterns. Alemtuzumab targets APCs, which include DCs, monocytes, and memory B cells, based on their CD52 expression. When monocytes repopulate, they encounter the circulating mAb that rapidly presents antigen to the antigen-specific lymphocytes (103, 104). Memory B cells often exhibit homeostatic expansion following treatment with alemtuzumab (105), which could complement ADA generation.

mAb dosage and schedule are other possible factors influencing ADA formation rates. Increased numbers of injections and higher mAb doses are associated with higher ADA risk, although some cases of chronic treatment and higher doses have lower immunogenicity (92, 106). For example, rituximab, a chimeric mAb anti-CD20, targets surface antigens on pre-B cells and B cells before their differentiation into PCs. As rituximab selectively depletes CD20 positive B cells, it does not affect mature PCs and does not have a propensity to elicit ADAs (107).

ASSAYS FOR IMMUNOGENICITY ASSESSMENT AND TOOLS FOR IMMUNOGENICITY REDUCTION

Pre-clinical Setting

Due to the growing importance of mAb immunogenicity, there has been a growing need for tools to assess immunogenicity and reduce the propensity of mAbs to induce ADAs. Great

efforts in tools such as *in silico* prediction algorithms and cell based experimental assays are facilitating immunogenicity assessment, especially during the initial development phases of the mAb (108).

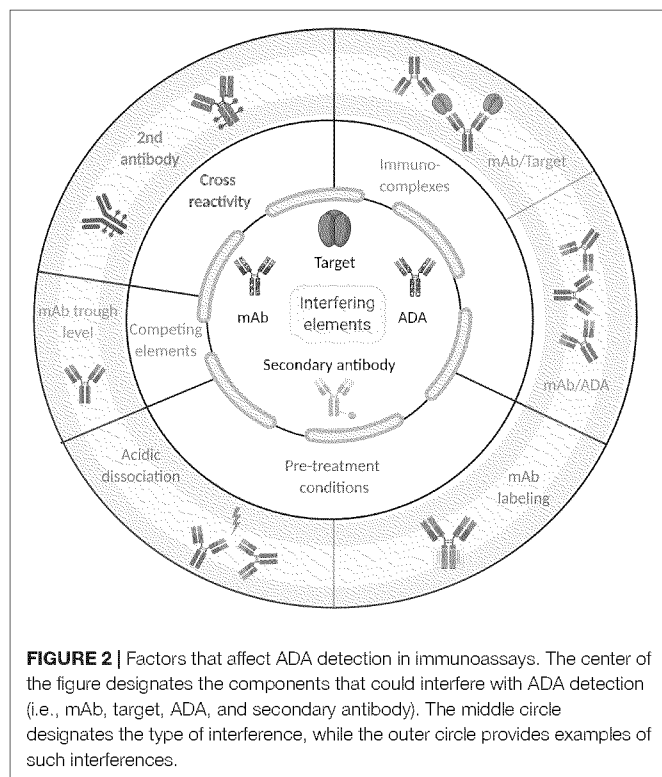
In silico CD4⁺ T cell epitope prediction models are often used to identify potentially immunogenic MHC-II peptide epitopes. These algorithms are based on the affinity of mAb-derived peptides to MHC-II (109–111).

With recent advances in proteomics and sequencing, several MHC-II peptide epitope databases have been constructed that provide a library of MHC-II binding data to enable immunogenicity prediction (112). Most algorithms that predict the immunogenic sequences recognized by T cells are later confirmed by assessing peptide binding to MHC molecules (88, 113). For example, a strong correlation was found between *in silico* evaluation of T cell epitopes from a recombinant Fc fusion protein, and the immunogenicity rate when administered to patients in a clinical trial (114). While such predictive algorithms are common used, they capture only a fraction of the system's complexity. Most CD4 + T cell epitope prediction algorithms are based on binding affinity and stability to MHC-II molecules (88, 110), but fail to consider other essential factors in the recognition of T cell epitopes. Among these factors are protease cleavage sites (115), T cell precursor frequency (116), and peptide and T cell competition (117).

Experimental tools are also used to make pre-clinical predictions about mAb immunogenicity risk. These include HLA binding assays, DC related assays, T cell stimulation assays, peripheral blood mononuclear cell (PBMC) stimulation assays, and various animal models (115). HLA binding and DC antigen presentation assays can evaluate potential T cell epitopes derived from the mAb, while T cell and PBMC stimulation assays examine whether a mAb can activate immune cells *in vitro* and *ex vivo* in terms of cell proliferation and cytokine release. For example, T cell epitopes in the variable regions of infliximab and rituximab were able to stimulate peripheral blood mononuclear cells (PBMCs) to secrete a variety of cytokines (61). In another study, the immunogenicity of secukinumab, an anti- interleukin-17A mAb used to treat plaque psoriasis, was assessed by examining T-cell proliferation (118).

Each of these experimental tools has limitations in assessing and predicting immunogenicity. While considered reliable and straightforward, most of the experimental assays are labor intensive and are impractical to implement with a large number of mAb candidates. These assays are often performed with cells derived from a naïve population, where the frequency of antigen-specific cells is relatively low and precludes a clear positive result due to low signal-to-noise ratios (88).

Other advancements are being made in the development of mAbs to which patients will be more tolerant. A previous study identified a set of naturally occurring human regulatory T cell epitopes (“Tregitopes”), present in the Fc and Fab domains of IgG, that induce tolerance when co-administered with other proteins (119). When incubated with PBMCs *in vitro*, Tregitopes activated CD4⁺ T cells and increased expression of regulatory cytokines, chemokines, and CD25/Foxp3. When were administered *in vivo* with protein antigens, Tregitopes inhibited



T cell proliferation, reduced effector cytokine expression, and induced antigen-specific adaptive tolerance. Co-administration of Tregitopes along with mAbs may be a useful tool for tolerization of mAbs.

Clinical Settings

Early and accurate ADA detection is extremely important for patients treated with biologics, especially for mAbs (120). ADA detection is required to provide the clinicians with sufficient information to monitor treatment and determine optimal intervention strategies (121). Detection of ADA against therapeutic mAbs is highly challenging since both the drug and the analyte are antibodies. Moreover, immunoassays are prone to biases due to the presence of the drug and immune-complexes in patients' serum. Historically, studies of the response following mAb administration and ADA prevalence have been inconsistent, partly due to the various assay formats used to monitor immunogenicity in clinical trials (122). Each available format has its limitations that can reduce the assay's utility in clinical and research settings, and also complicate interpretation of the data. Some assays have poor dynamic range and may generate false-negative results because of interfering interactions with the active drug, or false-positive results due to other antibodies like rheumatoid factor (123). **Figure 2** shows the competing factors which affect accurate measurement of ADAs.

An ELISA-based bridging assay is one of the most commonly used assays for ADA screening, where the mAb drug is used to first capture ADA present in the patient sera, and the latter are detected by adding additional labeled mAb as a secondary probe. Bridging ELISA assays are used for ADA detection of a large

variety of mAbs, and some include an acidic step to dissociate ADA from the mAb. The excess mAb is then captured or removed, and free ADA can be detected. These assays often have significantly higher background and suffer from low sensitivity due to the disassociation of antibodies. Bridging assays can also result in false-negatives, as they are more likely to "miss" low affinity IgM ADAs present in early stages of the immune response (124). Most ELISA-based bridging assays are also sensitive to the mAbs' trough levels (levels of circulating mAb at sampling time). ADA and mAbs tend to form high molecular weight immune-complexes, making ADA detection more challenging (125). To overcome this challenge, several drug-tolerant assays have been developed to measure ADA levels in the presence of high mAb concentrations (126). Most of these assays also use an acidic treatment step. Several other techniques have been reported to evaluate serum ADA levels. These assays include radio-immunoassays (127), Biotin-drug Extraction with Acid Dissociation (BEAD) (128), Precipitation and Acid dissociation (PANDA) (129), Affinity Capture Elution ELISA (ACE) (130), and Homogenous Mobility Shift Assay (HMSA) (131); these assays have been reviewed in detail elsewhere (126). While these assays presumably detect all serum ADA, they primarily provide qualitative measures to assist healthcare providers deciding on appropriate patient interventions, and many (if not all) studies underestimate actual ADA levels. These assays also lack standardization that could enable comparisons of ADA levels across health centers. The great diversity in these assays poses tremendous difficulty in studying ADA levels between different mAbs, across studies of the same mAb, and across different assays.

In a clinical context, it is important both to assess ADA levels in patient serum, and also to assess the presence of neutralizing antibodies that interfere with biological and clinical activity of the mAb. The neutralizing effect of ADAs can be assayed by testing whether ADAs in serum inhibit binding of the mAb to its target (132). Several cell-based assays were developed to detect *nt*ADA in patients' serum. One of these assays is a functional ADA cell-based assay that was developed to quantify the activity of TNF α antagonists. This assay assesses both drug activity and *nt*ADA levels (133), but correlations between the clinical outcome and assay results were not thoroughly tested. Another assay developed for *nt*ADA detection is the reporter gene assay, which is based on excretion of IL8 by HT29 cells due to TNF α stimulation (77). When the assay was applied to sera samples with low-level ADA, it detected *nt*ADA even prior to clinical LOR to the mAb, which allows the prediction of clinical LOR with high probability.

While these assays are accurate and sensitive, they require an active cell line, which complicates assay implementation. We recently reported on a newly developed quantitative bio-immunoassay for quantifying ADA specific to TNF α antagonists. The bio-immunoassay was further modified to easily assess the neutralization capacity of ADA using an *in vitro* assay (96). This assay can be readily used in a clinical setting that performs routine ADA measurements.

Other clinical approaches to reduce immunogenicity include active interference of the T cell responses to mAbs, thereby inducing individual tolerance of the immune system ("tolerization").

For example, administration of methotrexate (MTX) with infliximab reduced ADA formation in RA patients (134). MTX also reversed high ADA levels in infantile Pompe disease patients treated with rituximab, when administered alongside bortezomib, a proteasome activity inhibitor that leads to cell death (135). Azathioprine is also an immunosuppressive drug that can be given in combination with infliximab or adalimumab to improve treatment and reduce immunogenicity and ADA formation (136–138). However, such non-specific immunosuppressive approaches have potentially harmful side effects that must be balanced with the patient's overall treatment plan.

CONCLUDING REMARKS

Monoclonal antibodies have the potential to treat a wide range of diseases and disorders, but they can be highly immunogenic and induce undesirable ADA responses. ADAs can reduce mAb drug efficacy by altering its bioavailability and/or accelerating clearance from circulation. While the molecular mechanisms of ADA generation are not fully understood, it is dependent on both patient and drug characteristics. While early ADAs were related to the murine origin of the first mAb therapeutics, ADAs also occur against fully human mAbs. Indeed, complete humanization cannot completely abrogate mAb immunogenicity and ADA formation. The questions of why and how ADA are generated also depend on variability of the reported immunogenicity rates, which emphasizes the need for standardized clinical assays

for ADA detection. Understanding the mechanisms of ADA generation and the major factors that influence immunogenicity of mAbs will help us design safer mAbs with lower drug rejection rates. Recent and ongoing efforts to study mAb immunogenicity at the molecular level is augmenting our understanding of these mechanisms that lead to ADA formation, which may help provide new guidelines to improve the safety and efficacy of mAb therapeutics.

AUTHOR CONTRIBUTIONS

AV-M, MG-G, BD, and YW wrote the sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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A Race Against Time—Changing the Natural History of CRIM Negative Infantile Pompe Disease

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We report the clinical course of the first prenatally diagnosed cross-reactive immunologic material (CRIM)-negative infantile Pompe disease (IPD) patient [homozygous for c.2560C>T (p.Arg854X) variant in the GAA gene] to undergo prophylactic immune tolerance induction (ITI) and enzyme replacement therapy (ERT) within the first 2 days of life. Both parents were found to be carriers of the c.2560C>T (p.Arg854X) variant through prenatal carrier screening. Fetal echocardiogram at 31 weeks of gestation showed left ventricular hypertrophy. An echocardiogram on the 1st day of life revealed marked biventricular hypertrophy. Physical exam was significant for macroglossia and hypotonia. A short course of Prophylactic ITI with rituximab, methotrexate, and intravenous immunoglobulin (IVIG) in conjunction with ERT at a dose of 20 mg/kg every other week was started on day 2 of life. The patient completed the ITI protocol safely and complete B-cell recovery, based on CD19 count, was noted by 3 months of age. The patient never developed anti-rhGAA IgG antibodies to ERT. Vaccinations were initiated at 9 months of age, with adequate response noted. Complete recovery of cardiac function and left ventricular mass was seen by 11 weeks of age. At 8 months of age, the patient developmentally measured at 75–90% on the Alberta Infant Motor Scale, walked at 11 months and continues to develop age-appropriately at 50 months of age based on the Early Learning Accomplishment Profile. ERT dosing was increased to 40 mg/kg every 2 weeks at 32 months of age and frequency increased to 40 mg/kg every week at 47 months of age. Patient continues to have undetectable antibody titers, most recently at age 50 months and urine Hex4 has remained normal. To our knowledge, this is the first report of successful early ERT and ITI in a prenatally diagnosed CRIM-negative IPD patient and the youngest IPD patient to receive ITI safely. With the addition of Pompe disease to the Recommended Uniform Screening Panel (RUSP) and its addition to multiple state newborn screening programs, our case highlights the benefits of early diagnosis and timely initiation of treatment in babies with Pompe disease, who represent the most severe end of the disease spectrum.

Keywords: infantile Pompe disease, CRIM negative, early diagnosis, immunomodulation, newborn screening, anti-drug antibodies

INTRODUCTION

Pompe Disease is an autosomal recessive glycogen storage disorder caused by a deficiency of the lysosomal enzyme acid alpha-glucosidase (GAA), resulting in progressive glycogen accumulation. Patients with a severe deficiency of GAA activity present in infancy with cardiomyopathy and skeletal myopathy. The diagnosis of infantile Pompe disease (IPD) is often delayed, the median age of diagnosis is 4.7 months since non-specific findings like cardiomegaly, respiratory distress, hypotonia, and failure to thrive do not typically present until 2 months of age, although signs of the disease are present at birth (1). Without enzyme replacement therapy (ERT) with recombinant human acid alpha-glucosidase (rhGAA), death is imminent, usually within the first 2 years of life secondary to cardiorespiratory failure (2). Treatment with ERT has resulted in significantly improved survival, yet long-term consequences of the disease like facial muscle weakness, speech disorders, and dysphagia as well as signal alterations of the deep white matter on brain MRI are now being recognized (3, 4).

Despite the improved clinical outcomes, the response to ERT is very heterogeneous. Various factors known to impact the response to ERT include age on ERT initiation, extent of preexisting pathology, degree of muscle damage, cross-reactive immunologic material (CRIM) status, and anti-rhGAA IgG antibodies (5). While ERT has changed the natural history of Pompe disease and has significantly improved the overall survival of patients with IPD, it is not able to reverse the underlying pathology. Prior studies in patients diagnosed via newborn screening and treated with ERT have demonstrated that even a delay of few days in treatment initiation can impact the long-term outcomes of patients with IPD (6). It is of utmost importance to initiate treatment prior to irreversible muscle damage in such a rapidly progressive disease.

CRIM status is determined based on a patient's endogenous GAA enzyme level which is influenced by the nature of pathogenic variants. In CRIM-negative patients, there exist two deleterious GAA mutations, which lead to absence of native GAA enzyme production and, therefore, lack of exposure of the developing immune system to the GAA protein. Consequently, these patients are not immune tolerant to GAA and mount a high and sustained antibody response to rhGAA. Often these responses neutralize either enzyme uptake into cells or the catalytic activity of the enzyme (5). Thus, it is not surprising that high and sustained antibody titers (HSAT) herald clinical decline in CRIM-negative patients, who are at the most severe end of the disease spectrum (7, 8). Despite advances in ERT treatment, cases of CRIM-negative IPD treated with ERT alone still result in invasive ventilation or death within the first 3 years of life (9, 10).

The management of CRIM-negative IPD has evolved significantly in recent years with the advent of immune tolerance induction (ITI), which helps reduce the immune response to ERT, but to date has not been administered early in the neonatal period. Initiation of ERT soon after birth in IPD is the goal as a delay of just 10 days has been associated with worse biological, physical, and developmental outcomes (6, 11). We present the first case of a prenatally diagnosed CRIM-negative IPD patient

to undergo prophylactic ITI and ERT with recombinant human GAA within the first 2 days of life, who at 50 months of age is meeting all her developmental milestones and attending a regular prekindergarten class.

METHODS

GAA mutation analysis on the amniocentesis sample was done at Bioreference Laboratories (Elmwood Park, NJ). Anti-rhGAA IgG antibody titer measurements were performed at Sanofi Genzyme Corporation (Cambridge, MA). Postnatal GAA variant analysis and urinary glucose tetrasaccharide biomarker (Glc₄/Hex₄) measurements were performed at the Duke University Hospital Biochemical Genetics Laboratory. T and B cell studies and immunoglobulin titers were performed at Mayo Clinic Laboratories (Rochester, MN). The patient's mother provided informed consent for the use of clinical data and images for publication. Data for CK levels, anti-rhGAA IgG antibody titers, AST and ALT from birth to 170 weeks were extracted and analyzed.

The ITI approach that included four doses of weekly rituximab (375 mg/m², intravenously), three cycles of methotrexate (0.4 mg/kg; three doses per cycle with first three ERT infusions, subcutaneously or orally), and monthly IVIG (500 mg/kg) was initiated along with ERT, as described previously (Supplementary Figure 1) (12, 13). The limited dataset on our patient has been previously published as part of a large cohort (13).

CASE PRESENTATION

Diagnosis

The patient is a 4-year-old CRIM-negative IPD patient diagnosed prenatally. Family history was significant for the demise of their first child at 2 days of life in the Dominican Republic, from cardiorespiratory failure of unknown etiology. Both parents are of Dominican Republican ancestry and are second cousins once removed. The mother of the patient underwent prenatal carrier screening, which revealed that she was a carrier of the c.2560C>T (p. Arg854X) variant in the GAA gene. The father was subsequently found to be a carrier of the same variant. Amniocentesis performed at 19 weeks gestation revealed a fetus with a 46, XX karyotype, and homozygous (c.2560C>T) GAA gene variant, confirming CRIM-negative IPD (14). Fetal ultrasound at 28 weeks gestation was negative for any obvious birth defects or cardiomyopathy. However, a fetal echocardiogram performed at 31 weeks gestation showed mild left ventricular hypertrophy affecting the interventricular septum.

Clinical Course in the Neonatal Period

The patient was born via repeat cesarean section to a 31-year-old G3P2001 mother at 38 weeks gestation. APGARs were 9 and 9 at 1 and 5 minutes, respectively and birth weight was 3.5 kg. The physical exam was significant for macroglossia and hypotonia. There was no hepatosplenomegaly. Postnatal molecular studies confirmed the homozygous c.2560C>T variant.

Respiratory Status

The patient was initially on room air, but after a few hours she had some desaturations and tachypnea to 80 breaths/min and was started on CPAP. Chest X-ray on the 1st day of life showed submaximal expansion of lungs, the possibility of cardiomegaly could not be ruled out. CPAP was required intermittently, finally discontinued by week three of life, and she has remained on room air since.

Gastrointestinal Status

The patient was initially kept NPO and on parenteral nutrition due to tachypnea. Trophic feeds were started via oral gastric tube (OGT) on the 4th day of life but she developed abdominal distension and was made NPO again. OGT feeds were restarted on the 7th day of life. On day eight of life, she was noted to have abdominal distension, greenish aspirates, and irritability. Abdominal X-ray revealed dilated bowel loops. Upper gastrointestinal endoscopy was normal. Feeding evaluation was requested and she was found to have normal suck-swallow coordination. Gradually oral feeds were restarted and she was taking full feeds by mouth by the 19th day of life.

Cardiac Status

Echocardiogram performed on day 6 of life revealed biventricular hypertrophy with prominent moderator band and muscle bundles (Figure 1). The left ventricular mass index (LVMI) was 65.4 gm/m^2 (15). Electrocardiogram showed normal sinus rhythm and biventricular hypertrophy. On 12th day of life, the patient was noted to have mild periorbital and pedal edema, the brain natriuretic peptide (BNP) rose from 556 to 778 pg/ml (normal range 1–100pg/ml) and echocardiogram showed a mild decrease in ejection fraction from the previous study with LVMI of 66 gm/m^2 . Inotropic treatment with milrinone was initiated. The echocardiogram on the 14th day of life revealed a LVMI of 69 gm/m^2 . The periorbital and pedal swelling resolved, cardiac function improved over the next few days and BNP came down to 129 pg/ml. Echocardiogram on the 22nd day of life showed a LVMI of 65 gm/m^2 .

Treatment Details

Prophylactic immune tolerance induction (ITI) was started on day 2 of life with a short course of IV rituximab, SC methotrexate, and intravenous immunoglobulin (IVIG) (12, 16). ERT with alglucosidase alfa was started on day 3 of life at a dose of 20 mg/kg followed by infusions once every 2 weeks as per package insert (13). The patient was discharged home from the NICU at 1 month of age and continued to receive ERT on an outpatient basis every 2 weeks.

The patient successfully completed four doses of rituximab at 22 days of life, nine doses of methotrexate at 32 days of life, and continued to receive monthly IVIG until 6 months of age. There were no infections around the time of ITI administration. The patient tolerated the ITI protocol safely with B-cell recovery, measured as CD19 count of 1,448 cells/ μl (370–2,306 cells/ μl), at 3 months of age. The patient started her immunization schedule at 9 months of age and antibody titers to Tetanus

toxoid and Diphtheria checked at 18 months of age revealed an adequate response.

Growth and Developmental Status

The patient has shown normal growth velocity and at 4 years of age is at the 90th percentile for both height and weight. Developmentally she was sitting by 6 months of age, measured at the 75–90% on the Alberta Infant Motor Scale at 8 months and walked independently by 11 months of age. Now at 50 months of age, she continues to grow and develop appropriately based on the Early Learning Accomplishment Profile (ELAP). Audiology evaluation revealed bilateral mixed hearing loss at 3 years of age and she has been fitted with hearing aids. She attends a regular prekindergarten class and does not require any special services.

Current Clinical Status

Based on the published literature on apparent clinical benefits of increased ERT dose and concerns of clinical plateau in our patient, ERT dosing was increased to 40 mg/kg every 2 weeks at 32 months of age and frequency increased to 40 mg/kg every week at 47 months of age (17, 18). Complete recovery of cardiac function and left ventricular mass was seen by 11 weeks of age (Figure 2). No evidence of arrhythmia was seen based on a 24 h Holter monitor at 6 months of age (19). She continues to have undetectable anti-rhGAA antibody titers and normal urinary Hex4 at 50 months of age (20). Her AST, ALT, and total CKs have also been in the normal range (Figure 3). Patient is able to walk, run, skip, gallop, hop on one foot alternatingly, hop on both feet, and climb up and down the stairs independently.

The patient has not experienced any infusion associated reactions to the ERT to date. She continues to receive ERT at 40 mg/kg every week, has been off all ITI medications, continues to be seronegative, and is managed on an outpatient basis at a multispecialty center.

Current Respiratory Status

Patient has been diagnosed with reactive airway disease and has required albuterol nebulizations when symptomatic. She has undergone adenoidectomy due to a history of snoring. She never required invasive ventilation and remains on room air with no other respiratory support.

Current Gastrointestinal Status

The patient eats an age-appropriate diet by mouth with no assistance. She has regular bowel movements and attained bowel control by 40 months of age.

Current Cardiac Status

Patient continues to be followed by a pediatric cardiologist. Complete recovery of cardiac function and left ventricular mass was seen by 11 weeks of age (Figure 2). No evidence of arrhythmia was seen based on a 24-h Holter monitor at 6 months of age (19). Electrocardiogram and echocardiogram done every 3 months continue to show normal sinus rhythm and normal cardiac function, with the last one at 50 months of age.

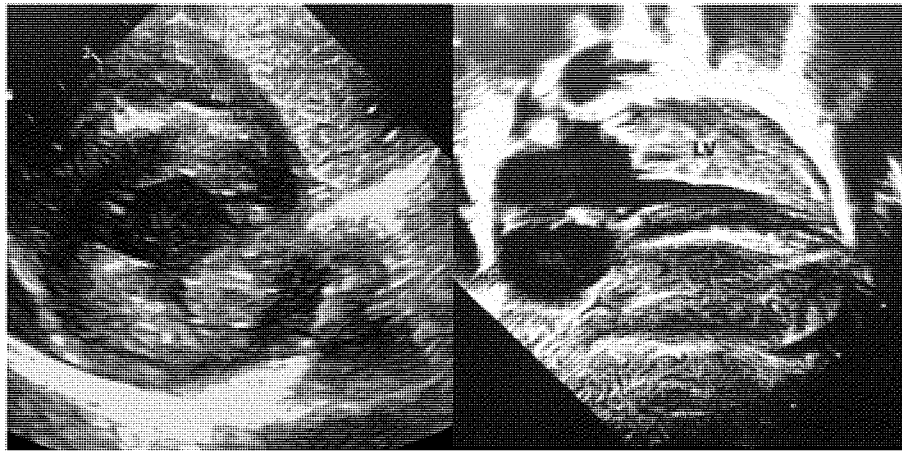


FIGURE 1 | Echocardiogram at 6 days of life, showing significant biventricular hypertrophy.

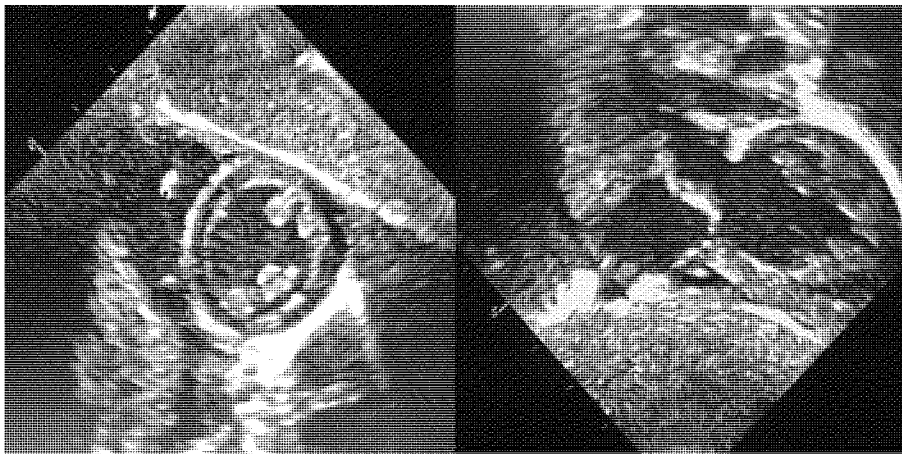


FIGURE 2 | Echocardiogram at 11-weeks of life, showing resolution of ventricular hypertrophy.

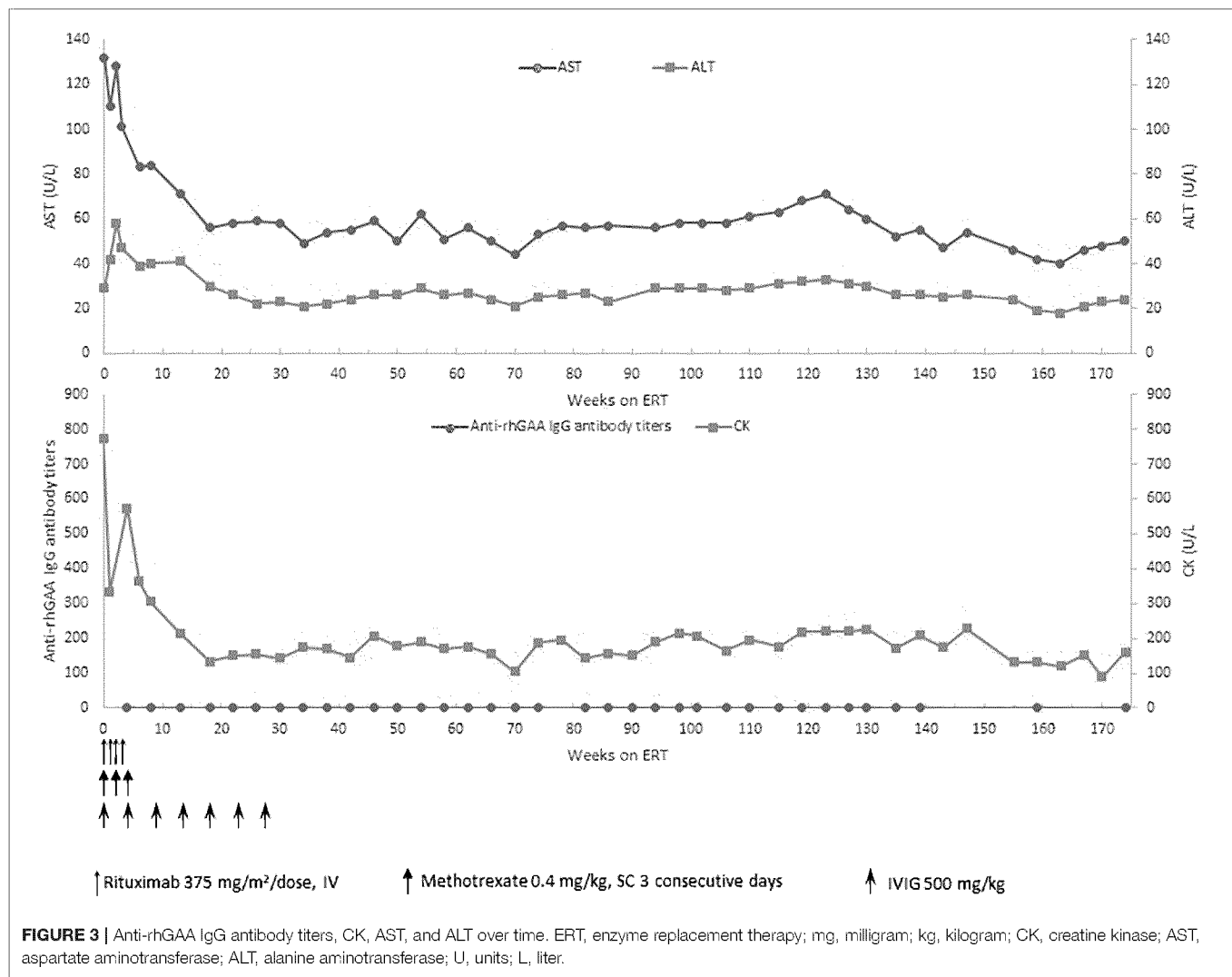
DISCUSSION

Medical advances have come a long way in the past 20 years in our understanding of IPD. Once considered a fatal diagnosis, the advent of ERT has made it possible to significantly extend the life span of affected patients, which is largely attributable to improvements in cardiomyopathy and skeletal muscle function (21).

Amongst the prenatally diagnosed IPD cases Hamdan et al., in 2008 reported a prenatally diagnosed patient due to hypertrophic cardiomyopathy seen at 32 weeks fetal echocardiogram, diagnosis confirmed at birth by enzyme assay and mutation analysis which revealed a homozygous mutation for c.1327-2A>G. The infant was treated with ERT from 18h of age and reportedly had a favorable outcome at 10 months. CRIM status was not determined and antibody titers were not reported (22).

Additionally, Abbot et al. (23) in 2011 reported a patient with prenatally diagnosed IPD due to family history with

CRIM-negative (R854X/R854X) IPD, who received standard dosing of alglucosidase alfa (Myozyme®) enzyme replacement therapy (ERT) from day 10 of life until she passed away at the age of 3 years 9 months. In the immediate neonatal period, there was cardiomegaly on chest X-ray, cardiac hypertrophy by echocardiogram, and development of a wide complex tachycardia. The available data at the time indicated that CRIM-negative patients had limited survival even with ERT. However, given the opportunity for very early treatment, the treating provider and family elected to initiate treatment with ERT, without immune modulation. It was believed that the baby would not mount an immune response due to the immaturity of the developing immune system. By 9 months of age, an echocardiogram was normal. Early motor development was within normal limits but by 2 years of age, her developmental progress had slowed. She seroconverted by the 4th month of ERT, and anti-rhGAA antibody titers peaked at 25,600 in the 27th month and remained moderately elevated at 6,400 during



the final 9 months of her life. Immunomodulatory therapy was considered but declined by family. She presented with cardiopulmonary arrest at 2 years 6 months and infection with Influenza A was confirmed. This led to a prolonged hospitalization with invasive respiratory support, and placement of tracheostomy and gastrostomy tube. Her developmental progress ceased, and she died suddenly at home from a presumed cardiac event at age 3 years 9 months.

Our patient is similar to the case described by Abbot et al. (23) with regards to the prenatal diagnosis of IPD as well as carrying the same homozygous GAA variant (R854X/R854X). This variant is one of the most frequently identified mutations in CRIM-negative alleles at up to 32.7% and commonly seen in the African American population. It is a nonsense mutation resulting in a premature stop codon (14). The key difference is that our patient was initiated on ITI in an ERT naïve setting at 2 days of life with rituximab, followed by ERT at 3 days of life. Our patient never developed an antibody response to rhGAA demonstrating tolerization to the ERT. Complete recovery of cardiac function

and left ventricular mass was seen by 11 weeks of age. Respiratory assistance in the form of intermittent CPAP was required during the first 3 weeks of neonatal life, but invasive ventilation has never been required so far. She has met all developmental milestones appropriately and now at 50 months of age is attending a regular prekindergarten class. A prior study suggested that ERT initiation at a very early age (<2 months) may help to diminish anti-drug antibodies to ERT (9). However, as evident from case described by Abbot et al. (23), patient initiated on ERT even within few days of life, are still at risk of developing high and sustained antibodies to ERT, resulting in suboptimal treatment response. In a retrospective study on CRIM-negative IPD treated with ERT monotherapy, Berrier et al. (5) described two CRIM-negative cases who were initiated on ERT within the 1st month of life and developed antibodies to ERT, leading to an eventual fatal outcome. Thus, the initiation of ERT at an early age does not prevent the development of antibodies to ERT.

To our knowledge, this is the youngest IPD patient initiated on immunomodulation with rituximab, methotrexate, and

IVIG. The data on the safety of rituximab in the pediatric population is limited. Prior studies have demonstrated that rituximab can lead to skewing of B cell subpopulation, persistent hypogammaglobulinemia, and can affect the response to routine vaccination (24–29). These cases likely needed rituximab for a longer duration. The ITI protocol used in our patient requires only 4 doses of rituximab. In a large cohort of IPD patients, this short 5 week course of ITI was successful in inducing immune tolerance to ERT in 88% of CRIM-negative IPD patients (13). Additionally, ERT is given concurrently with ITI. This is different from other suggested protocols in literature which required delay in ERT initiation by 3 weeks for induction of immunomodulation; such delay in treatment initiation can negatively impact the long-term clinical outcome of IPD patients (30). Our patient tolerated ITI without any adverse events. She had full B cell reconstitution following completion of ITI and normal immunoglobulin levels at the most recent follow-up. She is up to date on age-appropriate vaccinations and demonstrated an adequate humoral response to routine vaccines.

The newborn screen program (NBS) has been a great public health achievement since its induction in the early 1960s but continues to evolve as medical advances make the diagnosis and treatment of certain conditions like IPD more feasible. Pompe Disease was formally included in the RUSP in March 2015 and has been added to the NBS in 21 states with additional states soon to follow (31). Although additional data may be necessary to understand the efficacy and efficiency of such universal screening practices, knowing that outcomes can be significantly improved by early induction of current IPD therapies, supports the addition of Pompe disease screening in the NBS of other states.

Our case exemplifies the integration of prenatal genetic diagnosis to the coordination of complex multidisciplinary care in the treatment of a rare, previously fatal genetic condition. Our

patient has thus far tolerated the therapy well. Our experience with planning for and the management of this patient as well as the clinical outcome will provide crucial information especially in light of the addition of Pompe disease to the RUSP. As an increasing number of states now screen for Pompe disease in the newborn setting, there must be no delay in the timely initiation of appropriate treatment and the use of ITI as indicated.

ETHICS STATEMENT

Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

PG and BS wrote the manuscript. FK, DM, and JM performed fetal and postnatal cardiac measurements and collection of data. PT provided prenatal details. LT-K collected and organized lab results. AD made the graphs. Review and revision of the manuscript was done by PG, AD, and PK. All authors contributed to and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.01929/full#supplementary-material>

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Quantification of Bevacizumab Activity Following Treatment of Patients With Ovarian Cancer or Glioblastoma

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Highly sensitive reporter-gene assays have been developed that allow both the direct vascular endothelial growth factor (VEGF) neutralizing activity of bevacizumab and the ability of bevacizumab to activate antibody dependent cellular cytotoxicity (ADCC) to be quantified rapidly and in a highly specific manner. The use of these assays has shown that in 46 patients with ovarian cancer following four cycle of bevacizumab treatment, and in longitudinal samples from the two patients that respond to bevacizumab therapy from a small cohort of patients with glioblastoma, that there is a reasonably good correlation between bevacizumab drug levels determined by ELISA and bevacizumab activity, determined using either the VEGF-responsive reporter gene, or the ADCC assays. One of the two primary non-responders with glioblastoma exhibited high levels of ADCC activity suggesting reduced bevacizumab Fc engagement *in vivo* in contrast to the other primary non-responder, and the two secondary non-responders with a decreasing bevacizumab PK profile, determined by ELISA that exhibited low to undetectable ADCC activity. Drug levels were consistently higher than bevacizumab activity determined using the reporter gene assay in serial samples from one of the secondary non-responders and lower in some samples from the other secondary non-responder and ADCC activity was markedly lower in all samples from these patients suggesting that bevacizumab activity may be partially neutralized by anti-drug neutralizing antibodies (NAbs). These results suggest that ADCC activity may be correlated with the ability of some patients to respond to treatment with bevacizumab while the use of the VEGF-responsive reporter-gene assay may allow the appearance of anti-bevacizumab NAbs to be used as a surrogate maker of treatment failure prior to the clinical signs of disease progression.

Keywords: bevacizumab, ADCC, glioblastoma, ovarian cancer, VEGF, bioassay, reporter gene assay

INTRODUCTION

The anti-vascular endothelial growth factor-A (VEGFA) monoclonal antibody bevacizumab (Avastin®) is used extensively to treat recurrent disease in patients with ovarian cancer and patients with glioblastoma who have failed first line therapy (1–3). Although, bevacizumab treatment results in a high initial response rate the effect is transient and most patient's tumors eventually progress (2, 3). The mechanisms of bevacizumab treatment failure are poorly understood (1) and an accurate assessment of the treatment response in individual patients is key to better understand the most effective means of optimizing bevacizumab treatment.

Current methods for quantifying the activity of human VEGF, or antibodies that neutralize its activity, such as bevacizumab, are bioassays based on the ability of anti-VEGF antibodies to inhibit the proliferation or migration of primary human umbilical vein endothelial cells (HUEC) or other cells expressing VEGFR receptors, following treatment of the cells with VEGF (4). Such assays can take several days to perform, are subject to a high degree of variation, and are difficult to validate. Reporter-gene assays based on the establishment of a stable cell line transfected with a luciferase reporter-gene placed under the control of a drug responsive chimeric promoter, provide highly sensitive and reproducible methods for quantifying drug activity (5–7) and although a VEGF-specific reporter gene assay has been developed previously (8) there is a need for an assay with improved sensitivity and dynamic range. Highly sensitive reporter gene assays are described herein that allow both the direct VEGF neutralizing activity of bevacizumab to be quantified with improved sensitivity and the ability of bevacizumab to activate antibody dependent cellular cytotoxicity (ADCC) to be quantified rapidly and in a highly specific manner. These assays have been used to better understand the action of bevacizumab in 46 patients with ovarian cancer following analysis of samples taken after four cycle of bevacizumab treatment and in longitudinal samples from in a small cohort of patients with glioblastoma presenting different types of response to treatment with bevacizumab.

MATERIALS AND METHODS

VEGF Responsive Reporter Cells

Human embryonic kidney HEK293 cells (ATCC Catalogue N° CRL1573) were transfected with a 5-fold tandem repeat of the upstream activation sequence (UAS), cCGGAGGACTGTCTCCGagtc, of gal-4 regulating transcription of the firefly luciferase (FL) reporter gene. The cells were also co-transfected with an expression vector encoding a chimeric transcription factor consisting of the gal4 DNA binding domain (nt:1–130) fused to the *trans*-activating domain of Elk-1 (nt:307–427), together with an expression vector for human VEGFR2. A clonal cell line was established that exhibited a high degree of VEGF responsiveness following treatment of the VEGF-responsive cells with increasing concentrations of VEGFA for 18 h at 37°C prior to quantification of VEGF-induced FL activity using the Bright-Glo® (Promega, Madison, WI,

United States) reagent and a GloMax® luminometer (Promega, Madison, WI, United States).

Quantification of Bevacizumab Activity

A fixed dilution or serial dilutions of the sample or bevacizumab standard to be tested were incubated for 30 min at 37°C with 25 ng/mL of VEGF. The samples are then incubated with the VEGF responsive reporter-gene cells for 18 h at 37°C prior to quantification of VEGF-induced FL activity as described in the preceding section.

Quantification of ADCC Activity

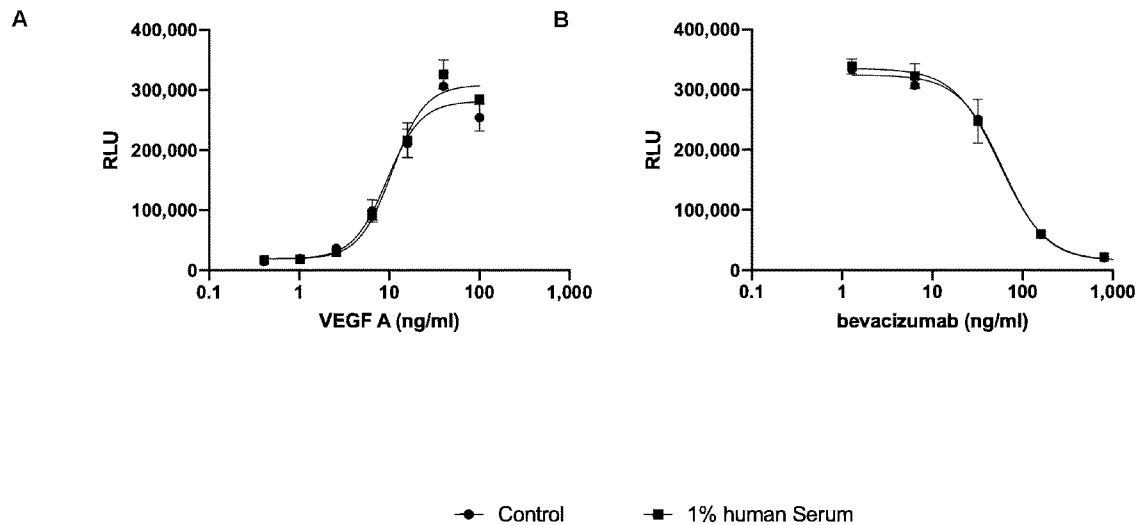
The novel engineered Jurkat ADCC effector cells expressing the V-158 wild-type variant of the FcγRIIIA receptor (CD16a) and the FL reporter-gene under the control of the principal transcription factors involved in FcγRIIIA signal transduction has been described previously (9). VEGF(–) target cells derived from human embryonic HEK293 cells, that do not express detectable levels of VEGF (Lallemant, unpublished results), were transfected with the costimulatory molecules CD80 and CD86 that were found to enhance the dynamic range of the ADCC assay for several different therapeutic monoclonal antibodies and the appropriate antigen positive target cells (Lallemant, unpublished results). VEGF (–) target cells expressing the costimulatory molecules CD80 and CD86 were again found not to express detectable levels of VEGF or ADCC activity in the presence of bevacizumab and the ADCC effector cells. The VEGF (++) target cells that express membrane-bound non-cleavable VEGFA were established by transfecting VEGF (–) target cells expressing the costimulatory molecules CD80 and CD86 with the gene encoding codon-optimized VEGFA fused to the coding sequence of the transmembrane region of TNFα bearing a mutation in the protease cleavage site as described previously (7).

Quantification of Bevacizumab Serum Concentrations by Capture ELISA

Microtiter plates were coated overnight with human recombinant VEGF₁₆₅ (R&D systems, catalog# 293-VE-001MG/CF) at a concentration of 0.15 µg/ml followed by blocking for 2 h with 3% BSA. For the standard curve recombinant bevacizumab (Avastin®) was used at concentrations ranging from 7 to 500 µg/ml. Test samples (diluted 1:100 in 0.1% blocking buffer) including negative controls were added and incubated for 1 h at 37°C. All wells, including test samples and standard curves, were then incubated with 100 µl of a monoclonal HRP-conjugated anti-bevacizumab antibody diluted 1:5000 (AbD Serotec, catalog# HCA 184P). Finally, ortho-phenylenediamine HRP substrate was added and the reaction was stopped with hydrochloric acid. Plates were washed four times with 0.05% PBS-TWEEN between each step. Plates were read at 492 nm and again at 620 nm and serum concentrations were determined using the standard curve. All procedures were optimized in the preceding assay validation steps.

Patient Population

A cohort of 46 patients with ovarian cancer and a second cohort of 6 patients diagnosed with glioblastoma according to



	VEGF	VEGF+ 1% Normal Human serum (NHS)	bevacizumab (Spike VEGF 30 ng/ml)	bevacizumab + 1% NHS (Spike VEGF 30 ng/ml)
Upper asymptote	281 516	308 488	324 536	335 370
Lower asymptote	12 915	13 412	14 526	13 639
Fold induction	21,8	23,2	22,3	24,4
Hill slope	2,3	2,3	-1,75	-1,7
IC ₅₀ (ng/ml)	9,4 +/- 2 ng/ml	10,71 +/- 2 ng/ml	59,79 +/- 12 ng/ml	56,06 +/- 18 ng/ml

FIGURE 1 | The VEGF responsive reporter-gene cell line was incubated for 18 h with increasing concentrations of VEGFA either alone or in the presence of 1.0% normal human serum prior to quantification of FL activity as described in the section “Materials and Methods” (A). Increasing concentrations of bevacizumab were mixed with 25 ng/ml of VEGFA for 30 min at room temperature prior to incubation for 18 h with VEGF responsive reporter-gene cells either alone or in the presence of 1.0% normal human serum and quantification of FL activity as described in the section “Materials and Methods” (B). The associated Table to the Figure shows the principal parameters of a 4PL plot determined using the Prism software.

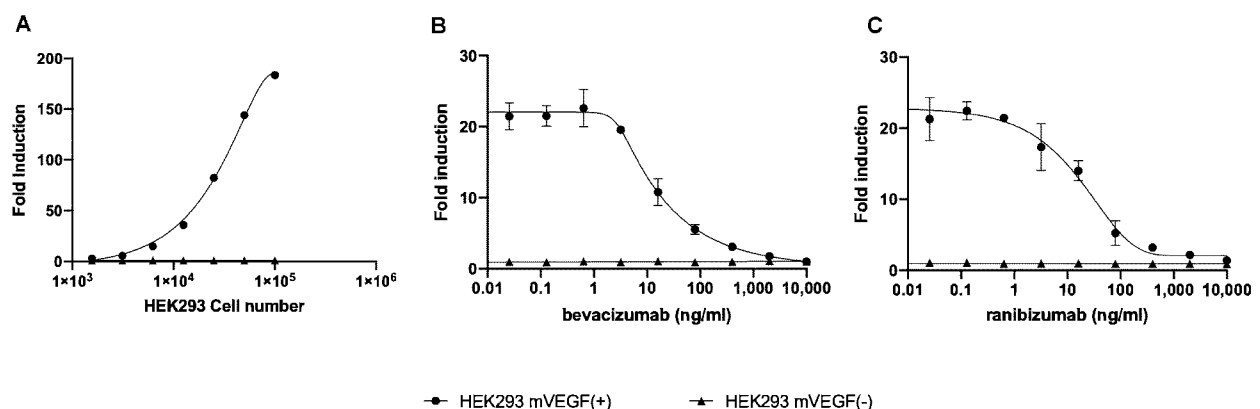


FIGURE 2 | Increasing concentrations of HEK293 mVEGF (+) target cells were incubated with 2.5×10^4 of the VEGF responsive reporter-gene cell line for 18 h at 37°C prior to the quantification of FL activity as described in the section “Materials and Methods” (A). Increasing concentration bevacizumab (B) or ranibizumab (C) were mixed with 10,000 mVEGF (+) target cells, sufficient to give an approximately 20-fold increase in the FL response of the VEGF responsive reporter-gene cell line, or 10,000 mVEGF (-) target cells for 30 min at room temperature prior to incubation for 18 h at 37°C with the VEGF responsive reporter-gene cell line and quantification of FL activity as described in the section “Materials and Methods”.

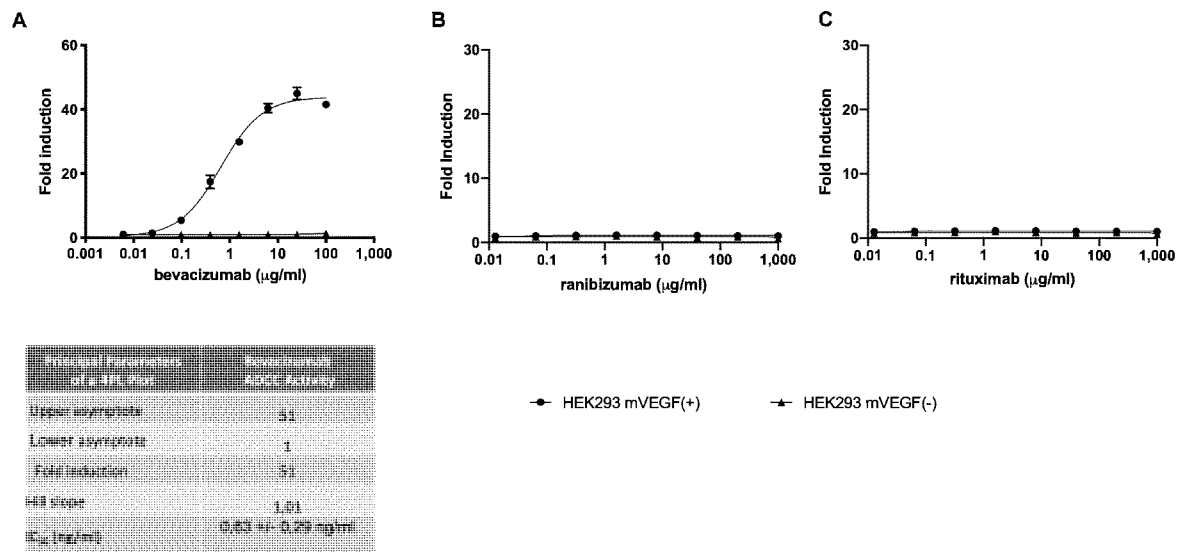


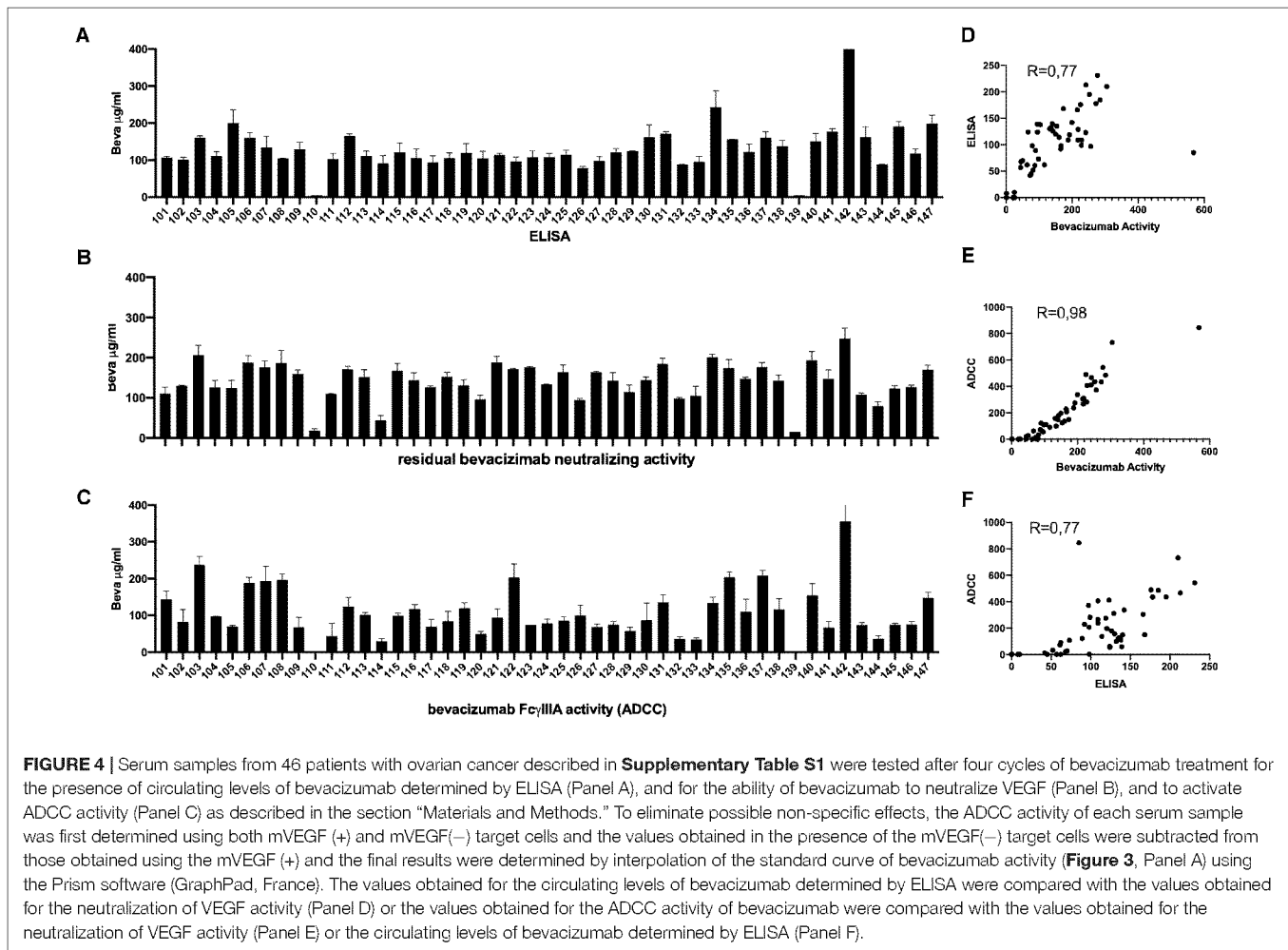
FIGURE 3 | ADCC effector cells (E) at a concentration of (1.2×10^5 cells/well), were incubated with mVEGF (+) or mVEGF(-) target cells (T) at an E:T ratio of 3:1 and increasing concentrations of bevacizumab (A) ranibizumab (B), or rituximab (C) for 4 h prior to the quantification of FL activity as described in the section “Materials and Methods”. The associated Table to the Figure shows the principal parameters of a 4PL plot of fold-induction of the ADCC activity of bevacizumab determined using the Prism software. To eliminate possible non-specific effects, the ADCC activity of each serum sample was first determined by interpolation of the standard curve of bevacizumab activity determined using both mVEGF (+) and mVEGF(-) target cells and the values obtained in the presence of the mVEGF(-) target cells were subtracted from those obtained using the mVEGF (+) target cells and using the Prism software (GraphPad, France).

the World Health Organization classification scheme (9) were both hospitalized at the Innsbruck University Hospital. Patients with glioblastoma were classified according to clinical and/or radiological outcomes as responders, who presented no tumor progression, primary non-responders presenting no response at all, and secondary non-responders, who exhibited an objective response followed by tumor progression. Patients were included in the study following written informed consent in accordance with the institution guidelines. This study was approved by the ethics committee of the University of Innsbruck: Approval number EKNR 1054/2017.

RESULTS

A reporter-gene cell line expressing FL under the control of an Elk-1-responsive chimeric promoter, one of the principal transcription factors involved in the VEGF signal transduction pathway (10), was developed that responds specifically to treatment of cells with VEGF (Figure 1A and Supplementary Figure S1). The reporter-gene cell line was used for the quantification of bevacizumab activity based on the ability of bevacizumab to neutralize soluble VEGFA as reflected by an inhibition of VEGF-induced FL activity (Figure 1B). The bevacizumab reporter-gene cell line described herein exhibited improved characteristics, including a dynamic range of approximately 20-fold and a IC₅₀ of approximately 60 ng/ml (Insert to Figure 1) relative to that published previously for a bevacizumab responsive reporter-gene assay based on a NFAT responsive promoter (3), and exhibited a stable response over

an extended number of passages in the presence of the selective agents (Supplementary Figure S2). The intra-assay and inter-assay precision for the bevacizumab reporter-gene cell line described herein ranged from 6 to 17% and 5 to 11%, respectively, for the coefficients of variation for the principal parameters of a 4PL plot (Insert to Supplementary Figure S3). The accuracy of the assay, determined from the ratio of the measured to the expected values for concentrations of bevacizumab ranging from 30 to 150 % of the expected values yielded a linear curve with a R^2 of 0.963 (Supplementary Figure S4). The presence of normal human serum was without effect on the assay results when tested at final concentrations ranging from 2.5 to 10.0 % (Supplementary Figure S5) encompassing the final concentration of human serum present in patient samples analyzed (Figure 1). The ability of the VEGF-responsive reporter-gene cell line to quantify the activity of membrane-bound non-cleavable VEGFA (Figure 2A) led to the observation that bevacizumab is able to neutralize membrane bound VEGF and provides a means of quantifying this activity using the mVEGF (+) target cells that express membrane-bound non-cleavable VEGF2A (Figure 2B). In contrast, bevacizumab did not exhibit any effect on FL reporter-gene activity in the presence of mVEGF (-) target cells (Figure 2B). Ranibizumab (Lucentis®), a derivative of bevacizumab lacking a Fc receptor was also shown to neutralize membrane bound VEGFA in the presence of mVEGF (+) but not mVEGF (-) target cells (Figure 2C). The ability of bevacizumab to activate ADCC was also quantified using an effector cell line expressing the FcγRIIIa receptor (CD16) that responds specifically to binding of the Fc moiety of an antibody to the FcγRIIIa receptor by activation of the FL reporter-gene (7) in



the presence of target cells that express non-cleavable membrane bound VEGFA (**Figure 3A**). No ADCC activity was observed when bevacizumab was tested in the presence of target cells that do not express membrane bound VEGFA (**Figure 3A**) or when ranibizumab (Lucentis®), a derivative of bevacizumab lacking a Fc moiety, was tested in the presence of target cells that express non-cleavable membrane bound VEGFA (**Figure 3B**) or when the effector cell line expressing the FcγRIIIa receptor and target cells that express non-cleavable membrane bound VEGFA were treated with increasing concentrations of the anti-CD20 antibody rituximab (**Figure 3C**).

Analysis of serum samples from a cohort of 46 patients with ovarian cancer following four cycles of bevacizumab treatment revealed (**Supplementary Table S1**) a reasonably good correlation between circulating drug levels determined by ELISA and the VEGFA neutralizing activity of bevacizumab (**Figures 4A,B**) and a close correlation between the VEGF2A neutralizing activity of bevacizumab and the ability of bevacizumab to activate ADCC (**Figures 4B,C**). Analysis of the results using the nonparametric Spearman rank correlation coefficient give a ρ value of 0.77 between circulating drug levels determined by ELISA and the VEGF2A neutralizing activity of

bevacizumab (**Figure 4D**), a ρ value of 0.98 between the ability of bevacizumab to activate ADCC and the VEGFA neutralizing activity of bevacizumab (**Figure 4E**), and a ρ value of 0.77 between the ability of bevacizumab to activate ADCC and circulating drug levels of bevacizumab determined by ELISA (**Figure 4F**). Analysis of longitudinal samples from a small cohort of patients diagnosed with glioblastoma (**Table 1**) according to the World Health Organization classification scheme (9) and presenting different types of response to treatment with bevacizumab, showed that overall, bevacizumab neutralizing activity correlated reasonably well with bevacizumab protein levels in most samples from patients irrespective of whether they were classified as responders, primary non-responders, or secondary non-responders although there was a tendency toward lower levels of bevacizumab neutralizing activity in samples from the two secondary non-responders (**Figures 5A,B**). A closer correlation was observed between the VEGFA neutralizing activity of bevacizumab and the ability of bevacizumab to activate ADCC (**Figures 5B,C**). In contrast, the host mediated ADCC activity of bevacizumab did not appear to be correlated solely with the level of circulating bevacizumab. Thus, the ADCC activity of bevacizumab was relatively low in most samples

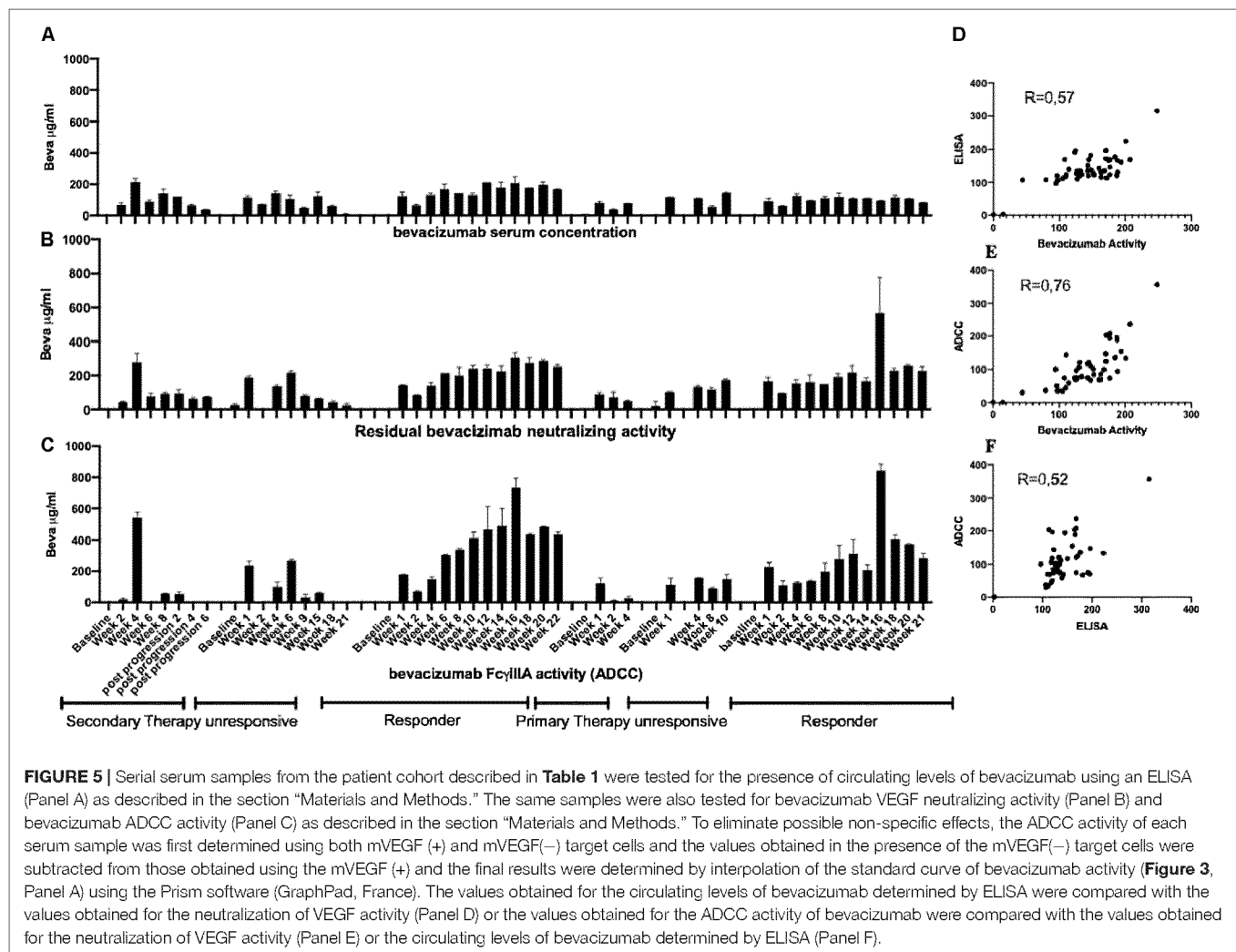


TABLE 1 | Patients with glioblastoma: Treatment schedule*.

Patient	Sex	Age	Recent or current use of corticosteroids	Temodal	Radiotherapy	Other previous chemo-therapy	Clinical response
IBK 1	F	38	Yes	Yes	Yes	No	Primary therapy unresponsive
IBK 2	M	61	Yes	Yes	Yes	No	Secondary therapy unresponsive
IBK 3	M	50	Yes	Yes	Yes	No	Primary therapy unresponsive
IBK 5	F	56	No	Yes	Yes	No	Responder
IBK 7	F	57	Yes	Yes	Yes	No	Responder
IBK 17	F	38	No	Yes	Yes	No	Secondary therapy unresponsive

*All patients with glioblastoma were treated with 15 mg of bevacizumab per kg of body weight every 3 weeks in addition to Temodal and Radiotherapy.

from the two patients classified as responders, relatively high in one of the two primary non-responders, and low to barely detectable in the other patient classified as a primary non-responder and the two secondary non-responders (**Figure 5**). Analysis of the results using the nonparametric Spearman rank correlation coefficient give a ρ value of 0.57 between circulating drug levels determined by ELISA and the VEGFA neutralizing activity of bevacizumab (**Figure 5D**), a ρ value of

0.76 between the ability of bevacizumab to activate ADCC and the VEGFA neutralizing activity of bevacizumab (**Figure 5E**), and a ρ value of 0.52 between the ability of bevacizumab to activate ADCC and circulating drug levels determined by ELISA (**Figure 5, Panel F**). We have shown that bevacizumab, but not the TNF α antagonist infliximab, also exhibits marked ADCC activity against human glioblastoma cells *in vitro* (**Supplementary Figure S6**).

DISCUSSION

Bevacizumab is used to target VEGF-dependent angiogenesis in patients with platinum-sensitive recurrent ovarian cancer (3) and is also used extensively to treat recurrent disease in patients with glioblastoma who have failed first line therapy (1, 2). Although bevacizumab treatment results in a high initial response rate the results are transient and most patient's tumors eventually progress (1–3). High grade glioblastomas produce large quantities of VEGFA that stimulates the proliferation of endothelial cells leading to the development of an abnormal vasculature (3). Bevacizumab is thought to act in part by reducing tumor-induced vascularization thereby limiting tumor growth (10). VEGFA exists in multiple isoforms, as a result of both alternative splicing of exons 6 and 7 and proteolysis, and the most common isoform VEGFA₁₆₅ is also present in a soluble form and as part of the extracellular matrix (11). The ability to quantify the activity of membrane bound VEGFA in addition to soluble VEGF as shown herein may facilitate a better understanding of the action of bevacizumab on tumor-induced vascularization in both gynecologic and neurologic tumors. In addition to reducing tumor-induced vascularization bevacizumab is also thought to exert direct anti-tumor activity against gliomas that express VEGF on their cell surface (12–15) and in animal models of ovarian cancer (16) and again the ability to quantify the activity of membrane bound VEGFA may also shed light on this process. There is also evidence to suggest that bevacizumab increases the sensitization of tumor cells to cytotoxic agents (13) and the ability to quantify the effect of bevacizumab on both soluble VEGF and membrane bound VEGF may also help elucidate the mechanisms of this process. Bevacizumab activity, determined using a VEGF responsive reporter-gene assay, correlated reasonably well with circulating levels of bevacizumab determined by ELISA in serial samples from the two patients classified as responders and were generally lower in serial samples from the two patients classified as secondary non-responders suggesting that treatment failure in secondary non-responders may be attributed at least in part to the presence of neutralizing anti-bevacizumab antibodies that could arise during bevacizumab treatment. Furthermore, the activity of circulating bevacizumab as determined by its ability to activate antibody-dependent cellular cytotoxicity was very low to undetectable in the serial samples from the two secondary non-responders. The microenvironment of glioblastomas contains numerous innate immune cells including microglia-macrophages and other immune cells resulting from alterations in the blood-brain barrier in addition to tumor cells (15). Studies using mouse models suggest that VEGF blockage can lead to an increased recruitment of monocytes as well as changes in dendritic cell subsets that may alter the adaptive immune response to the tumor (16). The anti-tumor activity of numerous monoclonal antibodies is mediated in part by the stimulation of cellular immunity (17) such as ADCC and antibody dependent cellular phagocytosis (ADCP). The two patients classified as responders both exhibited readily detectable levels of ADCC activity that overall correlated reasonably well with circulating levels of bevacizumab protein determined by ELISA and bevacizumab activity determined using the VEGF-responsive reporter-gene assay. To our knowledge this

is the first report that bevacizumab exhibits ADCC activity both *in vitro*, including against human glioblastoma target cells, and in samples of serum from patients. No ADCC activity was observed when bevacizumab was tested in the presence of target cells that do not express membrane bound non-cleavable VEGFA or when a derivative of bevacizumab lacking a Fc receptor (ranibizumab, Lucentis®) was tested in the presence of target cells that express membrane bound VEGFA, attesting to the validity of the results. Remarkably, a high level of bevacizumab ADCC activity was observed in one patient classified as a primary non-responder but not in samples from the other patients also classified as a primary non-responder or the two patients classified as secondary non-responder. Although it is well established that the neutralizing antibody response to the variable region of therapeutic antibodies can limit their efficacy (18) our results suggest that for antibodies that act in part by the activation of cellular immunity an immune response to the Fc moiety of the antibody may also limit their efficacy. Although it is difficult to draw any firm conclusions from these results due to the restricted number of samples tested from two small cohorts of patients included in this pilot study, they do show that it is indeed possible to quantify both the direct VEGF neutralizing activity of bevacizumab and the host mediated ADCC of activity bevacizumab in samples from patients with ovarian cancer or glioblastoma treated with bevacizumab and that clear differences are observed between samples from individual patients.

Although bevacizumab treatment was found not to confer an increase in overall survival in newly diagnosed patients with glioblastoma nor to confer an overall survival advantage in combination therapy (19), bevacizumab treatment can prolong progression-free survival and is used extensively in routine clinical practice in the United States and some other countries (20). An accurate assessment of the treatment response in individual patients is key to a better understanding of the most effective means of optimizing bevacizumab treatment.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/Supplementary Material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Geschäftsstelle der Ethikkommission der Medizinischen Universität Innsbruck Innrain 43 A-6020 Innsbruck. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CL and RF-M performed the VEGF reporter-gene and ADCC assay and analysis of the data under the direction of MT who also wrote the manuscript. MA, SI, TC, AG-W, FDP, and

FD supervised the clinical study. MA and SI performed the PK analysis under the direction of FD. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.515556/full#supplementary-material>

FIGURE S1 | Illustration of the interaction of VEGFA with its cell-surface receptor and activation of the cytoplasmic signal transduction pathway resulting in phosphorylation and activation of the chimeric Elk-1::Gal4 transcription factor in the nucleus. The chimeric transcription factor consisting of the transactivating domain of Elk-1 fused to the DNA binding domain of Gal4 then binds to the 5-fold

tandem repeat of the Gal4 upstream activation sequence (UAS) resulting in activation of the firefly luciferase reporter-gene.

FIGURE S2 | The stability of the VEGF responsive reporter-gene cell line was determined by testing VEGFA induced FL activity at regular intervals for 20 passages by incubating the VEGF responsive reporter-gene cells for 18 h with increasing concentrations of VEGFA prior to quantification of FL activity as described in the section "Materials and Methods".

FIGURE S3 | Inter and intra-assay precision was determined by incubating increasing concentrations of bevacizumab with 4 individual samples of VEGFA at a final concentration of 25 ng/ml for 30 min at room temperature on a single microtiter plate (A) or with a single sample of VEGFA at a final concentration of 25 ng/ml and incubated for 30 min at room temperature on 4 individual microtiter plates (B) prior to incubation for 18 h at 37°C with the VEGF responsive reporter-gene cell line and quantification of FL activity as described in the section "Materials and Methods." The associated Table to **Figure 2** shows the principal parameters of a 4PL plot determined using the Prism software together with intra-plate or inter-plate percentage coefficients of variation (% CV).

FIGURE S4 | The accuracy and linearity of the reporter-gene assay was determined by incubating concentrations bevacizumab corresponding to 25, 50, 100, 125, and 150% of the expected value with VEGFA at a final concentration of 25 ng/ml for 30 min at room temperature prior to incubation for 18 h at 37°C with the VEGF responsive reporter-gene cell line and quantification of FL activity as described in the section "Materials and Methods".

FIGURE S5 | Increasing concentrations of bevacizumab were mixed with 25 ng/ml of VEGFA for 30 min at room temperature prior to incubation for 18 h with VEGF responsive reporter-gene cells either alone or in the presence of 2.5, 5, or 10% normal human serum and quantification of FL activity as described in the section "Materials and Methods" (B). The associated Table to **Figure 1** shows the principal parameters of a 4PL plot determined using the Prism software.

FIGURE S6 | ADCC effector cells (E) at a concentration of (1.2×10^5) cells/well, were incubated with human U87 glioblastoma target cells (T) at an E:T ratio of 3:1 and 100 µg/ml of bevacizumab or 100 µg/ml of infliximab for 4 h prior to the quantification of ADCC activity as described in the section "Materials and Methods".

TABLE S1 | Patients with ovarian cancer: Treatment schedule*.

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Conflict of Interest: CL, RF-M, and MT were employed by Svar life Science France.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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