DEVELOPMENT OF VACCINES AGAINST EMERGING PATHOGENS

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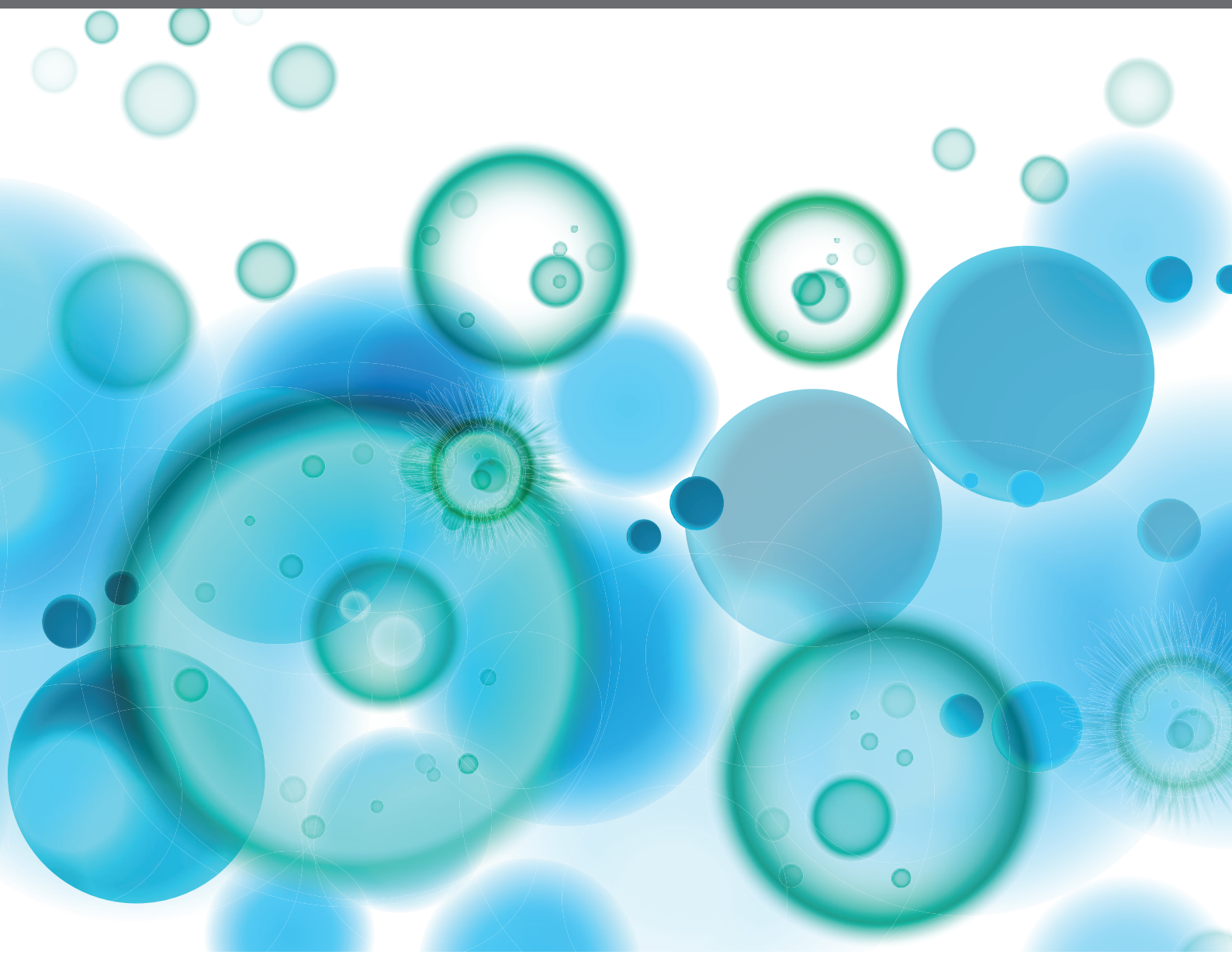
PUBLISHED IN: Frontiers in Immunology



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ISSN 1664-8714
ISBN 978-2-88974-162-5
DOI 10.3389/978-2-88974-162-5

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DEVELOPMENT OF VACCINES AGAINST EMERGING PATHOGENS

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Citation: Ewer, K., Lambe, T., van Doremalen, N., eds. (2022). Development of Vaccines Against Emerging Pathogens. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88974-162-5

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frontiers in Immunology

REVIEW
published: 03 September 2020
doi: 10.3389/fimmu.2020.02130

Viral Emerging Diseases: Challenges in Developing Vaccination Strategies

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In the last decades, a number of infectious viruses have emerged from wildlife or re-emerged, generating serious threats to the global health and to the economy worldwide. Ebola and Marburg hemorrhagic fevers, Lassa fever, Dengue fever, Yellow fever, West Nile fever, Zika, and Chikungunya vector-borne diseases, Swine flu, Severe acute respiratory syndrome (SARS), Middle East respiratory syndrome (MERS), and the recent Coronavirus disease 2019 (COVID-19) are examples of zoonoses that have spread throughout the globe with such a significant impact on public health that the scientific community has been called for a rapid intervention in preventing and treating emerging infections. Vaccination is probably the most effective tool in helping the immune system to activate protective responses against pathogens, reducing morbidity and mortality, as proven by historical records. Under health emergency conditions, new and alternative approaches in vaccine design and development are imperative for a rapid and massive vaccination coverage, to manage a disease outbreak and curtail the epidemic spread. This review gives an update on the current vaccination strategies for some of the emerging/re-emerging viruses, and discusses challenges and hurdles to overcome for developing efficacious vaccines against future pathogens.

Keywords: vaccines, emerging infectious diseases, viruses, epidemics, pandemics, antibody-dependent enhancement, SARS-CoV-2, COVID-19

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Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: *08 June 2020*
Accepted: *06 August 2020*
Published: *03 September 2020*

Citation:
Trovato M, Sartorius R, D'Apice L, Manco R and De Berardinis P (2020) Viral Emerging Diseases: Challenges in Developing Vaccination Strategies. Front. Immunol. 11:2130. doi: 10.3389/fimmu.2020.02130

INTRODUCTION

Since the start of this century, a certain number of new or neglected pathogens have emerged from wildlife reservoirs and spilt over into human populations, causing severe diseases (1–3). Factors such as urbanization, globalization, travels, international commerce, aging, and climate changes have contributed to favor emergence, spread, and transmission of pathogens. Contacts among humans and potential zoonotic reservoirs are increasing, the number of travelers and their movements is growing, the aged population are more susceptible to infections, and the geographic distribution of pathogens within a previous endemic zone is changing (4, 5).

During the last decades, the global community faced several outbreaks of emerging and re-emerging infectious diseases, with high threats to the health security, biodefense, and economy worldwide (6, 7). The occurrence of significant disease outbreaks—such as SARS (severe acute respiratory syndrome) originating in China in 2002 (8), the 2009 H1N1 swine flu pandemic from Mexico (9), MERS (Middle East respiratory syndrome) that occurred in Saudi Arabia in 2012 (10), the West African outbreak of Ebola virus (EBOV) in late 2013 (11), the Zika virus (ZIKV) outbreak originating in Brazil in 2015 (12), the 2018 health emergence in Nigeria caused by Lassa virus (13), and the ongoing Coronavirus disease 2019 (COVID-19) pandemic (14)—has renewed interests in

developing strategies to faster prevent, treat, and/or control emerging and re-emerging viruses with high epidemic potential. Usually, there is little or no knowledge about identity, epidemiology, and pathogenesis of a new infectious agent appearing for a first time in a certain geographic area (as in case of novel coronaviruses or new influenza variants), as well as the potential to spread out from the zoonotic reservoir, making hard to predict if, where, and when a disease outbreak will occur. The World Health Organization (WHO) and the National Institutes for Allergy and Infectious Diseases (NIAID) published a list of pathogens to be prioritized for research and development, given their epidemic potential. This non-exhaustive list comprises viruses, bacteria, protozoa, and fungi, causing diseases for which efficient countermeasures do not actually exist, or require new therapeutics (15, 16).

As proven by historical records, vaccination has played a pivotal role in reducing morbidity and mortality from devastating infectious diseases, successfully leading to disease eradication (i.e., smallpox), and generally decreasing infectious disease burdens. Even in presence of therapeutic options, vaccines are the valuable means to prevent infections and overall represent the much wanted achievement. However, even with worldwide efforts, getting a vaccine to the public takes time, and side effects, dosing issues, and manufacturing problems can all cause delays. Thus, we have to use this time with great concern. Generally speaking, in case of newly emergent diseases, conventional strategies might raise some issues. The unpredictable identity of largely unknown emerging pathogens, the lack of appropriate experimental animal models, and the time and costs for faster developing, producing, licensing, and globally distributing effective vaccine candidates are some of the major challenges to overcome in case of pandemic threats. Hence, new and/or alternative approaches in vaccine design and development are required to rapidly face outbreak situations (17).

This review will discuss the current vaccination strategies for some of the emerging and re-emerging viruses, as well as the approaches that might be suitable in face of global pandemic threats.

EMERGING AND RE-EMERGING VIRAL INFECTIOUS DISEASES

Emerging and re-emerging pathogens represent a constant epidemic threat to humanity not only for the public health consequences but also for the economic, social, and political effects they may globally provoke. Therefore, a major public awareness and preparedness would be fundamental in fighting emerging infectious diseases. The terms "emerging and re-emerging infectious diseases" mainly refer to two major categories of infectious diseases: newly emergent infections, caused by novel pathogens; and re-emerging infectious diseases, caused by microbes reappearing after previous control, and/or eradication (1). Almost 60% of emerging infectious diseases are zoonoses, with the great majority of them originating in wildlife, and the number is constantly increasing. Climate changes have been related to the emergence of vector-borne diseases in severe environmental conditions, but this is a most debated issue, as well as the contribution of agricultural practices (18). In addition, the chances of infectious disease spreading could also include livestock/wildlife animal markets and consumption of those. A study where Australia was used as a model of urbanization has proposed a relation among increasing pandemic threats and urbanization: it ascribes the increased threat of pandemic to the high number of major city residents, the exponential intensification of international air traffic, and the commuter mobility network (19).

Epidemic Versus Pandemic

Basically, an epidemic is an event that occurs when there is an increase, often sudden, in the frequency of a disease above what is normally expected in that population, in that area; while pandemic (from: παν = all, and δεμοσ = people) refers to an epidemic that spreads over several countries or continents at the same time, usually affecting a large number of people (20).

In the last decades, a certain number of viruses came to light for the first time or reappeared, giving rise to significant epidemics and pandemics (**Figure 1** and **Table 1**).

Epidemic outbreaks of viral diseases were mostly caused by flaviviruses generally transmitted by vectors, including West Nile virus (WNV) (21, 22), ZIKV (23, 24), Yellow Fever virus (YFV) (25, 26), and Dengue virus (DENV) (27, 28). Vector-borne diseases, including Chikungunya fever caused by Chikungunya alphavirus (CHIKV) (27, 29), are extremely difficult to eradicate because viruses are maintained in nature by propagation among vectors and hosts, without human–human contact. Moreover, dry and hot climate conditions seem to foster mosquitoes to bite humans than animals, increasing the risk of spreading diseases with a devastating impact (30). Today, most areas of the world are endemic for at least one flavivirus, with DENV being the most prevalent, and approximately 50–100 million people are infected each year. Among viral hemorrhagic fevers, Lassa fever (LF) is a rodent-borne acute disease caused by Lassa virus (LASV) (31), endemic in many West African countries, including Nigeria that experienced a high mortality rate in the 2018 outbreak (32). Ebola virus disease (EVD) and Marburg virus disease (MVD) are caused by members of the Filoviridae family, EBOV (33), and Marburg virus (MARV) (34), respectively. The 2013–2016 Ebola outbreak in West Africa was the largest since the virus was first discovered in 1976, with a case fatality rate for *Zaire ebolavirus* of 75% (35), while the largest recorded MVD outbreak occurred in Angola in 2004 (36).

Concerning pandemics, flu pandemics were reported three times during the twentieth century; genome analysis of pandemic influenza viruses dated 1918 (H1N1), 1957 (H2N2), and 1968 (H3N2) demonstrated that all viral strains fully or partially originated from non-human reservoirs, and that the ultimate origin of HA (hemagglutinin) genes are from avian influenza viruses (37), with the 1918 strain likely being the ancestor of the subsequent epidemic variants. Hence, the 1918 influenza pandemic has been called the mother of all pandemics (38). During the 2009 pandemic, caused by (H1N1)pdm09 virus, it has been estimated that 0.001–0.007% of the world's population died of respiratory complications associated with the viral infection

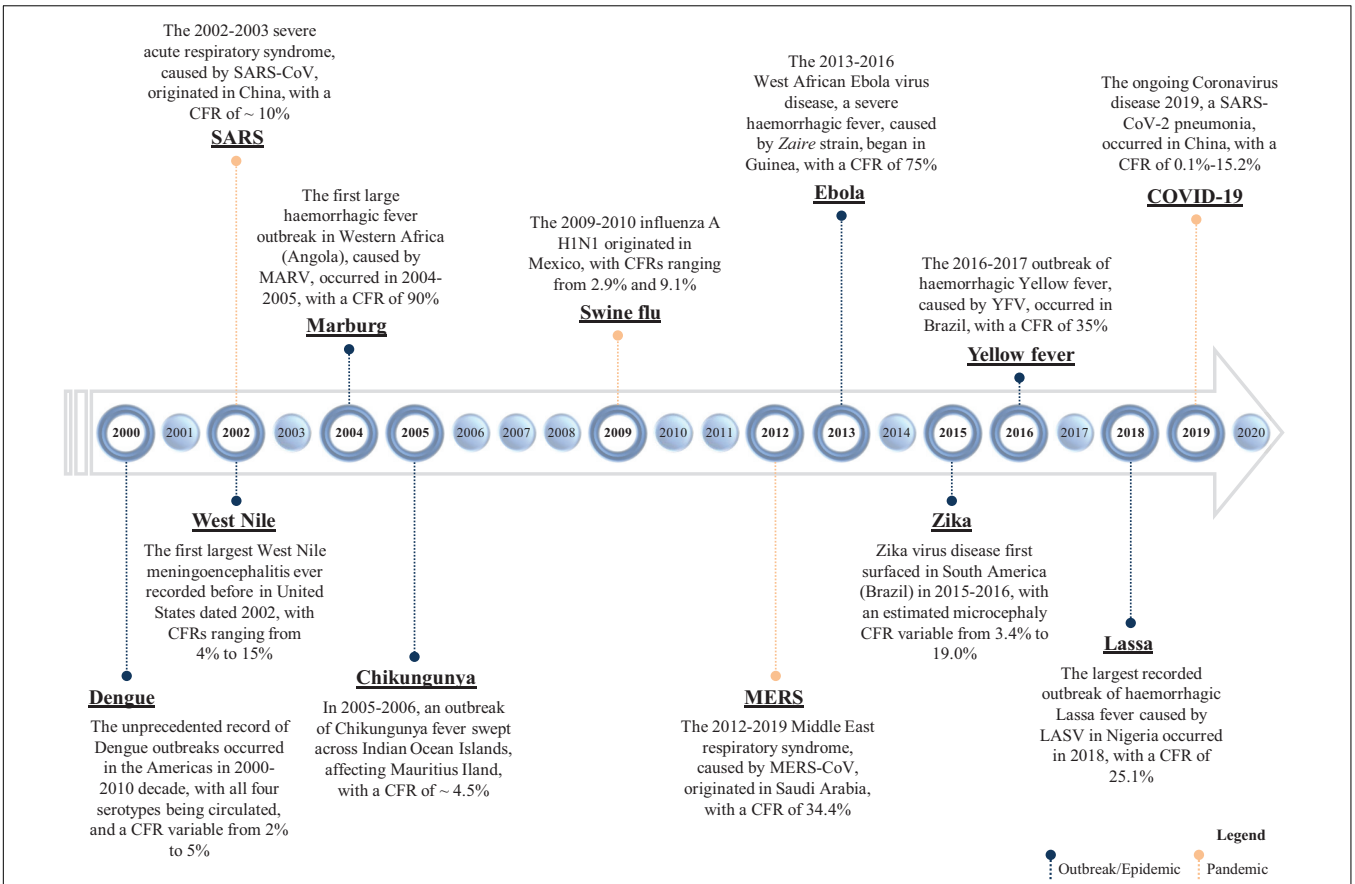

FIGURE 1 | Timeline of emerging and re-emerging viral diseases. The year on the timeline is the year of the emergence or re-emergence of the schematically reported viral epidemic outbreaks within a certain geographic area; the overall given values of CFR (case fatality rate) refer to "the proportion of cases of a specified condition that are fatal within a specified time," according to Dictionary of Epidemiology (228). SARS-CoV, severe acute respiratory syndrome coronavirus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; MERS-CoV, Middle East respiratory syndrome coronavirus; MARV, Marburg virus; YFV, Yellow Fever Virus; and LASV, Lassa virus.

TABLE 1 | Emerging and re-emerging viral diseases.

Disease	Virus	Family/Genus	Reservoir/spill-over hosts	Transmission	References
West Nile fever (WNF)	WNV	Flaviviridae *Flavivirus*	Mosquitoes; birds/horses, dogs, rabbits	Mosquitoes	(22)
Zika fever	ZIKV	Flaviviridae *Flavivirus*	Mosquitoes; NHPs; domestic animals	Mosquitoes; vertical transmission	(23)
Yellow Fever (YF)	YFV	Flaviviridae *Flavivirus*	Mosquitoes; NHPs	Mosquitoes	(24)
Dengue fever (DF)	DENV	Flaviviridae *Flavivirus*	Mosquitoes; NHPs	Mosquitoes	(25)
Chikungunya fever	CHIKV	Togaviridae *Alphavirus*	Mosquitoes; NHPs	Mosquitoes	(25)
Lassa fever (LF)	LASV	Arenaviridae *Mammarenavirus*	Multimammate mouses	Rodent-to-human	(26)
Ebola virus disease (EVD)	EBOV	Filoviridae *Ebolavirus*	Fruit bats/NHPs; antelopes	Human-to-human	(27)
Marburg virus disease (MVD)	MARV	Filoviridae *Marburgvirus*	Bats/NHPs; humans	Human-to-human	(28)
Swine flu	A(H1N1)pdm09	Orthomyxoviridae *Influenzavirus A*	Pigs	Human-to-human	(29)
Severe acute respiratory syndrome (SARS)	SARS-CoV	Coronaviridae *Coronavirus*	Bats/palm civets	Human-to-human	(30)
Middle East respiratory sindrome (MERS)	MERS-CoV	Coronaviridae *Coronavirus*	Bats/dromedary camels	Human-to-human	(30)
Coronavirus disease 2019 (COVID-19)	SARS-CoV-2	Coronaviridae *Coronavirus*	Bats; likely malayan pangolins	Human-to-human	(31)

NHPs, non-human primates.

during the first 12 months, after the first reported case (39). The age of deceased people was below 65 years in almost 80% of cases, a peculiarity compared with the seasonal influenza epidemic. The mortality rates observed in 1968 and 1918 flu were of 0.03% and 1–3% due to H3N2 and H1N1, respectively, and ranging from 2.9 and 9.1% in 2009 (40). The 2009 H1N1 pandemic has been commonly referred to as swine flu for the swine origin of the virus, first isolated in Mexico and United States in April 2009. The viral genome sequencing indicated that the virus contains a combination of genes never reported in swine or humans before. It has been demonstrated that the swine has become a reservoir of H1 viruses with the potential to cause future pandemics (37). A (H1N1)pdm09 virus monovalent vaccine was produced in late 2009 (41), but the virus has not been eradicated and it continues to circulate as a seasonal variant, causing hospitalization, and death (42).

In November 2002, a first case of SARS was reported in Guangdong (China), and after 7 months, the coronavirus (CoV) causing the disease, named SARS-CoV, spread in 37 countries, giving rise to lower respiratory tract infections, with a poor outcome in 10% of cases (43, 44).

Middle East respiratory syndrome-CoV, the causative agent of MERS, was isolated in 2012. The coronavirus has caused isolated MERS outbreaks thereafter, becoming endemic in Arabian Peninsula, with a case fatality rate of 34.4% (43–45).

On March 11, 2020, WHO has declared the Coronavirus disease 2019 (COVID-19) outbreak a global pandemic. The disease is caused by a novel coronavirus, known as SARS-CoV-2, that shares almost 88% of the genome with that of SARS-CoV (46, 47). Actually more than 5.9 million (as of August 1, 2020) of people are infected, with an overall case fatality rate of 0.1–15.2% (48).

VACCINE PLATFORMS

In case of global public health emergencies, governmental and private organizations, vaccine developers, and regulatory authorities should all massively collaborate in selecting and funding the most suitable vaccine platform and strategy to quickly act and curtail disease outbreaks. At the outset of a disease outbreak, gaps in knowledge of identity, pathogenesis, epidemiology of the new emerging pathogen, time required to study the immune responses correlating with the outcome of the viral infection, and the lack of appropriate preclinical models susceptible to infection for testing a vaccine candidate pose several barriers and impediments to expedite vaccine design and development, and thus to ensure global vaccination coverage in time.

In the fight against newly emergent viruses, vaccine design might benefit from a range of platform technologies, including nucleic acid vaccines, viral-vector vaccines, and recombinant protein-based vaccines (likely to be administered with adjuvants) (17, 49). Compared with conventional vaccines, such as live attenuated and inactivated vaccines, molecular-based platforms might offer a more versatile tool against new emergent viruses, allowing a more fast, low-cost, and scalable vaccine manufacturing. Essentially, these platforms rely on the use of a system to deliver and present a new antigen (or a synthetic gene) to rapidly target an emergent pathogen. Theoretically, once a platform has previously met safety and efficacy requirements to be moved and advanced into the market, a candidate vaccine against a new virus might profit from the same system, production, and purification protocols, only replacing the disease target antigen (or inserted gene), thus streamlining the vaccine discovery.

Inactivated and Live Attenuated Vaccines

In inactivated vaccine, the virus is rendered uninfectious using chemicals, such as formaldehyde or heat. This technology, conceived in the nineteenth century, is used for few vaccines still in use (i.e., inactivated polio, whole cell pertussis, and hepatitis A) (50). Live attenuated vaccines are obtained by passing the virus through animal or human cells until it picks up mutations that make it unable to cause the disease (i.e., measles, mumps, chickenpox, etc.); the attenuated smallpox was used for the massive vaccination campaign that successfully eradicated the infection (51), and currently, attenuated influenza viruses are used as vaccines against the seasonal influenza (52). The advantages of live attenuated vaccines are the intrinsic adjuvant properties, the ability to infect cells (**Figure 2**), and to activate the innate immune response. Interestingly, a safe SARS-CoV-2 inactivated vaccine (PiCOVacc) has been recently described as being able to induce specific neutralizing antibodies (NAbs) in experimental animal models (53), and a phase III clinical trial (NCT04456595) will soon assess efficacy and safety of this candidate in health care professionals (**Table 2**).

Nucleic Acid Vaccines: mRNA and DNA Vaccines

Nucleic acid vaccines include either mRNA or plasmid DNA (pDNA) vaccines (**Figure 2**).

Two types of mRNA vaccines were developed: conventional non-replicating mRNA vaccines and self-amplifying vaccines (or viral replicons). The *in* vitro enzymatic transcription (IVT) of a DNA template plasmid, containing the promoter sequence for the DNA-dependent RNA polymerase, provides a mature mRNA molecule, with the open reading frame that encodes the target antigen, the 5′ and 3′ flanking untranslated regions (UTRs), the 5′ cap, and the terminal poly(A) tail. Self-amplifying RNA (SAM) vaccines are commonly based on alphavirus genomes, where genes coding for the structural proteins are replaced with that encoding the target antigens, while the RNA replication machinery sequences are conserved, allowing intracellular antigen-encoding RNA amplification and higher antigen expression levels than the conventional mRNA vaccines (17, 54). Once the mRNA vaccine is delivered to the host cells and reaches the cytoplasm, it is translated *in vivo* by the host cellular machinery, providing the corresponding post-translationally modified antigen (**Figure 2**), thus mimicking the *in vivo* natural infection. mRNA vaccines activate the innate immune system, triggering host immune sensing receptors, and successively promoting adaptive immune responses (55). Several

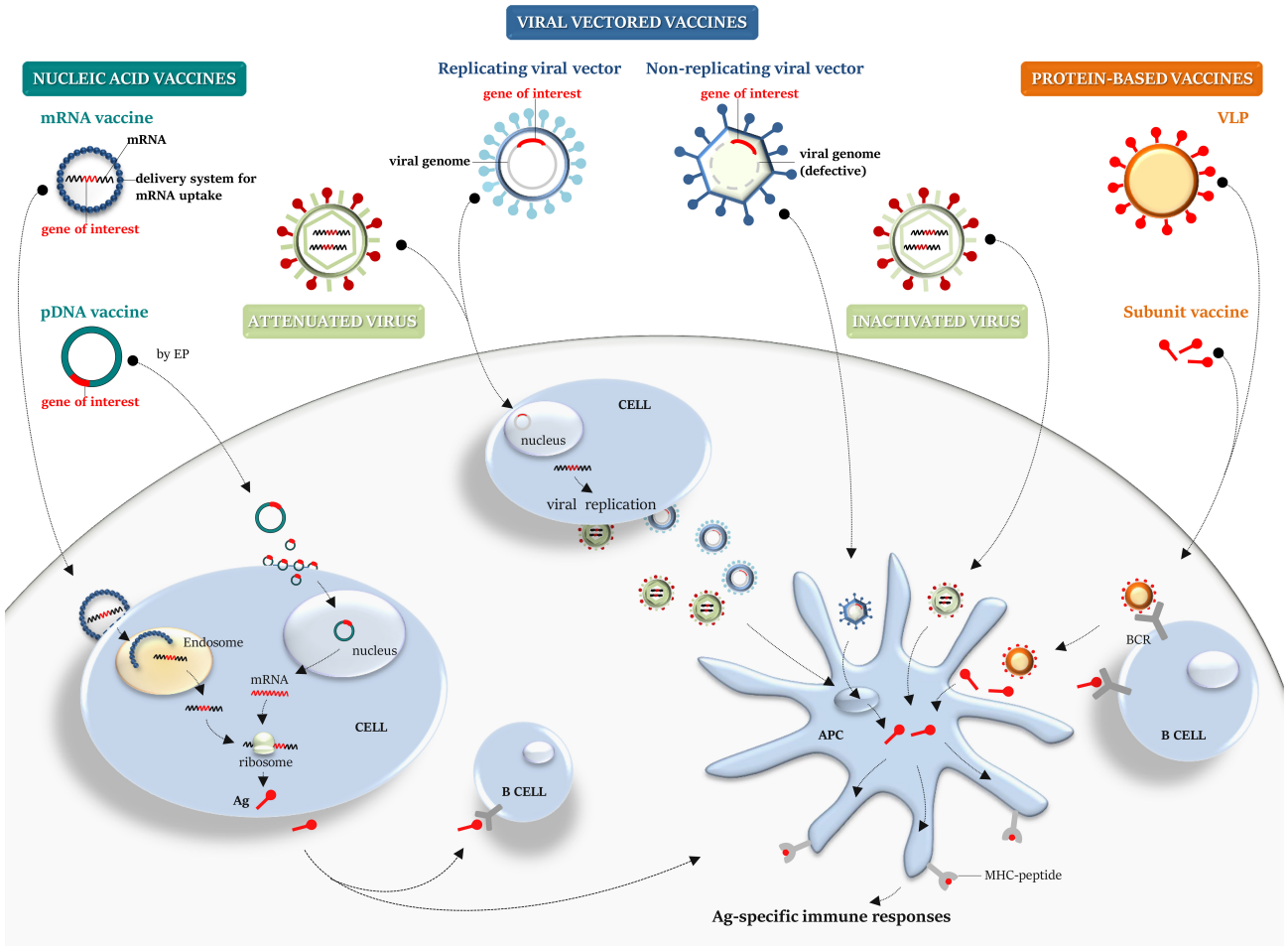

FIGURE 2 | Platforms for vaccine manufacturing: a graphical overview. Nucleic acid, viral-vector, protein-based, live attenuated and inactivated vaccines are schematically illustrated. *Nucleic acid vaccines*: conventional non-replicating mRNA vaccine, containing the target gene sequence, can be encapsulated into a delivery system to aid its cellular uptake. Once released from endosome into the cytosol, it is translated by the host cellular machinery into the target antigen. A pDNA carrying a gene target reaches the nucleus to achieve transcription and translation into the cytosol. pDNA can be internalized by somatic cells (i.e., myocytes) and then the secreted antigen can be taken up by APCs or naïve B cell, priming immune responses. *Viral vectored vaccines*: defective viral vector, carrying a transgene cassette, can be employed as a system to deliver a transgene and allow the expression of the heterologous antigen within the infected cell. A recombinant replicating viral vector retains the ability to replicate and produce progeny virus particles that can then infect cells, leading to transgene expression and Ag processing and presentation. *Protein-based vaccines*: recombinant subunit vaccine or a VLP can be taken up by APCs for MHC presentation and B-cell recognition through BCR. *Virus vaccines*: compared with an inactivated virus, a live attenuated virus retains the ability to replicate and infect cells, mimicking the natural infection. APCs, antigen-presenting cells; MHC, major histocompatibility complex; Ag, antigen; pDNA, plasmid DNA; EP, electroporation; BCR, B-cell receptor; and VLP, virus-like particle.

technological innovations have allowed to overcome some of the concerns associated with instability, half-life, inefficient *in vivo* delivery, and high innate immunogenicity of mRNA platform (56). mRNA vaccines do not produce infectious particles and potentially do not integrate into the host genome, reducing safety issues, and no anti-vector immunity is elicited. They can be quickly produced (likely within the time required to get genomic information from the new emergent virus), saving time and cutting costs. Thus, the mRNA platform offers a promising attractive alternative to conventional vaccines, should a disease outbreak occur.

No RNA vaccine has been yet licensed for humans, but encouraging results from preclinical and human clinical trials have shown that mRNA vaccines are able to induce safe and long-lasting immunity against different infectious viral diseases, including Zika (57), influenza (58–61), Ebola (61), Dengue (62), and other viral diseases (17). A SARS-CoV-2 mRNA-based vaccine entered clinical phases just 2 months after the identification of the viral genome sequence (NCT04283461), and a phase III study (NCT04470427) will assess its effectiveness to prevent COVID-19 (63–65). A clinical study (NCT04449276) is currently evaluating a similar vaccine in healthy adults (**Table 2**).

The DNA-based strategy, like the mRNA-based technology, offers a valuable platform to design and deliver any target of choice, due to safety profile, stability, ease of gene manipulation, and large-scale vaccine manufacturing, in short

TABLE 2 | Current vaccine platforms in clinical trials.

Virus	Platform	Antigen	Vaccine	Phase	Trial N.	Sponsor
WNV	Inactivated virus	Whole virus	HydroVax-001	I	NCT02337868	NIAID
	Viral vector	prM and E in YFV	ChimeriVax-WN02	II	NCT00442169	Sanofi
	pDNA	prM and E	VRC-WNVDNA017-00-VP	I	NCT00106769	NIAID
ZIKV	Inactivated virus	Whole virus	TAK-426	I	NCT03343626	Takeda
			ZPIV		NCT02937233	NIAID WRAI
			ZPIV		NCT02963909	NIAID WRAI
	Live attenuated virus	Whole virus	rZIKV/D4Δ30-713	I	NCT03611946	NIAID
	mRNA	prM and E	mRNA-1893	I	NCT04064905	Moderna
			mRNA-1325		NCT03014089	Moderna
	pDNA	prM and E	VRC-ZKADNA085-00-VP	I	NCT02840487	NIAID
			VRC-ZKADNA090-00-VP	II	NCT03110770	NIAID
	Viral vector	prM and E in MV	MV-ZIKA	I	NCT02996890	Themis Bioscience
	Viral vector	M and E in Ad.26	Ad26.ZIKV.001	I	NCT03356561	Janssen
DENV	Inactivated virus	Whole virus	TDENV-PIV	I/II	NCT02421367	GlaxoSmithKline
	Live attenuated virus	Whole virus	TDV	III	NCT02747927	Takeda
	Live attenuated virus	Whole virus	TetraVax-DV-TV003	III	NCT02406729	Butantan Institute
	pDNA	prM and E	D1ME100	I	NCT00290147	U.S. Army
	Viral vector	prM and E in YFV	Dengvaxia	III	NCT02993757	Sanofi
					NCT02948933	Sanofi
CHIKV	Live attenuated virus	Whole virus	VLA1553	I	NCT03382964	Valneva
	mRNA	prM and E	VAL-181388	I	NCT03325075	Moderna
	Viral vector	NC + E in MV	MV-CHIK	II	NCT02861586	Themis Bioscience
LASV	pDNA	GPC	INO-4500	I	NCT03805984	Inovio Pharmaceuticals
	Viral vector	GP and NP in MV	MV-LASV	I	NCT04055454	Themis Bioscience
MARV	pDNA	MARV/EBOV-GP	VRC-EBODNA023-00-VP	I	NCT00997607	NIAID
	Viral vector	MARV-GP in ChAd3	cAd3-Marburg	I	NCT03475056	NIAID
		MARV/EOBV-GP in MVA Multifilo + Ad.26	MVA-BN(R)-Filo + Ad26.ZEBOV	I	NCT02891980	NIAID
SARS-CoV	Inactivated virus	Whole virus	SARS-CoV	I	NCT00533741	NIAID
	pDNA	S	VRC-SRSDNA015-00-VP	I	NCT00099463	NIAID
MERS-CoV	pDNA	S	GLS-5300	I/II	NCT03721718	Inovio Pharmaceuticals
	Viral vector	S in ChAdOx1	ChAdOx1 MERS	I	NCT03399578	Oxford University
		S in MVA	MVA-MERS-S	I	NCT03615911	University Hamburg-Eppendorf
SARS-CoV-2	Inactivated virus	Whole virus	SARS-CoV-2	I/II	NCT04352608	Sinovac
				I/II	NCT04383574	Sinovac
				III	NCT04456595	Sinovac and Butantan Institute
	LNP-mRNA	S	mRNA-1273	I	NCT04283461	Moderna/NIAID
				II	NCT04405076	Moderna/NIAID
				III	NCT04470427	Moderna/NIAID
			CVnCoV	I	NCT04449276	CureVac AG

(Continued)

TABLE 2 | Continued

Virus	Platform	Antigen	Vaccine	Phase	Trial N.	Sponsor
	pDNA	S	INO-4800	I	NCT04336410	Inovio Pharmaceuticals
				I/II	NCT04447781	Inovio Pharmaceuticals
			AG0301-COVID19	I/II	NCT04463472	AnGes, Inc.
			GX-19	I/II	NCT04445389	Genexine, Inc.
	Viral vector	S in Ad5	Ad5-nCoV	II	NCT04341389	CanSino Biologicals
				I	NCT04313127	Beijing Institute and CanSino Biologics
		S in ChAdOx1	ChAdOx1 nCoV-19	I/II	NCT04324606	Oxford University
	Subunit	RBD-dimer	Recombinant new CoV vaccine (CHO cells)	II	NCT04466085	Anhui Zhifei Longcom Biopharmaceutical
		S	SARS-CoV-2 rS	I/II	NCT04368988	Novavax
		RBD	KBP-COVID-19	I/II	NCT04473690	Kentucky BioProcessing, Inc.
	VLP	Coronavirus-Like Particle	CoVLP	I	NCT04450004	Medicago

To retrieve the listed clinical trials, we visited the "https://clinicaltrials.gov" website and used the following keywords: virus name; biological; study phase (selecting the most advanced study); vaccine platform. prM, pre-membrane; E, envelope; NC, nucleocapsid; GPC, glycoprotein precursor; GP, glycoprotein; S, spike; RBD, receptor binding domain; MV, Measles virus; MVA, Modified vaccinia Ankara; rVSV, recombinant Vesicular Stomatitis Virus; Ad.26, Adenovirus type 26 vector; ChAd3, Chimpanzee adenovirus type 3 vector; VLP, virus-like particle; CHO, Chinese Hamster Ovary cells; LNP, lipid nanoparticles; NIAID, National Institute of Allergy and Infectious Diseases; and WRAI, Walter Reed Army Institute.

time at low costs. Thus, it might be a promising solution to overcome the hurdles of vaccine clinical development in the time a given unknown virus starts to spread in a certain area. A DNA vaccine is essentially based on a pDNA backbone with an inserted eukaryotic expression cassette. A pDNA can be used to encode viral antigens, which can lead to antigen-specific immune responses (66, 67), on cellular uptake and *in vivo* long-term gene expression, potentially providing advantages over mRNA vaccines in terms of protein coding capacity, and amount and extent of antigen production. Unlike mRNA, pDNA needs to cross both plasma and nuclear membranes to enter into a cell target, reach the nucleus, and achieve transcription (**Figure 2**). Advances in pDNA delivery devices (i.e., use of gene gun; *in vivo* electroporation, EP), and delivery systems (i.e., encapsulation in LNPs; adsorption to polymers), have greatly enhanced molecular stability, delivery efficiency, uptake, and antigen expression. In addition, the use of optimized pDNA formulations and encoding molecular adjuvants, to be administered in prime-boost strategies or simultaneously with other vaccine platforms, has generally improved the low protective immune-stimulatory profile of pDNA (67). However, some potential safety concerns should be considered, including long-term persistence upon administration, which could eventually lead to genomic integration events, antibodies against bacteria-derived plasmids that could potentially trigger autoimmune diseases, and unwanted side effects due to encoded and co-delivered molecular adjuvants (17, 67).

Even though no DNA vaccine has been yet licensed for use in humans (four for veterinary use), this platform has shown great promise for several emerging viral diseases, including Ebola and Marburg (68), MERS (69), West Nile (70), Dengue (71), Chikungunya (72), and other viral diseases (17), and more recently for COVID-19 (73). Currently, DNA-based vaccine candidates, encoding the S protein from SARS-CoV-2, have moved into clinical phase I/II development (63, 65, 74) (**Table 2**).

Viral-Vector Vaccines

Recombinant viral vector-based platform employs either live replicating often attenuated or non-replicating viruses as vector vaccines (**Figure 2**). Viral vector vaccines represent the biotechnological evolution of live attenuated and inactivated vaccines: a viral backbone devoid of the replication machinery to be used as a shuttle to express *in vivo* the chosen target antigen. Several viral backbones have been exploited to generate viral-vector vaccines. Targeted deletion of replication genes represents the non-empirical way of virus attenuation, allowing the generation of a wide array of viral vectors, engineered by insertion of a transgene cassette.

The modified virus Ankara (MVA) is an attenuated form of the Vaccinia Virus (VACV), derived from more than 570 passages in chick embryo fibroblasts, a method that empirically modifies the viral genome, without affecting the immunogenicity (75). It is able to infect multiple cell types but cannot replicate inside the infected cells, ruling out the safety concerns related to the use of live vaccines.

One of the drawbacks in the use of a viral vector vaccine is that multiple immunizations lead to the host response against the structural viral proteins, limiting the efficacy of vaccination, as demonstrated in a study based on cellular immune response. To overcome this limitation, the heterologous prime-boost regimen has been introduced in several clinical trials, where two different viral vectors or a pDNA prime-viral vector boost were tested (76). Risks of integration into the host genome do potentially exist, as some viral vectors enter to the nucleus of cells to achieve transcription and replication. A major restrain in the production of viral vector vaccines is the time-consuming manufacturing; several attempts to accelerate vaccine production are in development, like selecting cell lines with higher yield or choosing the best promoter for transgene expression to reduce vaccine doses (77).

Among the available viral vectors, the adenoviruses are the most used in priming the immune response, being able to

induce humoral and cellular responses (78). A pre-existing anti-vector immune response jeopardized the vaccine response in adenoviral-based clinical trials (79). To avoid pre-existing immunity, adenoviral vectors of non-human origin or rare serotypes have been used as vaccine platform. The use of chimpanzee adenoviral vectors proved to be safe and effective in clinical trials conducted against Ebola (80) and Respiratory Syncytial Virus (RSV) (81). Vesicular Stomatitis Virus (VSV), a single-stranded negative sense RNA virus that naturally infects livestock, represents an attractive safe alternative over other viral vectors due to low risk of pre-existing immunity, lack of DNA molecules during replication, and ability of VSV-based vaccines to induce effective humoral responses (82).

For humans, two viral-vector vaccines are available: Imojev, a Japanese encephalitic virus (JEV) vaccine; and Dengvaxia, a Dengue vaccine (both from Sanofi Pasteur). Both are produced using the chimeric YFV as vector: two of the YFV genes have been replaced by genes encoding the pre-membrane (prM) and the envelope (E) protein of JEV or DENV, and the chimeric viruses are propagated in cell culture (83, 84). Conversely, several viral vector vaccines have been licensed for veterinary use because of the less stringent regulatory requirements (76). To face COVID-19, an Adenovirus type 5 vector expressing SARS-CoV-2 S protein (Ad5-nCoV) has been advanced into phase II trial (NCT04341389), while a phase III study (ISRCTN89951424) is currently investigating the chimpanzee adenoviral vector (ChAdOx1 nCoV-19), expressing the same protein (63, 65, 74) (**Table 2**).

Recombinant Protein-Based Vaccines

Recombinant protein-based vaccines consist of immunogenic proteins from the target pathogen. Once identified, recombinant proteins can be produced on a large scale, in bioreactors, using heterologous expression systems, like bacteria, yeast, plants, insect, or mammalian cell lines, depending on the post-transcriptional pattern of modification required (85). Vaccines based on recombinant proteins represent a safe platform because they do not contain pathogen-derived genetic information, and the manufacturing does not require manipulation of live pathogens. They might represent a platform of choice when a fast response to an epidemic is on demand, as the vaccine production can start once the genome of the new virus has been sequenced, even before the virus isolation.

Protein-based vaccines can be obtained producing recombinant virus subunits (SUVs) that can be administered in combination with adjuvants to improve the host immune response against the recombinant viral antigens (86). Recombinant proteins derived from viral capsid can self-assemble into virus-like particles (VLPs), high ordered and repetitive structures devoid of the viral genome. VLPs display antigenic epitopes in their original conformation in high copy number, they retain the size and geometrical organization of the original virus (mainly icosahedral or rod shape), preserving the viral immunogenicity due to the ability to crosslink B cell receptor on B cell surface (87), and to be taken up by antigen-presenting cells (APCs) (88, 89) (**Figure 2**). Several strategies have been proposed to improve dendritic cell (DC) uptake, by expressing targeting molecules such as antibodies directed against endocytic receptors, and to augment immunogenicity, through simultaneous delivery of maturation stimuli, like TLR agonists (90, 91). When not able to self-assemble into a VLP, the selected antigen can be expressed as chimeric protein: several VLP platforms are available for the display of heterologous antigens on the viral coat proteins. Recombinant VLPs from plant virus, like Tobacco mosaic virus (92), or alpha mosaic virus (93), are easily produced, competing for speed and cost of production with VLP platform based on mammalian viruses (94). The most used VLP platform is the HBcAg-VLP, the core antigen from hepatitis B virus (HBV) (95). It is also possible to chemically attach the heterologous antigen to a preformed VLP by using conjugation methods (96). Although this strategy could increase the manufacturing costs, it might be suitable when the expression of recombinant antigens affects the VLP assembly.

To date, VLP-based vaccines that have been licensed for human use include Cervarix (Merck & Co., Inc.) and Gardasil (GlaxoSmithKline Biologicals), used in prophylaxis against human papilloma virus (HPV), formed by the L1 major viral capsid protein; Engerix-B (GlaxoSmithKline Biologicals) and Recombivax-HB (Merck & Co., Inc.), consisting of HBV surface antigen (HBsAg), with a lipoprotein-like structure; and Flucelvax (Flucelvax Tetra in EU and Flucelvax Quadrivalent in United States), consisting of surface antigens from four influenza strains, recommended for individuals at high risk. A recombinant hepatitis E virus (HEV) vaccine, named Hecolin HEV 239 (Xiamen Innovax Biotech Co., Ltd.), containing the capsid protein from genotype 1 Chinese viral strain, has been licensed for use in China.

Currently, several recombinant protein-based vaccines against SARS-CoV-2 are under preclinical and clinical evaluation (64, 74) (**Table 2**). It is worth mentioning that Kim and colleagues designed and developed a SARS-CoV-2 subunit vaccine within 4 weeks of the identification of SARS-CoV-2 S protein N-terminal domain S1 sequence. Delivery of recombinant subunit vaccines by microneedle array resulted in potent antibody response in mice (97), and vaccination with a SARS-CoV-2 Spike S1-Fc fusion protein induced antibody responses in small animal models and NAbs in monkeys (98).

VACCINES FOR VIRAL INFECTIOUS DISEASES: STATE OF ART

In **Table 2** are listed the vaccine candidates that currently moved into clinical trials for preventing the viral infectious diseases discussed in the following section.

WNV

West Nile virus includes five lineages; among them, lineage 1 was classified as the most virulent, while lineage 2 is considered more attenuated. However, during a serious outbreak in Hungary in 2008, the sequencing of lineage 2 showed some genetic mutations that demonstrated the increased virulence of this strain and its explosion throughout the central Europe (99, 100), causing renewed interest in the development of a vaccine against WNV.

20 years after the epidemic that hit the United States, no WNV vaccine has been yet released for human use, while four vaccine formulations are on the market for veterinary use, three based on the whole inactivated virus (WN Innovator, Vetera WNV, and Prestige WNV), and one on recombinant vaccine expressing WNV prM/E into a canarypox backbone (Recombitek Equine WNV) (101, 102). These vaccines completely protect horses from viral infection but require subsequent administrations and several booster doses overtime.

For the development of a vaccine for humans, many different platforms were used in preclinical studies, and many of them entered into phase I/II trials, including hydrogen peroxide–inactivated whole virus (HydroVax) vaccine (NCT02337868) (103), a recombinant truncated form of WNV E protein (104), recombinant chimeric live attenuated viral vectors, employing YFV (105), or MVA (106) delivering WNV prM/E proteins (NCT00442169), pDNA vaccines encoding prM/E (NCT00106769) (70, 107). All the Envelope-based vaccines induced NAbs against both WNV lineages 1 and 2, but some candidates are unable to generate long-lasting antibody responses, requiring multiple administrations (103, 108, 109). Thus, further improvements are needed for the development of next-generation vaccines (110). Recently, a WNV replication-deficient vaccine candidate with a deletion of the non-structural protein NS1 has been shown to protect mice from a highly lethal viral challenge, after a single dose, without adverse effects (111).

ZIKV

During the 2015 outbreak in Brazil, an abnormal microcephaly number and other birth defects in newborns were reported (112). For this reason, vaccination of pregnant and of reproductive-age women became an urgency. Shan and colleagues developed a candidate vaccine, using a live attenuated viral strain containing a deletion in the 3′ region of the virus genome. This vaccine induced strong and protective antibody response, after a single injection in mice and macaques, and reduced viral RNA in placental and fetal tissues in infected mice (113). The immunized mice also developed a robust T-cell response (114). Although promising, this attenuated virus-based formulation does not meet the safety standards required to be used to vaccinate pregnant women, whose prophylaxis requires a vaccine that fulfill higher safety standards.

A number of different replication-deficient viral vectors have been recently developed and are currently under evaluation. Immunization of mice with a vaccine based on MVA delivering the ZIKV prM and the E structural proteins (MVA-ZIKV) elicited NAbs and potent ZIKV-specific $CD8^+$ T-cell responses, mainly with an effector memory phenotype (115). A rhesus adenovirus serotype 52 vector (RhAd52), expressing ZIKV prM and E proteins, induced high titer of ZIKV-specific antibodies after the first prime, offering complete protection against subcutaneous ZIKV challenge, in mice (116), and rhesus monkeys (117). These adenoviral-based vaccines induced antibodies that were also maternally transmitted (118). In addition, Abbink et al. using the rhesus macaque model demonstrated that a complete anti-ZIKV immunity can only be achieved through vaccination with a combination of different vaccine platforms (117). ZIKV vaccine candidates currently in phase I clinical trials include inactivated and live attenuated vaccines, mRNA and pDNA vaccines, and recombinant viral-vectored vaccines, mainly targeting the prM and E proteins (119). A DNA-based vaccine encoding the prM signal sequence from JEV and ZIKV E proteins moved into phase II (NCT03110770), showing immunogenicity and safety in humans (120).

YFV

A protective and efficacious vaccine against YFV is currently available. To date, the main type of YF vaccine produced on a large scale is based on the live attenuated 17D virus vaccine. This vaccine is obtained after numerous passages of Asibi virus strain in mouse and chicken embryo that generate a strain with accumulated mutations in the envelope protein. These mutations affect the virus binding to the host receptor, reducing its neurotropism and vicerotropism, and mosquito transmissibility (121). Because the vaccine is produced in chicken embryo, there are issues related to manufacturing costs and vaccine availability. The interruption of vaccination coverage against YF in endemic countries has caused major outbreaks in Africa and South America in 2015 and 2016, which exhausted the 17D vaccine stockpiles leading to the use of an emergency "fractional dose" campaign in the Democratic Republic of Congo (122). Thus, the fluctuating demand for doses during outbreaks makes the accessibility to the vaccine still a problem to be solved.

DENV

The need for a vaccine against DENV has become an urgency only in recent decades. Dengue fever is caused by four distinct virus serotypes, DENV1–4, able to circulate simultaneously in endemic areas, making extremely difficult the development of a broad protective vaccine. Recently, the Food and Drug Administration approved the first Dengue vaccine by Sanofi-Pasteur, named CYD-TDV or Dengvaxia (123, 124), a tetravalent live attenuated virus vaccine on YFV backbone, whose release has generated controversy due to evidence that the administration can increase the risk of a more severe form of the illness in people with a pre-existing immunity toward other DENV strains (125, 126). For this reason, the use of Dengvaxia is strictly limited, depending on age (between 9 and 16) and serostatus of recipients to vaccinate (exclusively individuals who had a previous DENV infection), generating concerns about its cost–benefit balance. Studies for the development of a safer vaccine are still ongoing, and candidate vaccines include a tetravalent Dengue purified inactivated virus vaccine, currently in phase I/II clinical trial (NCT02421367), and two live attenuated tetravalent chimeric TDV (DENVax), and TVD 003/005 (TetraVax-DV) vaccines, currently in phase III clinical trials (NCT02747927; NCT02406729) (127).

CHIKV

No vaccine is actually available to prevent CHIKV infection. Among the candidates in ongoing studies, two of them achieved and completed phase I or II trials: VLA1553 and MV-CHIK vaccines. VLA1553 candidate (by Valneva) is a live CHIKV (La Réunion isolate LR2006 OPY1) attenuated by a partial

deletion of the gene encoding the non-structural replicase complex protein. This vaccine induced immunity lasting over 20 months after a single shot immunization (NCT03382964). MV-CHIK vaccine is a live attenuated measles-vectored CHIKV vaccine that induced CHIKV-specific NAbs and shown to be well tolerated by all the participants (NCT02861586) (128). Recently, Moderna Therapeutics tested a vaccine based on engineered mRNA encoding CHIKV structural polyproteins (mRNA-1388) in a phase I clinical trial. As shown in preclinical studies, this formulation induced strong immune responses after one single injection, totally protecting mice from developing the disease (129).

LASV

Currently, there is no vaccine for LASV infection. Among the difficulties to tackle in the development of effective vaccines, there are the high genetic diversity of LASV strains and the absence of established correlates of protection. The high titer of antibodies does not prevent the viral replication, suggesting that protection to LASV is probably cell mediated (130). Vaccine platforms under advanced development include a DNA-based vaccine (INO-4500 from Inovio), moved into phase I (NCT03805984) (131), and a live attenuated vaccine based on measles virus, expressing LASV glycoprotein and nucleoprotein (MV-LASV). The MV-LASV vaccine gave promising results in preclinical animal models, being able to activate innate immunity, adaptive T-cell and B-cell responses (132), and it has been advanced to phase I clinical trial (NCT04055454), aimed at evaluating the optimal dose.

EBOV

Although the identification of EBOV dates back to 1976 (133), only few studies on vaccine candidates and four clinical trials were conducted before the West African outbreak in late 2013. Vaccines against Ebola virus have been extensively reviewed previously (134–136).

MARV

Several vaccine platforms have been tested in preclinical animal models and shown to be able to protect animals from MARV infection and to induce both humoral and cellular immune responses. These include VLPs (137), DNA vaccines (68), recombinant adenoviral vectors (138), and rVSV (139, 140). Many works have emphasized the use of a multivalent vaccine formulation to achieve protection against different filoviruses. Vaccination with a single dose of a trivalent formulation based on rVSV expressing glycoproteins from EBOV, *Sudan ebolavirus* (SUDV), and the Angola strain of MARV elicited antibodies specific for the three glycoproteins in non-human primates (NHPs) and a balanced T-cell response sufficient to protect against the viral challenges (141). Similarly, VLPs delivering a trimeric hybrid glycoprotein from MARV, EBOV, and SUDV fully protected vaccinated animals from MARV challenge, inducing specific NAbs (142). Using an enhanced DNA-based platform encoding the envelope glycoprotein from MARV and EBOV, Shedlock and colleagues showed that a polyvalent-filoviral vaccine candidate, delivered by *in vivo* EP, elicited in preclinical models robust NAbs and cytotoxic T cells, completely protecting animals from the viral challenge, after a single dose administration (68). Actually, a multivalent phase I study (NCT02891980) is evaluating safety and immunogenicity of two heterologous and two homologous prime-boost regimens using a MVA multi-filo and Ad26 Zaire Ebola (Ad26.ZEBOV) vaccines (143) in healthy volunteers, with the aim to analyze the protective response to different filoviruses.

CURRENT STATUS ON CORONAVIRUS DISEASES

Coronaviruses are a group of single-stranded RNA viruses that have been present in humans for at least 500–800 years and all originated in bats (144, 145). Earlier than 2019, six coronaviruses had been known to cause diseases in humans: HCoV-229E, HCoV-043, HCoV-Nl63, HCoV-HKN1, SARS coronavirus (SARS-CoV), and MERS coronavirus (MERS-CoV) (146). In late 2019 and early 2020, a novel coronavirus was discovered to be the cause of a rapidly spreading outbreak of respiratory disease, including potentially fatal pneumonia, in Wuhan, China. The virus, provisionally designated 2019-nCoV and later given the official name SARS-CoV-2, owing to its similarity to SARS-CoV (then named SARS-CoV-1), was isolated and the viral genome sequenced. SARS-CoV-2 was characterized as a beta-coronavirus (147). The disease caused by the virus was officially named Coronavirus Disease 2019 (COVID-19) by WHO.

Coronaviruses are capable of adapting quickly to new hosts through the processes of genetic recombination and mutation *in vivo*. Point mutations alone are not sufficient to create a new virus. However, this may occur when the same host is simultaneously infected with two coronavirus strains, enabling recombination of genomic fragments of hundreds or thousands of base pairs long and thus making a new virus (148, 149). This susceptibility enabled the emergence, in approximately two decades, of three new human coronavirus species with epidemic potential: SARS-CoV-1, MERS-CoV, and SARS-CoV-2. Coronaviruses enter cells *via* binding to a host receptor followed by membrane fusion. The angiotensin-converting enzyme 2 (ACE2) was identified as the cell receptor for SARS-CoV (150), and recently also for the new SARS-CoV-2 (151), while MERS-CoV binds the dipeptidyl peptidase 4 (DPP4) receptor, also known as CD26 (152). The S protein is used for virus–cell receptor interaction during viral entry (153). Transmission of the virus during the viremic stage of disease is primarily *via* respiratory secretions (droplets) or direct contact. SARS-CoV-2 is extremely contagious, with an estimated basic reproduction number (R0) of 2.24–3.58 (154). In contrast, the R0 for both SARS-CoV-1 and MERS-CoV is less than 1 (155). It soon became apparent that infected individuals might be capable of transmitting the virus during the prodromal period (156).

Prevention

Social distancing strategies (quarantine and community containment) represent the only efficacious means of controlling coronavirus spread in the absence of effective drugs or vaccine

against the pathogens. Of importance, for preventing the spread of the disease caused by contact with patients or contaminated fomites, hygiene measures are also mandatory, such as washing hands with soap and water or with alcohol-based preparations. Indeed, coronaviruses are able to survive on various surfaces for few days but can be inactivated by disinfection (157). Finally, because it has been demonstrated that the overlap between human and animal ecosystems have given to coronaviruses the opportunity to cross the species barrier, to prevent future zoonotic diseases, a coordination with veterinary experts as well as stricter laws governing the trade of wild animals would be necessary.

Vaccines

Humans are extremely exposed to these pathogens because these viruses had not previously circulated in the human population, as testified by the absence of antibodies against coronavirus in healthy people. In addition, the innate immune response has demonstrated to be insufficient in controlling coronavirus infection because decreases in viral load are coincident with the specific antibody response (158, 159). In this context, vaccines represent a much expected resource. A hopeful premise is represented by the successful containment of coronavirus epidemics in farm animals by vaccines, based on either killed or attenuated virus (160), and concerning SARS-CoV-2 by the finding that specific antibodies are detectable in 100% of patients with COVID-19, 17–19 days after symptom onset (161), and that the magnitude of antibody titers positively correlated with viral neutralization potency (162).

After the SARS outbreak, several vaccines were formulated based on various strategies, as recombinant S protein-based vaccines, attenuated and whole inactivated vaccines, as well as vectored vaccines. Pre-clinical data showed animal protection from challenge with SARS-CoV-1. However, sterilizing immunity was not always achieved (163). In few cases, the use of live virus as a vaccine resulted in complication including lung damage, eosinophil infiltration, and liver damage in animal models. Moreover, a study of vaccination with inactivated SARS-CoV-1 in NHPs reported enhancement of disease caused by specific epitopes on the S protein [reviewed in (64)]. Another issue is related to the length of a protective immune response. Both humoral and cellular responses have been found important for lasting protection. In long-term studies of recovered SARS patients, antibody responses waned after approximately 6 years, while T-cell responses persisted, suggesting that the latter is required for long-lasting immunity.

Concerning MERS-CoV, the vaccines proposed target the S protein (164–166), including mucosal vaccine for intranasal administration (167). However, cases of enhanced lung diseases were also reported in preclinical models of vaccination in mice (168). New MERS-CoV vaccines in development also include live attenuated, protein subunit, and DNA vaccines (169, 170). Recently, a small animal model that replicates MERS-CoV transmission has been developed (170) and will help the pre-clinical studies.

Following the alarming data and casualties provoked by COVID-19, a strong effort by the research community is going on at the moment, and WHO has been informed of dozens of vaccines in preparation using different platforms, as mentioned in section "Vaccine Platforms." Some of these candidate vaccines are already in phase I/II clinical trials, while others have been advanced to phase III studies (63, 65, 74) (**Table 2**). However, it is possible that a SARS-CoV-2 vaccine will not be available for another 12–18 months. Recently, a rhesus macaque model that recapitulates SARS-CoV-2 infection has been developed to study immunopathogenesis and test vaccine candidates (73, 171).

Passive Immunotherapy

Therapy based on passive administration of anti-coronavirus antibodies, isolated from patient sera, also represents a much wanted option for the treatment of coronavirus diseases (172), and a global effort is pursued in this direction to treat patients before the achievement of a validated vaccine. In addition, researchers are trying to produce in laboratory specific and protective anti-coronavirus antibodies. In the case of SARS outbreak, a monoclonal antibody (MAb) with neutralizing activity, being able to block receptor association, was identified and described (173). Moreover, neutralizing MAbs have also been produced to fight MERS-CoV infection. In a collaborative study by US and Chinese researchers, MAbs targeting the receptor (CD26/DPP4) binding domain of MERS-CoV spike glycoprotein were reported (174). Japanese researchers have also investigated anti-CD26 MAb for MERS-CoV and have identified the humanized MAb YS110 as a promising candidate (175). Finally, in the case of SARS-CoV-2 outbreak, Dutch researchers claimed the identification of a human MAb named 47D11 able to block SARS-CoV-2 infection (176). Recently, a MAb able to cross-neutralize SARS-CoV-2 has been identified from memory B cells of a SARS-CoV-infected individual. The antibody, named S309, engages the S receptor-binding domain, recognizing a highly conserved protein/glycan epitope distinct from the receptor-binding motif (177). More recently, other potent neutralizing antibodies were isolated by different research institutions (178–180).

Amidst the gamut of high-affinity antibodies with the potential to neutralize human pathogenic viruses, single-domain antibodies, referred to as nanobodies or Nbs (15 kDa), and nanobody-based human heavy chain antibodies (75 kDa) derived from camelids might be harnessed as useful therapeutics for the ongoing COVID-19 pandemic (181). Camelid heavy-chain-only antibodies (HCAbs) are composed of two heavy chains with a single variable domain (VHH) as the target-binding module. Recombinant VHHs, devoid of the effector domains, act as single-domain antibodies and harbor advantageous features over conventional antibodies (higher thermal and chemical stability, higher solubility, smaller size, lower susceptibility to steric hindrances, ease of manufacturing, and simple structure) to have been recently proposed as prospective therapeutic candidates against various infectious pathogens (181). VHHs isolated from a llama subcutaneously immunized with perfusion-stabilized SARS-CoV-1 and MERS-CoV S proteins have been recently characterized and shown to be able to neutralize S pseudotyped viruses *in vitro*, interfering with the host cell receptor binding (182). Interestingly, SARS-CoV-1 S-directed

VHH cross-reacted with SARS-CoV-2 Receptor Binding Domain (RBD) and neutralized SARS-CoV-2 S pseudoviruses *in vitro* as a bivalent human IgG Fc-fusion format, underscoring the potential of VHHs to treat coronavirus diseases (182).

VACCINE HURDLES: FLAVIVIRUS CROSS-REACTIVITY AND ANTIBODY-DEPENDENT ENHANCEMENT

Flavivirus Cross-Reactivity

Because of the global spread of diseases caused by flaviviruses, understanding the cross-reactivity of anti-viral immunity among these viruses is of crucial importance for predicting the evolution of viral disease outbreaks.

Recently, the analysis of PBMCs isolated from individuals infected by DENV or vaccinated with DENV TV005 or YF17D vaccines, and pulsed with a pool of antigens from autologous and heterologous flaviviruses, indicated that both CD4 and CD8 T-cell responses were specific, with little or no cross-reactivity, despite the high level of homology (183). Individuals pre-exposed to DENV infection developed T-cell responses against non-structural ZIKA proteins rather than structural envelope protein, suggesting that previous flaviviral infections biased the T-cell response toward more cross-reactive non-structural epitopes (184). Studies enrolling mothers who gave birth to microcephalic babies after ZIKV infection, showed serological evidence of a pre-existing anti-Dengue response, suggesting that vaccination against DENV does not protect against ZIKV microcephaly (185). However, cross-reactive antibodies between ZIKV and DENV have been described, mainly targeting the structural dimeric envelope protein (186, 187). The antigenic sequences are both linear and quaternary, with NAbs mainly recognizing the latter. The high-conserved E protein fusion loop induces cross-reactive but weak NAbs that can be a marker of worst outcome during subsequent flaviviral infections (188). A research concerning ZIKV-specific B-cell responses in three DENV-experienced donors showed that 5 months after the infection, the pool of antibodies comprised both poorly NAbs derived from pre-existing DENV-induced memory B cells, associated with an enhanced ZIKV infection *in vitro*, and potent ZIKV-specific antibodies originated *de novo* (189, 190). The possibility that WNV-specific antibodies may drive the infection by other flaviviruses is still controversial, even if cross-reactivity was demonstrated. Plasma samples from convalescent human WNV patients were shown to enhance ZIKV infections by antibody-dependent enhancement (ADE) phenomenon (191); conversely, mice previously infected with ZIKV and challenged with WNV showed enhanced protection toward the second infection (192).

The immunological Flavivirus cross-reactivity, the ADE phenomenon (discussed below), genetic mutations that increase the virulence, potential pre-existing immunity concerns, combined with the necessity to increase cost-effectiveness of marketable products are among the issues that have limited the development of successful vaccines until now. The use of T-cell inducing vaccines or proteins with mutations into conserved Envelope fusion-loop epitopes might be useful to overcome the cross-reactivity hurdle (193).

ADE: Antibody-Dependent Enhancement

Known as ADE of viral infection, ADE is a phenomenon occurring when antibodies facilitate virus entry into the host cells, driving viral replication and increasing infectivity, with subsequent severe outcomes.

Among the several stumbling blocks in realizing a safe vaccine, ADE is a phenomenon largely underestimated, but that can produce severe adverse effects, rendering vaccinated individuals more predisposed to develop harsh symptoms after infection (194). The first report of ADE dates 1964 (195). The molecular mechanisms disclosed the involvement of FcγR (196) and complement receptors (197). When an antiviral antibody (induced by vaccination or viral infection) with no neutralizing or sub-neutralizing activity is produced, it can act like a bridge between the virus and the FcγR expressed on the surface of immune cells, leading to viral uptake (**Figure 3**), as demonstrated for DENV, ZIKV, WNV, Influenza, SARS-CoV, MERS-CoV, and EBOV (194). The role of complement receptor has been demonstrated in EBOV response: two antibodies directed against epitopes in close proximity bind the C1q, forming an immune complex able to enhance the virus entry into a target cell (198), whereas in an animal model of MERS-CoV, C3a and C9 protein level increase was observed after passive immunization (199). The first licensed vaccine against DENV (CYD-TDV-Dengvaxia) caused hospitalizations in two large multicenter phase III trials; after result revision, it has been estimated that in seronegative individuals, it can produce adverse effects (194), and WHO recommendations are to vaccinate only seropositive individuals in endemic areas of age older than 9 years. Using a mathematical model of DENV transmission to formulate hypothesis on vaccine trial results, it was speculated that "Seronegative recipients gain transient protective cross-reactive immunity akin to that observed for natural infection," increasing the risk of severe disease after infection, while vaccination of seropositive subjects results in boosting the immune response, producing a protection comparable with the one obtained in individuals who has had two natural infections (200).

The most severe adverse effect after vaccination was registered when a formalin-inactivated vaccine against RSV produced an increase of severe illness in vaccinated infant (hospitalization: 80% RSV vaccinated *vs.* 5% vaccinated against parainfluenza) and two deaths (201). Afterward, a role for the Th2 response was hypothesized in generating the RSV-mediated ADE (202), and it was demonstrated that the formalin-inactivated virus produced ADE in monkeys (203), suggesting that the carbonyl groups on formaldehyde-inactivated RSV were responsible for the Th2 response in mice (204). Moreover, the observation that formalin inactivation produced an alteration of antigens, leading to the production of non-NAbs, whose avidity did not mature, and the activation of complement were also reported for a measles vaccine (205). The low-avidity non-NAbs are produced in absence of TLR activation (and affinity maturation), and they

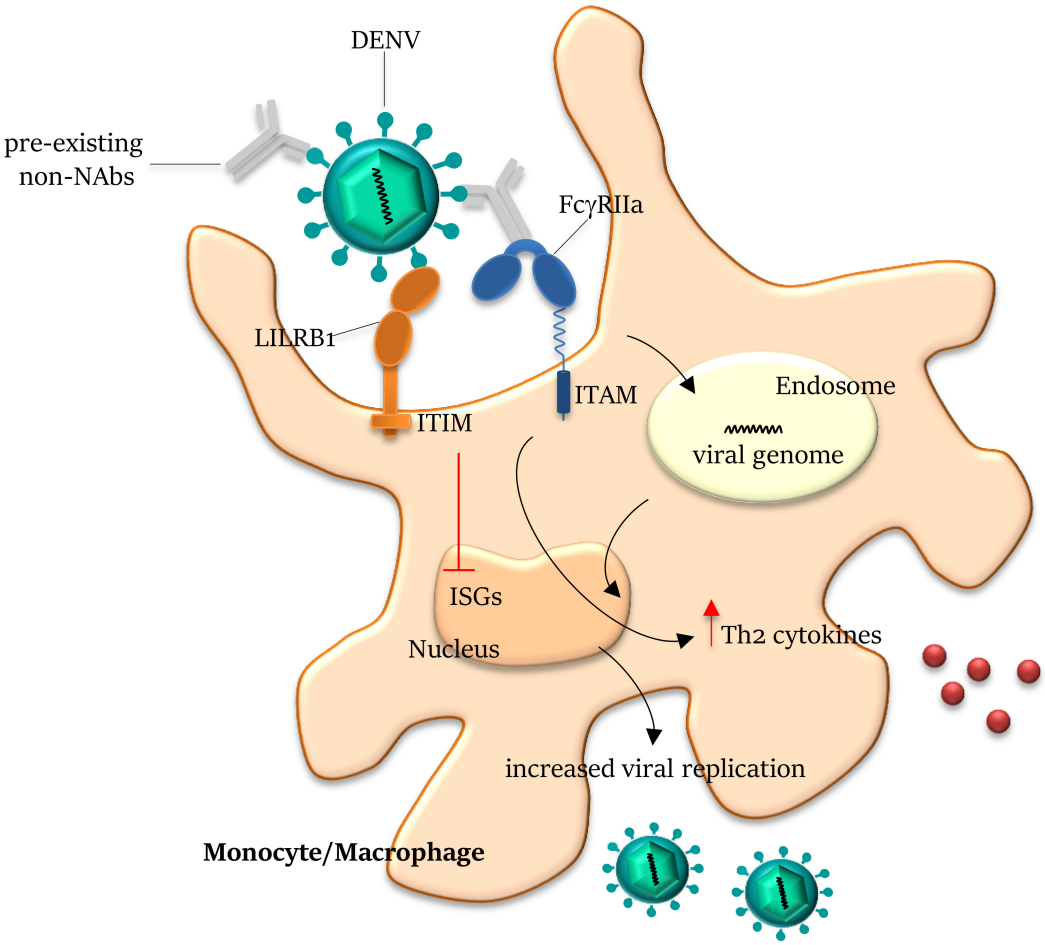

FIGURE 3 | Antibody-dependent enhancement on Dengue infection. Antibodies generated from a previous DENV infection can recognize but do not neutralize another DENV serotype and can lead to antibody-dependent enhancement (ADE) of entry of the latter virus into host cells. The pre-existing non- (or sub-) neutralizing antibodies bind DENV through the Fab domains and mediate viral entry into FcγR-expressing cells. On engagement by the Fc domains, the virus–antibody immune complex is internalized by the activating FcγRIIa within the endosome. Co-ligation of FcγRIIa and LILRB1 (leukocyte immunoglobulin-like receptor-B1) to opsonized DENV drives the inhibitory signal cascade *via* immunoreceptor tyrosine-based inhibition motif (ITIM) pathway, abrogating the expression of ISGs (Interferon Stimulated Genes). Ligation of FcγRIIa to immune complex also increases Th2 cytokine production and reduces IFNγ, inhibiting the JAK/STAT signaling pathway, overall resulting in the suppression of the antiviral response and increase of viral replication. NAbs, neutralizing antibodies; and ITAM, immunoreceptor tyrosine-based activation motif.

trigger complement activation (206), enhancing viral infection. To induce potent NAbs, the TLR activation has been obtained using a Th1-polarizing adjuvant (207), in association with the candidate vaccine exposing the epitopes of interest.

Antibody-dependent enhancement has been reported also in many studies focusing on the development of SARS and MERS vaccines, demonstrating that vaccination with the whole S glycoprotein can increase the susceptibility to viral infection with a mechanism not linked to the virus receptor expression on the host cells (208), and especially when antibodies are induced with low titer (209). While for many flaviviruses the mechanism of ADE has been explained through evidences that antibodies developed during a primary infection can enhance entry of a heterologous virus *via* Fc-receptor during a secondary infection, for MERS-CoV and SARS-CoV, it has also been proposed that NAbs that strongly bind the RBD region of the S surface protein can induce conformational changes that enhance the virus entry *via* canonical viral-receptor-dependent pathways, mimicking viral receptor binding (210, 211), and antibodies targeting a specific region of the S protein enhanced the viral infection in a SARS model of NHPs (212). The high sequence homology and the similarity in structure shared among SARS-CoV, MERS-CoV, and SARS-CoV2 S glycoproteins raises reasonable concerns about the development of COVID-19 vaccines based on the S protein.

OTHER OPTIONS FOR PANDEMIC CONTAINMENT

In potential pandemic settings, the clinical development of vaccines is the main aim. However, apart from technical reasons,

the vaccine production might be delayed also for economic considerations and safety issues. Other strategies may be based on self-disseminating vaccines and induction of trained immunity.

To control zoonosis, the formulation of self-disseminating vaccines acts at the level of animal, insect, or environmental reservoir, to directly interfere within the animal-to-human transmission (213). They are essentially based on replicating viral vectors engineered to express the disease antigen and to target a certain animal population (214). Global vaccination of animals could be achieved to effectively contain an emerging pathogen within the wildlife reservoir, avoiding its global spread. Feasibility concerns, costs, and safety issues should be considered when using this strategy to control reservoirs linked to the emergence of high-risk pathogens. In addition, which animal pathogen will cause a human disease is generally unpredictable. It is interesting to underline that a vaccination of great apes with an engineered specific CMV-based vector has been proposed as a strategy to potentially interrupt (or at least decrease) the zoonotic transmission of Ebola virus to humans, being able to protect animals from the lethal viral challenge (213, 215).

Trained immunity-based vaccines (TIbV) might be formulated to stimulate broader anti-infectious responses compared with conventional vaccines for their capacity to increase innate immunity and enhance adaptive responses (216). This strategy exploits the ability of innate immune cells (monocytes, macrophages, NK cells) to undergo extensive metabolic and epigenetic reprogramming, following certain vaccinations or infections, and to become primed for a quite long period of time to respond more potently to autologous or heterologous re-infection, mounting the so-called "innate immune memory." Triggering of pattern recognition receptors (PRRs) by microbial effector stimuli results in increased production of pro-inflammatory cytokines and/or reactive oxygen species, and in enhanced immune responses, regardless the primary stimulation (217).

Many infectious stimuli are considered potent activators of trained innate immunity, including β-glucan and chitin (components of fungal cell wall), LPS (a component of the cell wall of Gram-negative bacteria), and the Bacille Calmette-Guérin (BCG) vaccine (218). Thus, TIbV should contain pathogen-associated molecular patterns (PAMPs) to target PRRs and subsequently induce trained immune cells. BCG vaccine, VACV, and live attenuated influenza vaccines, together with immunostimulants, could be ascribed to this category of vaccines (216). It is worth mentioning that a whole-cell killed bacterial vaccine might have played a role in preventing pneumonia and mortality during the 1918 Influenza pandemic (219). Recently, a work by Berg and colleagues showed that BCG vaccination is associated with the flattening of the curve in the spread of COVID-19, suggesting that BCV vaccine might serve as a protective factor against the disease (220). However, it should also be noted that an enhanced immune response mediated by reprogrammed immune cells could contribute to the development or maintenance of inflammatory, neuroinflammatory, and chronic metabolic disorders (221). The phenomenon of "trained immunity" occurring in the brain is known as microglial priming. Exposure of primed microglial cells to a second stimuli can cause an augmented inflammatory response, leading to neuroinflammation and production of neurotoxic molecules. The hyperglycemia condition that characterizes type 2 diabetes could long term affect the cellular metabolism of monocytes and macrophages, leading to increased cytokine production and subsequent diabetes complications, including atherosclerosis. An augmented activation of innate cells may also result in the induction and maintenance of chronic inflammatory disorders, including rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, or sarcoidosis (221).

CONCLUSION

The COVID-19 pandemic experience, combined with the previous viral disease outbreaks, should give blueprints for rapidly responding to the emergence of high-risk pathogens in the future.

It is a common belief that vaccines would be the only means of providing long-term immunity and preventing viral diseases. Despite the great progress made in vaccine research, we are still unable to produce successful vaccines in a timely manner. Human trials take a long time and given a huge list of vaccine candidates, it is hard to choose the most promising one. While the WHO proposed a Solidarity Vaccine trial to test all the candidates in rolling trial until they fail, to increase the chances of succeeding, some vaccine stakeholders are considering extreme alternatives for emergency use: intentionally infect young healthy volunteers at low risk in controlled "human challenge trials" to define which vaccine will work (222). Although these approaches are already used for studying Influenza (223) and Dengue diseases (224), it is hard to ethically accept this option without a validated therapy. Vaccines go through regulatory pathways before the final approval and licensure. In epidemic or pandemic settings, we need to carefully develop a vaccine, as quickly as possible, that adequately proved to be safe and effective (225).

Scientists need to fill the gaps in understanding the epidemiology of novel viruses, to identify potential zoonotic reservoirs or spill-over hosts, and the way of transmission of pathogens. Once the pathogen is identified, preclinical models need to be developed to study virus–host interactions and early test vaccine candidates, defining the immune correlates of protection. Pathogen-specific epitopes need to be identified to guide structure-based vaccines that will elicit protective antibodies, minimizing the induction of non- or weakly NAbs that would promote ADE of viral infection (226). Moreover, data sharing and collaboration among academia, government, and companies will be essential to coordinate a strategic approach in face of next pandemic threats (227).

AUTHOR CONTRIBUTIONS

All authors equally contributed to this work and read and approved the final manuscript.

FUNDING

This work was supported by PRIN 2017 "NanoTechVax Tackling biological barriers to antigen delivery by nanotechnological vaccines" Prot. 20173ZECCM, and Consiglio Nazionale delle Ricerche (CNR), Italy: Laboratori Congiunti Bilaterali Internazionali (Scienze Biomediche), Project: "New vaccines against poverty-related and neglected tropical diseases." MT was supported by postdoctoral fellowship from CNR, Italy: Laboratori Congiunti Bilaterali Internazionali (Scienze Biomediche), Project: "New vaccines against poverty-related and neglected tropical diseases."

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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.

frontiers in Immunology

ORIGINAL RESEARCH
published: 25 September 2020
doi: 10.3389/fimmu.2020.585133

Successive Immunization With Epitope-Decreasing Dengue Antigens Induced Conservative Anti-Dengue Immune Responses

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Repeated homologous antigen immunization has been hypothesized to hinder antibody diversification, whereas sequential immunization with heterologous immunogens can educate B cell differentiations towards conserved residues thereby facilitating the generation of cross-reactive immunity. In this study, we developed a sequential vaccination strategy that utilized epitope-decreasing antigens to reinforce the cross-reactivity of T and B cell immune responses against all four serotypes dengue virus. The epitope-decreasing immunization was implemented by sequentially inoculating mice with antigens of decreasing domain complexity that first immunized with DENV1 live-attenuated virus, following by the Envelope protein (Env), and then Env domain III (EDIII) subunit protein. When compared to mice immunized with DENV1 live-attenuated virus three times, epitope-decreasing immunization induced higher TNF-α $CD8^+$ T cell immune response against consensus epitopes. Epitope-decreasing immunization also significantly improved neutralizing antibody response to heterologous serotypes. Moreover, this sequential approach promoted somatic hypermutations in the immunoglobulin gene of antigen-specific memory B cells in comparison to repeated immunization. This proof-of-concept work on epitope-decreasing sequential vaccination sheds light on how successively exposing the immune system to decreasing-epitope antigens can better induce cross-reactive antibodies.

Keywords: dengue virus, vaccine development, sequential immunization, immunoglobulin diversification, epitope-decreasing

OPEN ACCESS

Edited by:
Neeltje van Doremalen, Rocky Mountain Laboratories (NIAID), United States

Reviewed by:
Srijayaprakash Babu Uppada, University of Nebraska Medical Center, United States
Marc Paul Girard, Université Paris Diderot, France

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Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: *19 July 2020*
Accepted: *16 September 2020*
Published: *25 September 2020*

Citation:
Hou J, Ye W, Loo HL, Wong LH and Chen J (2020) Successive Immunization With Epitope-Decreasing Dengue Antigens Induced Conservative Anti-Dengue Immune Responses. Front. Immunol. 11:585133. doi: 10.3389/fimmu.2020.585133

INTRODUCTION

A major challenge in dengue vaccine development is to induce robust and protective cross-reactive immunity against all four serotypes of dengue viruses (DENV). DENV 1–4 are antigenically distinct, but closely related viruses (1, 2) with up to 70% sequence homology (3, 4). In endemic countries, co-circulation of multiple serotypes of DENV is prevalent, and therefore, chances of getting either co-infection with multiple serotypes or sequential encounter with heterotypic dengue viruses is a common phenomenon (5).

The current licensed DENV vaccine, Dengvaxia, is a tetravalent vaccine composed of four DENV serotypes. However, the vaccine has limited overall efficacy (approx. 60%) against acute dengue, with only 50% and 35–42% for DENV1 and DENV2, respectively (6–8). Besides antigen selection, optimization and vaccine types in vaccine development, the immunization regimen is also critical. Traditionally, vaccination regimens employ multiple inoculations of immunogens, such as inactivated virus and cocktails of antigens. However, such regimens might not be optimal. Conserved epitopes may be masked by highly variable regions (9, 10), and the presence of these more accessible and non-immunogenic epitopes can "distract" B cell responses (11). Indeed, in a monkey DENV vaccination model, interference between DENV serotypes and immunodominance of certain epitopes led to dominance of neutralizing antibody titers against DENV4 (12). Instead, sequential immunization with a series of directional immunogens with decreasing epitope modifications have been shown to elicit heterologous neutralizing responses against HIV-1 (13). Additionally, our recent publication clearly demonstrated sequential immunization induced stronger and broader T and B immunity against four DENV serotypes than tetravalent-formulated immunization (14). The underlying mechanism of this strategy relies on directing B cell education. The successive boosting with epitopes of decreasing complexity forced B cells to rearrange the immunoglobin and promoted antibody avidity to recognize the conserved epitopes through somatic hypermutations.

One concern for sequential vaccination in DENV vaccine development is the phenomenon of antibody-dependent enhancement (ADE) (15–17). The ADE hypothesis suggests that pre-existing antibodies generated in response to a primary infection may have insufficient antibody avidity or concentration to neutralize secondary infection by a different dengue serotype. During secondary dengue infection, such weakly neutralizing antibodies may promote the infection of Fc receptor-bearing cells leading to virus amplification, cytokine storm and subsequent plasma leakage (18). The occurrence of ADE thus raises safety concerns about whether incomplete protection against all four dengue serotypes prior to complete vaccination can increase disease severity (19). The results from a phase 2b trial of CYD tetravalent dengue vaccine in Thai schoolchildren requiring multiple vaccination doses showed no increased risk for severe diseases during the course of the vaccinations (6). Consistently, clinical trials on monovalent chimeric dengue vaccine (20) and bivalent CYD dengue vaccine (21) both showed a lack of adverse events and viremia after heterotypic dengue vaccine inoculation. These findings demonstrated the safety and feasibility of sequential vaccination.

In this study, we examine whether introducing a series of directional DENV immunogens with decreasing epitope modifications sequentially can improve both T cell and B cell immune responses against four DENV serotypes in mice model. Our results show that epitope-decreasing vaccination potentially induces higher cellular immune responses targeting conserved epitopes compared to repeated immunization with a priming immunogen. We further study the immunoglobulin diversification in antigen-specific B cells after each immunization to understand the evolutionary dynamics in different immunization approaches.

MATERIALS AND METHODS

Mice and Immunization Regimens

C57BL/6J (B6) mice were used for the experiment. Mice were bred and housed at the Animal Facility, National University of Singapore (NUS). All procedures and care were approved by the NUS Research Ethics Committee under Protocol R13-6157. All ethical regulations regarding animal research were complied with.

Table 1 depicts the immunization schedules of two vaccination strategies. For repeated immunization, 1×10^6 PFU Dengue 1 live virus (strain 2402DK1) (DENV1) in 50 µl volume was used per injection, repeated three times. For epitope-decreasing immunization, 1×10^6 PFU DENV1/50 µl, 10 µg/50 µl DENV1 extracellular domain Envelope protein (Env) (CTK Biotech), and 10 µg/50 µl DENV1 Env protein domain III (EDIII, in-house production) were administered at the first, second, and third dose, respectively. Two groups of 8-week old female B6 mice (5 mice per group) were immunized 3 times intramuscularly with 2 weeks apart each dose under general anesthesia. Two weeks after the final dose, the mice were sacrificed for terminal analysis.

Intracellular Cytokine Staining

Splenocytes from immunized mice was assessed for cytokine production by intracellular cytokine staining as described previously (22). Briefly, 1 million splenocytes were stimulated with a peptide cocktail (23) or each serotype virus (DENV1/2402DK1, DENV2/3295DK1, DENV3/863DK1 and DENV4/2240DK1). Cells were surface stained with anti-CD4 and -CD8 et al. primary antibodies followed by intracellular staining with anti-TNFα monoclonal antibodies. Data were acquired on LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo (version 10.6.0 Tree Star).

Dengue Plaque Reduction Neutralization Test (PRNT)

Neutralizing antibody titer (nAb) was determined by PRNT as previously described (24) on four strains DENV1/2402DK1, DENV2/3295DK1, DENV3/863DK1 and DENV4/2240DK1. The highest serum dilution that resulted in 50% or more plaques reduction compared to the virus control wells was considered as the neutralizing endpoint titer ($PRNT_{50}$).

EDIII-Specific Binding Antibody ELISA Assay

Ninety-six-well plates were coated with 1µg/ml in-house produced recombinant EDIII protein and kept at 4°C

TABLE 1 | Immunization schemes.

Group	Immunization	1st shot	2nd shot	3rd shot
1	Repeated	DENV1	DENV1	DENV1
2	Epitope-decreased	DENV1	DENV1/Env	DENV1/EDIII

overnight. The plates were washed 5 times with PBST (0.05% Tween 20) and blocked with 5% BSA at 4°C overnight. After washing, serum samples were added to plates in dilution from 1:200 to 1:25,600 and incubated for 2 h in 37°C. Secondary HRP-labeled anti-mouse IgG diluted to 1:5000 was added to plates and incubated for 1 h at 37°C. TMB substrate was added and the absorbance was read at 450nm. The cut-off threshold was set at least two times higher than the result of negative sera sample. The titer was determined by the last dilution giving value above the cut-off threshold.

B Cell Assays

The antigen-specific B cell responses were probed by fluorochromes labeled DENV1 and DENV2 E proteins as previously described (24). Cell were analyzed on an X20 flow cytometer (BD Biosciences) and data processed using FlowJo version 10.6.0 (Tree Star).

Antigen-Specific Immunoglobulin Repertoire Sequencing by RNA-Seq

A total of 10,000 DENV-specific B cells (either $DENV1^+$ or $DENV2^+$ or $DENV1^+DENV2^+$) were sorted on FACS Aria II cell sorter (BD Biosciences). The RNA extraction, cDNA synthesis and target gene amplification, sequencing library preparation as described previously (14). The libraries were multiplexed and subjected to MiSeq V3 2×301 bp sequencing.

Raw sequences were processed using the toolkit "pRESTO" (version 0.5.13) (25). Briefly, the paired-ends MiSeq data was firstly assembled into a full-length B cell receptor (BCR) sequences, followed by removing the low-quality reads, annotating Ig isotype, masking the primer regions and yielding the final sequences comprised of unique sequence with at least two representative reads. The IMGT/High database of mouse immunoglobulin repertoire was used as reference to perform V (D)J alignment using IgBLAST in tool "Change-O" (version 0.4.6) (26). The V segment genotypes were inferred using package "TIgGER" (version 0.2.10) (26). Ig sequences were assigned into clonally related lineages and the full germline sequences were built after preforming automated detection of the clonal assignment threshold by using package "SHazaM" (version 0.2.1) (26). Mutations were defined as nucleotides that were different from the inferred germline sequence. The clonal diversity of the repertoire was analyzed using the general form of the diversity index, as proposed by Hill (27) and implemented in the package "Alakazam" (version 0.3.0) (26). The somatic hypermutation targeting models were computed by the SHazaM software (version 0.2.1) (26). The raw data has been deposited in Gene Expression Omnibus (GSE154371).

Statistical Analysis

The statistical analysis of T and B cell responses and nAb titer were performed using two-sided Mann-Whitney test in GraphPad Prism 7.0 software (GraphPad Software Inc.). The statistical comparisons between strategies at indicated doses on Ig repertories mutation frequency were calculated using unpaired two-sided Wilcoxon test in R.

RESULTS

Epitope-Decreasing Sequential Immunization Induced Potent T Cell Response to Conserved Epitopes

We compared the T cell immune responses between mice that were immunized with three doses of live DENV1 virus (repeated immunization) and mice that were sequentially immunized with live DENV1 virus, DENV1 Env protein and DENV1 EDIII subunit protein (epitope-decreasing immunization). Two weeks following the last immunization, splenocytes were harvested and stimulated with a mixture of either consensus DENV peptides or DENV1-4 and analyzed $CD8^+$ T cells for TNFα production. In contrast to repeated immunization, epitope-decreasing immunization induced higher level of both homotypic (DENV1) and heterotypic (DENV3/DENV4) specific TNFα-producing $CD8^+$ T cells (**Figure 1A**). Importantly, compared with repeated immunization, epitope-decreasing immunization reinforced antigen-specific $CD8^+$ T cells responding to consensus DENV peptides stimulation (**Figure 1A**). This indicated that epitope-decreasing immunization potentially narrowed down T cell response to specific and conservative epitopes.

Next, we compared two groups on the neutralizing antibody (nAb) titer and anti-EDIII binding antibody titer in serum samples of immunized mice after 3 doses. As expected, both immunization regimens elicited strong homologous nAb responses against DENV1. Heterotypic nAb responses against DENV2, DENV3, and DENV4 were also observed, although at lower titers compared to anti-DENV1 (**Figure 1B**). Notably, epitope-decreasing immunization significantly boosted anti-DENV2 nAb response to near 2 times than the titer obtained from repeated immunization. Moreover, binding ELISA assay demonstrated epitope-decreasing immunization induced higher IgG titer against the conserved EDIII domain compared to repeated immunization (**Figure 1C**) with near significance.

To further investigate and quantify homo- and heterotypic antigen-specific B cell responses following two immunizations, we utilized DENV1/E-AF647 and DENV2/E-AF548 to stain for DENV1 and DENV2-specific B cells in the spleen two weeks after the last immunization dose. Both repeated immunization and epitope-decreasing immunization induced comparable levels of homotypic $DENV1^+$ or heterotypic $DENV2^+$ single positive, and cross-reactive ($DENV1^+$ and $DENV2^+$ double-positive) B cells (**Figure 1D**).

Cumulatively, these results suggest that epitope-decreasing immunization strategy is beneficial for inducing T cell responses and promoting nAb responses that target conserved immunodominant regions, such as the EDIII domain.

Epitope-Decreasing Sequential Immunization Promoted Immunoglobulin Mutation Frequency

To compare the diversity of immunoglobulin (Ig) repertoires between repeated and epitope-decreasing immunizations, we performed Ig-RNA sequencing on sorted $DENV1^+$ and/or

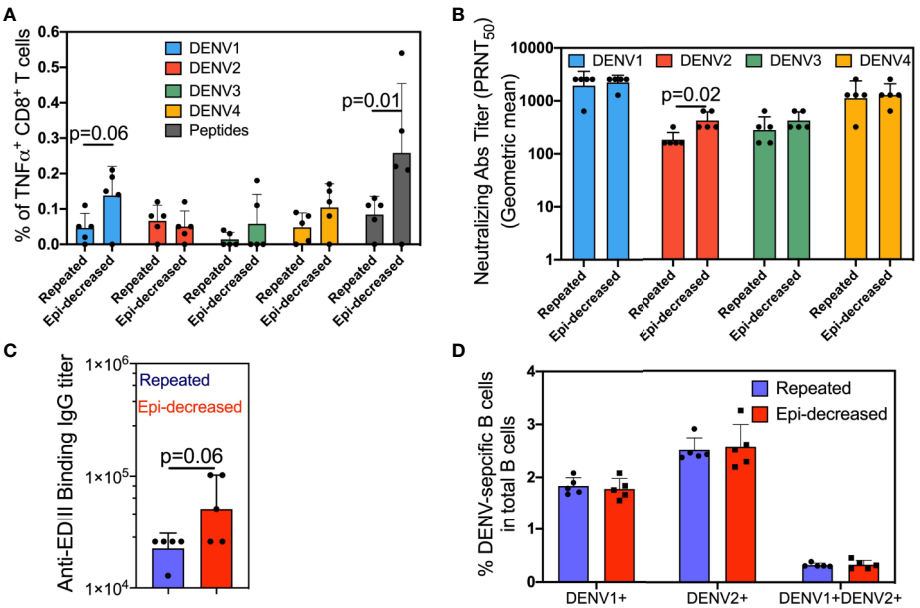

FIGURE 1 | The DENV-specific cellular and humoral immune responses. **(A)** Splenocytes were stimulated with each of the four DENV (DENV1/2402DK1, DENV2/3295DK1, DENV3/863DK1 and DENV4/2240DK1, at M.O.I = 1) or consensus envelope and capsid peptide pool (at a final concentration 5 μg/ml per peptide) or medium as control for 6 h at 37°C in the presence of BFA. Cells were surface stained for CD3 and CD8, and intracellularly stained for TNFα. The plots show the percentage of TNFα producing $CD8^+$ T cells. Each dot represents one mouse. The bar plots show the mean value and SD, and the colors indicate antigens used for stimulation. The p value denotes the comparison results between the indicated comparisons were calculated by Mann-Whitney test. **(B)** The neutralization antibody titers two weeks after the 3rd immunization were measured by plaque reduction neutralization test (PRNT) assay. Four serotypes of dengue virus were separately incubated with serially diluted sera to measure the neutralization capability of reactive antibodies. The serotype specific neutralizing antibodies were determined by 50% plaques reduction compared to the virus control wells. The data shows as geometric mean titers ± geometric SD in the bar plot. Each dot represents one mouse. The p values between the indicated comparisons were calculated by Mann-Whitney test. **(C)** Anti-DENV Env EDIII Ig binding Ab titers in the sera after the 3rd immunization specific were measured by ELISA. The data shown are geometric mean titers ± geometric SD. Each dot represents one mouse. The p value shows the Mann-Whitney comparison result between two groups. **(D)** Two weeks after last immunization, splenocytes were stained with Alexa conjugated DENV1 and DENV2 E proteins and appropriate antibodies. The antigen specific $DENV1^+$, $DENV2^+$ and $DENV1^+DENV2^+$ B cells were assessed. The bar plots show the mean value and SD. Each dot represents one mouse. The p values were calculated by Mann-Whitney test.

TABLE 2 | The summary of RNA-seq results.

Group	Original Sequences	Assembled Sequences	Filtered Sequences	IgBlastClones	Final Repertoire
Repeated	2,926,869	2,764,584	123,356	30,758	28,134
Epi-decreasing	2,705,628	2,557,028	121,402	30,270	27,133

$DENV2^+$ B cells. The sequencing details are described in **Table 2**. As DENV-specific B cells differentiated from IgM^+/IgD^+ to IgG^+, the Ig heavy chain variable gene usage decreased. The IgG^+ B cells predominantly used immunoglobulin heavy chain variable region genes (IGHV) 1, 3, 5, 7, and 14. Distinctively, IGHV13 (specifically, IGHV13-2) was only induced in epitope-decreasing immunization strategy. Based on the clone frequencies, the IGHV3-1 usage in IgG isotype was more prominent following epitope-decreasing immunization. IGHV3-6 usage was elevated in $DENV^+$ B cells that expressed either IgD or IgM within epitope-decreasing immunization strategy (**Figure 2A**). Interestingly, the Ig diversification induced by each immunization was comparable (**Figure 2B**).

Somatic hypermutations (SHM) analysis revealed that repeated immunization boosted IgG WRC/GYW hot spot mutation, whereas epitope-decreasing immunization promoted IgG SYC/GRS cold spot mutation. Additionally, epitope-decreasing sequential immunization induced higher WRC/GYW and WA/TW hot spots mutation frequencies than repeated immunization in IgM isotype (**Figure 2C**).

Together, these results suggest that epitope-decreasing immunization, through antigen-driven progression, reinforces some mutations through somatic hypermutations to generate high specificity and affinity antibody that recognize conserved domains.

DISCUSSION

A comprehensive understanding of how vaccine elicits protective and broadly cross-reactive immune responses is critical when handling pathogens that deceive and escape the immune memory by continually changing their antigenic characteristics.

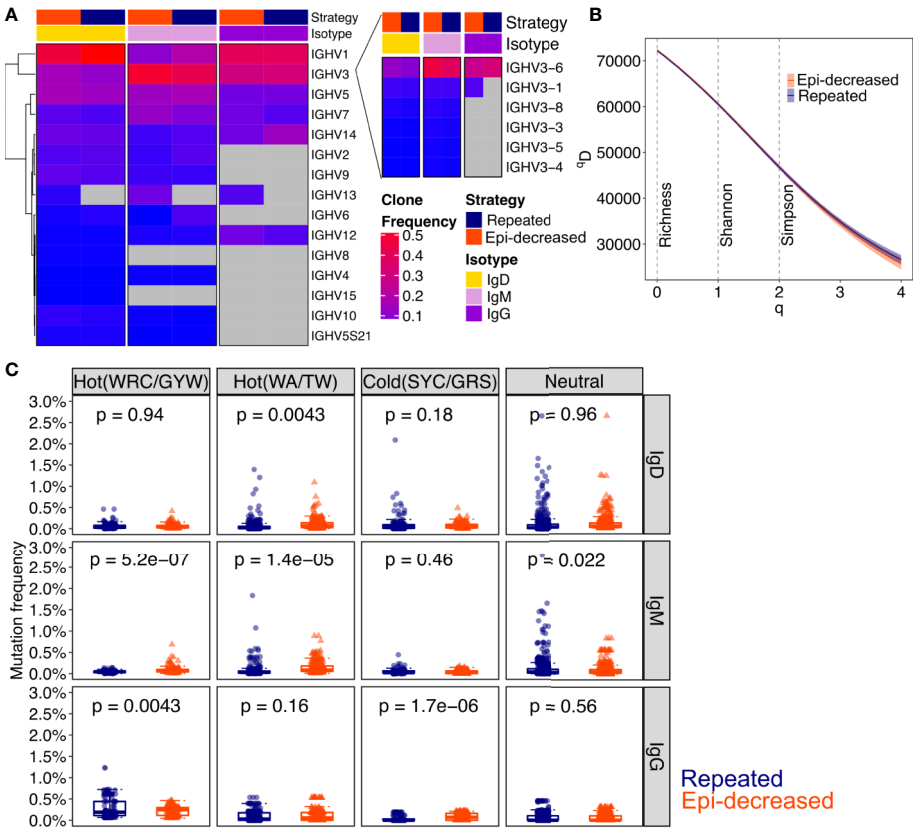

FIGURE 2 | The characteristic of immunoglobulin and somatic hypermutation induced by different strategies. **(A)** Heatmap shows the V_H family usages by clone frequencies with red to blue to grey corresponding to from high, low to absence. Strategy indicates the immunization and isotype indicates IgD, IgM and IgG. The Chi-square test was performed for statistical analysis. **(B)** The clonal diversity analysis was performed by using the generalized Hill's diversity index. The diversity index (^qD) was calculated over a range of diversity orders (q) and plotted as a smooth curve. The ^qD values depict the level of diversity for a given value of q. The lower ^qD values represent lower diversity. Shaded area represents 95% percentiles. The Richness diversity index, which equates to q = 0, the Shannon diversity index, q = 1, and the Simpson diversity index, q = 2, were plotted as dashed vertical lines. **(C)** The bar plots for the levels of somatic hypermutation (SMH) in hot- and cold spots with Ig isotypes. SMH targeting profiles were analyzed for 5-mer motifs from both immunization strategies. The WRC/GYW hotspot motifs, WA/TW hotspot motifs, SYC/GRS cold spot motifs and neutral spots are shown. Each dot represents a 5-mer motif and each box covers the 25th – 75th percentiles of the mutability rates of the 5-mer motifs in its corresponding groups, with the horizontal bar indicating the median. The p values show the statistical significance by Wilcoxon test analysis for indicated groups.

Here, we investigate the effect of epitope-decreasing sequential immunization strategy on the generation of cross-reactive responses against DENV1-4. Epitope-decreasing immunization can be considered as a form of supervised learning, which guides the immune response to focus on conserved domains.

As neutralization of DENV1 is generally weaker compared to other dengue serotype, we investigated the T cell and B cell responses induced by repeated live DENV1 virus vaccination compared to sequential vaccination with DENV1 live virus, followed by DENV1 Env protein and finally by DENV1 EDIII subunit protein in mice model. In agreement with previous reports, as the complexity of the immunogens decreased, the immune responses elicited were guided toward immunodominant targets (13). In this study we show that epitope-decreasing immunization can reinforce specific T cell immune responses on consensus epitopes. Additionally, it also induces heterotypic humoral immunity as shown by nAb capable of cross-neutralizing DENV2 and DENV3. This presumptively is due to antibodies generated through epitope-decreasing immunization having greater binding capacity for EDIII domains.

The immunoglobulin repertoire sequencing results revealed that successive boost with epitope-decreased antigens generated similar Ig diversity as repeated immunization strategy. This suggest that repeated homologous immunization does not effectively increase Ig diversity as not all exposed surfaces of the DENV1 virus are antigenic epitopes. Consequently, epitope-decreased sequential immunization not only did not lead to a loss of Ig diversity, but also help to navigate T and B cells to focus on the conservative epitopes.

The sequential immunization approach presumptively can educate the memory cell to recognize homological domains that have a high probability of harboring conserved epitopes (11). Through stepwise boosts with antigens of decreasing epitope

complexity, shared domains are emphasized thereby allowing the development of cross-reactivity. Sequential immunization serves to train the memory immune response to concentrate on "familiar" domains that already existed in the memory subset, thus directing antibody evolution. On the other hand, the consistent immunogens used in repeated immunization may burden the naïve or memory cells due to antigenic variation and frustrate memory maturation impeding cross-reactive Ab formation. In a realistic way, the principle of sequential immunization generally aligns with the reality for individuals living in dengue endemic areas, whose immune responses may become protective after multiple heterotypic exposures. Moreover, through this specific epitope decreasing approach, we were able to find a similar affect based on the use of sequential immunization but avoid the potential side effect of vaccine-induced ADE, which will pave the way for a safe and effective use of the vaccine and to combat the virus.

Finally, this study sheds light on how we can manipulate the immune system and supervise it to focus immunity on specific conserved domains to achieve the goal of broad cross-reactivity.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by National University of Singapore (NUS) Research Ethics Committee under Protocol R13-6157.

AUTHOR CONTRIBUTIONS

JH and JC designed this study and drafted the manuscript. WY drafted and revised the manuscript. JH, HL and LW conducted all assays.

FUNDING

This work was supported by the National Research Foundation of Singapore through the Singapore–MIT Alliance for Research and Technology's (SMART) Interdisciplinary Research Group in Infectious Disease Research Program.

ACKNOWLEDGMENTS

We thank Hwee Cheng Tan for preparing the dengue viruses, and Farzad Olfat for administrative support.

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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.

frontiers in Immunology

ORIGINAL RESEARCH
published: 29 September 2020
doi: 10.3389/fimmu.2020.564627

Chimeric Virus-Like Particles and Capsomeres Induce Similar CD8$^+$ T Cell Responses but Differ in Capacity to Induce CD4$^+$ T Cell Responses and Antibody Responses

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Despite extensive research, the development of an effective malaria vaccine remains elusive. The induction of robust and sustained T cell and antibody response by vaccination is an urgent unmet need. Chimeric virus-like particles (VLPs) are a promising vaccine platform. VLPs are composed of multiple subunit capsomeres which can be rapidly produced in a cost-effective manner, but the ability of capsomeres to induce antigen-specific cellular immune responses has not been thoroughly investigated. Accordingly, we have compared chimeric VLPs and their sub-unit capsomeres for capacity to induce CD8$^+$ and CD4$^+$ T cell and antibody responses. We produced chimeric murine polyomavirus VLPs and capsomeres each incorporating defined CD8$^+$ T cell, CD4$^+$ T cell or B cell repeat epitopes derived from *Plasmodium yoelii* CSP. VLPs and capsomeres were evaluated using both homologous or heterologous DNA prime/boost immunization regimens for T cell and antibody immunogenicity. Chimeric VLP and capsomere vaccine platforms induced robust CD8$^+$ T cell responses at similar levels which was enhanced by a heterologous DNA prime. The capsomere platform was, however, more efficient at inducing CD4$^+$ T cell responses and less efficient at inducing antigen-specific antibody responses. Our data suggest that capsomeres, which have significant manufacturing advantages over VLPs, should be considered for diseases where a T cell response is the desired outcome.

Keywords: malaria, vaccine, T cells, virus-like particle, capsomere, murine polyomavirus, chimeric, *Plasmodium yoelii*

OPEN ACCESS

Edited by:
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University of Oxford, United Kingdom

Reviewed by:
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Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: *22 May 2020*
Accepted: *25 August 2020*
Published: *29 September 2020*

Citation:
Pattinson DJ, Apte SH, Wibowo N, Rivera-Hernandez T, Groves PL, Middelberg APJ and Doolan DL (2020) Chimeric Virus-Like Particles and Capsomeres Induce Similar CD8$^+$ T Cell Responses but Differ in Capacity to Induce CD4$^+$ T Cell Responses and Antibody Responses. Front. Immunol. 11:564627. doi: 10.3389/fimmu.2020.564627

INTRODUCTION

The annual mortality rate of malaria is currently estimated at 405,000 people of whom 67% are children under 5 years of age (1). Eliminating the causative *Plasmodium* spp. parasite will likely require an effective vaccine (2) but conventional sub-unit vaccines strategies have thus far proved to be suboptimal. The identification and development of vaccine delivery platforms which induce long-lasting robust cellular and antibody immune responses is a global health priority. A specific goal is a vaccine against the pre-erythrocytic (sporozoite/liver) stage of *Plasmodium* sporozoites which would prevent both the clinical symptoms which develop during the blood stage, and the transmission of the diseases which occurs during the sexual stage.

The most advanced malaria vaccine candidate, RTS,S (also known as Mosquirix™), is a virus-like particle (VLP) comprising multiple copies of the *P. falciparum* circumsporozoite protein (*Pf* CSP) B cell repeats and some $CD4^+$ and $CD8^+$ T cell epitopes fused with recombinant hepatitis B surface antigen (RTS) and co-expressed with free hepatitis B surface antigen (S) (3), co-administered with AS01 adjuvant (4). However, although early clinical studies showed some protective efficacy in the first year after vaccination, it is now established that RTS,S induced protection is low and wanes quickly (5, 6). Significant research efforts have been directed at either improving the vaccine platform, or incorporating additional antigens to broaden the protective immune response. Additionally, ease and cost of manufacturing is an important consideration.

VLPs are formed when recombinant viral structural proteins assemble into a highly repetitive array which resembles the native form of the virus. These units contain no genomic material so are incapable of replication, but they are highly immunogenic and particularly good at inducing antibody responses without the need for additional adjuvants (7–9). VLP vaccines that have been licensed include hepatitis B virus and human papillomavirus where protection is mediated through neutralizing antibodies (10, 11). In those VLPs, the entire structural protein comes from the pathogen itself and their structure resembles the cognate native virus targeted by the vaccine. However, instead, a generic VLP independent of the pathogen target can be used to produce a chimeric VLP with antigenic epitopes inserted into regions of a flexible carrier virus, thereby making a vaccine platform which can theoretically target any organism [reviewed in (12–14)]. Chimeric VLPs have been shown to be effective at inducing robust antibody responses (12–14), but their efficacy in generating cellular responses has not been comprehensively investigated. *In vitro* studies have shown that chimeric hamster polyomavirus and SV40 VLPs incorporating T cell epitopes from mucin 1 and influenza, respectively, can induce activation of epitope-specific $CD8^+$ T cells (15, 16). Further evidence of $CD8^+$ T cell induction was reported using hamster polyomavirus in a mouse study which demonstrated *in vivo* clonal proliferation of transferred epitope-specific $CD8^+$ T cells, tumor growth inhibition and protection from lymphocytic choriomeningitis virus (17). We recently reported that immunization of mice with murine polyomavirus incorporating defined T cell epitopes derived from the *P. yoelii* CSP antigen (*Py*CSP) induced robust $CD8^+$ T cell responses as well as high antibody titres, but poor $CD4^+$ T cell responses (18).

Murine polyomavirus VLPs are generated when VP1 structural proteins form pentameric capsomeres (19) which can then be chemically induced *in vitro* to self-assemble into a highly repetitive VLP containing 72 capsomeres (7, 9, 20). The crystal structure of the VP1 protein has been previously described (21) as well as the predicted structures of chimeric capsomeres and VLPs with the Group A Streptococcus J8 peptide epitopes (7). The formation of VLPs from the subunit capsomere components adds time and cost to vaccine production (22). Also, the introduction of foreign epitopes could interfere with the structural formation of the VLPs. These shortcomings could be overcome if the chimeric capsomeres themselves were sufficiently immunogenic so that the subsequent VLP production steps were not required. To address this, our colleagues constructed a truncated MuPy-VP1 which prevented the formation of VLPs, and further modified the protein with the addition of multiple epitope insertion sites to increase the resultant antigen to vector ratio (8). These truncated capsomeres were, however, less effective at inducing of antibody responses and required co-administration with adjuvants (7, 8, 23) including nanoparticles such as silica, poly (D,L-lactic-co-glycolic acid) (PLGA) and poly caprolactone (PCL) (23, 24). In another study, it was shown that antibody responses induced by capsomeres could be enhanced to levels similar to those of VLPs without adjuvants, but this required a 20–40 times increase in the capsomere dose (7, 25). However, IFN-γ cellular responses induced by capsomeres were similar to those of VLPs, even at low doses in a HPV16 L1 model (25). Additionally, both HPV16 L1 VLPs and their component capsomere induced robust cytotoxic $CD8^+$ T cell responses which were capable of tumor regression in the absence of adjuvants (26).

Herein, we extended those studies to comprehensively compare chimeric murine polyomavirus VLP and capsomere constructs incorporating defined $CD8^+_{(280-288)}$ (27) and $CD4^+_{(59-79)}$ (28) T cell and B cell repeat epitopes (29) from *Py*CSP for capacity to induce robust $CD8^+$ and $CD4^+$ T cell responses and antibody responses. In addition, we evaluated the potential benefit of co-administration of poly(I:C) adjuvant with VLPs, and of including a heterologous DNA prime-boost regimen.

MATERIALS AND METHODS

Oligonucleotides, Peptides and Plasmid DNA

For genomic insertion of epitopes into VLPs and capsomeres, complementary oligonucleotides for the *Py*CSP $CD8^+_{280-288}$ T cell (SYVPSAEQI), $CD4^+_{59-79}$ T cell (YNRNIVNRLLGDALNGKPEEK), and B cell repeat (QGPGAPQGPGAP) peptide epitopes were codon-optimized for *E. coli* expression then synthesized by GeneWorks (Adelaide, Australia).

For peptide stimulation in T cell assays- *Py*CSP $CD8_{280-288}$ (SYVPSAEQI), $CD4_{59-79}$ (YNRNIVNRLLGDALNGKPEEK)

and B cell (QGPGAPQGPGAP) peptides were purchased from Mimotopes Pty Ltd (Victoria, Australia).

For plasmid DNA immunizations, plasmid DNA encoding full-length *Py*CSP (pVR2516) or empty vector without insert (pVR1020; Vical Inc, CA, USA) were commercially purchased from PureSyn Inc. (Malvern, PA, USA).

Plasmid Construction

The plasmid pGEX-4T-1 (GE Healthcare Biosciences, UK) with the murine polyomavirus VP1 sequence (accession number M34958) was obtained from the Protein Expression Facility (PEF, University of Queensland). For the VLP platform, the VP1 sequence was modified by PEF by inserting an *Afe*I restriction enzyme site flanked with $Glycine_4$-Serine linker sequences at position 293; this was designated pGEX-VP1-S4-G4S (7). For the capsomere platform, the VP1 sequence was truncated to remove the first 28 and last 63 amino acids, and restriction enzyme sites were inserted at positions 28, 85, 293, and 380; this construct was designated VP1ΔNΔC (8). Human codon-optimized *Py*CSP $CD8^+_{(280-288)}$ and $CD4^+_{(59-79)}$ T cell epitopes and the B cell repeat epitope sequences were individually inserted into the *Afe*I site in pGEX-VP1-S4-G4S and into positions 28, 293 and 380 of VP1ΔNΔC using standard molecular biology techniques and constructs were confirmed by Sanger sequencing.

Expression and Purification of Chimeric Capsomeres and VLPs

Wild-type pGEX-VP1-S4-G4S and VP1ΔNΔC, or the chimeric constructs detailed above, were separately transformed into chemically competent *E. coli* Rosetta DE3 pLysS bacteria (Novagen, CA, USA). The GST-tagged VP1 proteins were expressed by bacteria culture in Terrific Broth and expression induced using 0.2 mM IPTG and purified as previously described (7, 20). Briefly, filtered supernatant from sonicated bacteria were purified using a 5 ml GSTrap HP affinity column (GE Healthcare, UK), then the GST tag cleaved using thrombin (GE Healthcare UK). Capsomeres were then isolated by size-exclusion chromatography using a Superdex 200 10/300 GL column (GE Healthcare, UK). Endotoxin levels were reduced to below 5 EU/ml using Vivapure Q maxi H ion exchange columns (Sartorius Stedim, Gottingen, Germany) (7). VP1 capsomeres were assembled into VLPs by dialysis against an assembly buffer (7, 20) and then against PBS (7); VP1ΔNΔC capsomeres were dialyzed only against PBS (7). The characterization of VLPs for this project has been previously reported (18). VLPs were analyzed using asymmetric flow field-flow fractionation coupled to multi-angle light scattering (AF4-MALS) and transmission electron microscopy to assess size distribution as previously described (30, 31).

Immunization of Mice

Female BALB/c mice ($n = 5$/group) aged 6–7 weeks (Animal Resources Center, WA, Australia) were immunized three times at 3-week intervals by (i) subcutaneous injection (s.c.) of pooled VLPs or pooled capsomeres (pools comprising 10 μg of each CD8, CD4 and B cell chimeric construct) on the lower back near the base of the tail; (ii) intramuscular injection (i.m.) of plasmid DNA (100 μg) into the anterior tibialis; or (iii) s.c. injection of pooled peptides (pools comprising 30 μg of each $CD8^+$, $CD4^+$ or B cell peptide epitopes) on the lower back near the base of the tail. Capsomeres and peptides were co-administered with 50 μg of high molecular weight poly(I:C) adjuvant (Invivogen, USA); and VLPs were administered with or without this adjuvant, in parallel groups. Mice were immunized in both homologous and heterologous prime/boost regimens involving two priming doses of *Py*CSP plasmid DNA followed by a booster dose of capsomere, VLP or peptide. Negative control groups included PBS, wild-type capsomere with poly(I:C), or wild-type VLP with poly(I:C). The positive control group received three doses of *Py*CSP plasmid DNA. All murine experiments were approved by the QIMR Berghofer MRI Animal Ethics Committee and were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004).

Splenocyte Harvesting and *in vitro* Stimulation

Ten days after the final immunization, spleens were harvested and single cell suspensions generated by mechanical disruption and red blood cell lysis. For ELISpot, cytometric bead array (CBA) and intracellular cytokine staining (ICS) assays, 5×10^5 splenocytes were then co-incubated with 1.5 x 10^5 gamma irradiated (16,666 cGy) mouse B cell lymphoma A20 cells (ATCC TIB-208) which had been either DNA-transfected, peptide-stimulated, or untreated. Transfections with plasmid DNA encoding *Py*CSP (pVR2516) or empty vector (pVR1020) was achieved using the AMAXA Nucleofector system (Lonza, Switzerland) using Kit V and program C-25 with 5×10^6 A20 cells per cuvette, following the manufacturer's protocol. Peptide stimulation with *Py*CSP $CD8^+_{(280-288)}$ and $CD4^+_{(59-79)}$ T cell epitopes, or these peptides combined with the B cell repeat epitope peptide. Cells were incubated in KD-MEM media comprised of Dulbecco's Modified Eagle's Medium (SAFC Global, USA) supplemented with folic acid (136 nM), L-asparagine (32 mM), L-arginine (67 mM), sodium bicarbonate (24 mM), HEPES (10 mM), β-2-mercaptoethanol (5 nM), L-glutamine (1.5 mM), penicillin (100 Units/L), streptomycin (100 mg/L), and 10% fetal calf serum.

IFN-γ ELISpot Assay

IFN-γ ELISpot assays were conducted as previously described (18, 32). Briefly, MSIPS4510 multiscreen ELISpot plate (Merck Millipore, Germany) well were pre-coated with 10 μg/ml anti-mouse IFN-γ antibodies (BD Biosciences, USA), blocked with KD-MEM containing 10% FCS, and washed. Then splenocyte/A20 cultures in quadruplicate were incubated at 37°C and 5% CO_2 for 40 h. Wells were washed and stained with 2 μg/ml biotinylated anti-mouse IFN-γ antibodies (BD Biosciences, USA), followed by 1 μg/ml streptavidin-HRP (BD Biosciences, USA). The assay was developed using AEC substrate (BD Biosciences, USA). Spots were counted using the AID ELISpot reader system (Autoimmun Diagnostika GmbH, Germany).

Cytometric Bead Array

Splenocyte/A20 cultures were incubated at in 96-well U-bottom plates in 200 μl of complete media at 37°C and 5% CO_2 for 72 h. Culture supernatant was collected and stored at −80°C prior to assay. Secreted IFN-γ, TNF, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, and IL-13 cytokines were analyzed using the mouse cytometric bead array flex kit (BD Biosciences, USA) following the manufacturer's protocol. Samples were acquired using a FACSArray instrument (BD Biosciences, USA) and data analyzed using the CBA array software (BD Biosciences, USA).

Intracellular Cytokine Staining

Splenocyte/A20 cultures in 200 μl of complete media supplemented with 0.1% Golgi Plug (BD Biosciences, USA) in 96-well U-bottom plates were incubated for 6 h at 37°C and 5% CO_2. Cells were stained with PE-Cy7 labeled anti-$CD8^+$ (53-6.7) and BV510 labeled anti-$CD4^+$ (RM4.5) antibodies before being fixed with 4% paraformaldehyde at room temperature (RT) for 15 min. Cells were then washed with permwash and stained with APC-labeled anti-IFN-γ (XMG1.2), PE-labeled anti-IL-2 (JES6-5H4) and FITC-labeled anti-TNF-α (MP6-XT22) antibodies diluted in Cytofix/Cytoperm (BD Biosciences, USA). All antibodies were purchased from Biolegend with the exception of anti-TNF-α which was purchased from eBioscience. Flow cytometric analysis was performed on a Fortessa 4 (BD Biosciences, USA). Post-acquisition data analysis was performed using FlowJo software version 10 (Treestar, USA).

ELISA

Sera was collected from mice 14 days after immunizations 1 and 2, and 5 days after the final immunization. Nunc Maxisorp plates (Thermo Fisher Scientific, USA) were coated overnight with either *Py*CSP B cell repeat peptide linked to a polystyrene binding tag (33) with a $glycine_4$ spacer (34) (Mimotopes, Australia) (5 μg/ml) or *Py*CSP recombinant protein (1 μg/ml) diluted in carbonate buffer. Wells were subsequently blocked with PBS containing 2% BSA. Triplicate wells of 2-fold serially diluted sera in PBS-BSA 0.1% were used for endpoint titrations, and sera diluted 1:400 for isotype screening. For IgG responses, wells were incubated with biotinylated donkey α-mouse IgG antibodies (Jackson ImmunoResearch Laboratories, USA) diluted 1:20,000 in PBS-BSA 0.1%, followed by incubation with streptavidin-HRP (BD Biosciences, USA) diluted 1:1,000 in PBS-BSA 0.1 and 0.2% Tween20. For IgG isotype responses, wells were incubated with HRP-conjugated goat anti-mouse IgG_1, rabbit anti-mouse IgG_{2a}, goat anti-mouse IgG_{2b}, or goat anti-mouse IgG_3 antibodies (Invitrogen, USA) all diluted 1:3,000 in PBS-BSA 0.1 and 0.2% Tween20. Wells were developed with tetramethylbenzidine (TMB) and stopped using TMB stop reagent (Sigma Aldrich, USA). Absorbance was measured at 450 nm using a VersaMax microplate reader (Molecular Devices, USA). Positivity was defined as OD_{450} value >3× standard deviations above the mean blank (no serum) values.

Indirect Fluorescence Antibody Test (IFAT)

Sporozoite-specific antibodies were assayed by Indirect fluorescence antibody test (IFAT) using a protocol modified slightly from that previously described (35). Briefly, cryopreserved *P. yoelii* 17XNL sporozoites (Sanaria Inc., MD, USA) were centrifuged at 10,000 × *g* for 5 min then resuspended to 10^5 sporozoites per ml of Medium 199 (Life Technologies, USA). Then, 10 μl was added to wells drawn on a microscope slide using a Barrier Pap pen and air dried at RT before long-term storage at −80°C. Prior to use, slides were thawed to RT in a desiccator cabinet. Pooled sera from each immunization group collected 5 days after the final immunization was diluted 1:400 in PBS with 2% BSA, then 10 μl was added to each well and incubated at 37°C in a humid box. Wells were gently washed with PBS then stained with 10 μl of FITC conjugated anti-mouse IgG antibodies (BD Biosciences, USA), diluted 1:30 in filtered PBS containing 0.005% Evans blue. Slides were incubated for 30 min in a humid chamber and then washed gently with PBS. A cover slide was mounted over PBS with 10% glycerol and slides viewed on an EVOS fluorescence microscope (Advanced Microscopy Group, USA) at x400 magnification.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 6.0 (GraphPad, CA, USA). Logarithmic transformed data of groups were compared by one-way analysis of variance (ANOVA) and Bonferroni's multiple comparison test. Statistical significance is reported as $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

RESULTS

Capsomere and VLP Construction

The genomic insertion of *Py*CSP peptide epitopes into the murine polyomavirus VP1 protein for generation of VLPs (7) or within the truncated VP1 proteins for capsomeres (8) was confirmed by Sanger sequencing. Size exclusion chromatography fractions post-GST cleavage showed the formation of capsomeres as previously described (8), and these were subsequently used for capsomere immunizations. The VLP forming proteins were assembled *in vitro* and analyzed by AF4-MALS analysis, which showed the mean radius of the chimeric VLPs [wild-type, 21.00 ± 1.27 nm; CD8 VLPs, 20.51 ± 0.67 nm; CD4 VLPs, 21.07 ± 0.61 nm; B cell VLPs, 20.85 ± 0.67 nm (mean radius ± SD)] with minimal amounts of aggregation (18). Transmission electron microscopy showed similar morphology between all VLP groups (18).

Capsomeres Induce Similar $CD8^+$ T Cell IFN-γ Responses to VLPs

Significant antigen-specific IFN-γ responses were induced by homologous immunization with both capsomeres and VLP, when compared to their respective negative control, as detected after *in vitro* stimulation with *Py*CSP DNA transfected A20s or with pooled *Py*CSP peptides (**Figure 1A**). The amount of detected IFN-γ was not significantly different between capsomeres or VLP immunized groups ($p > 0.05$). The peptide immunizations also

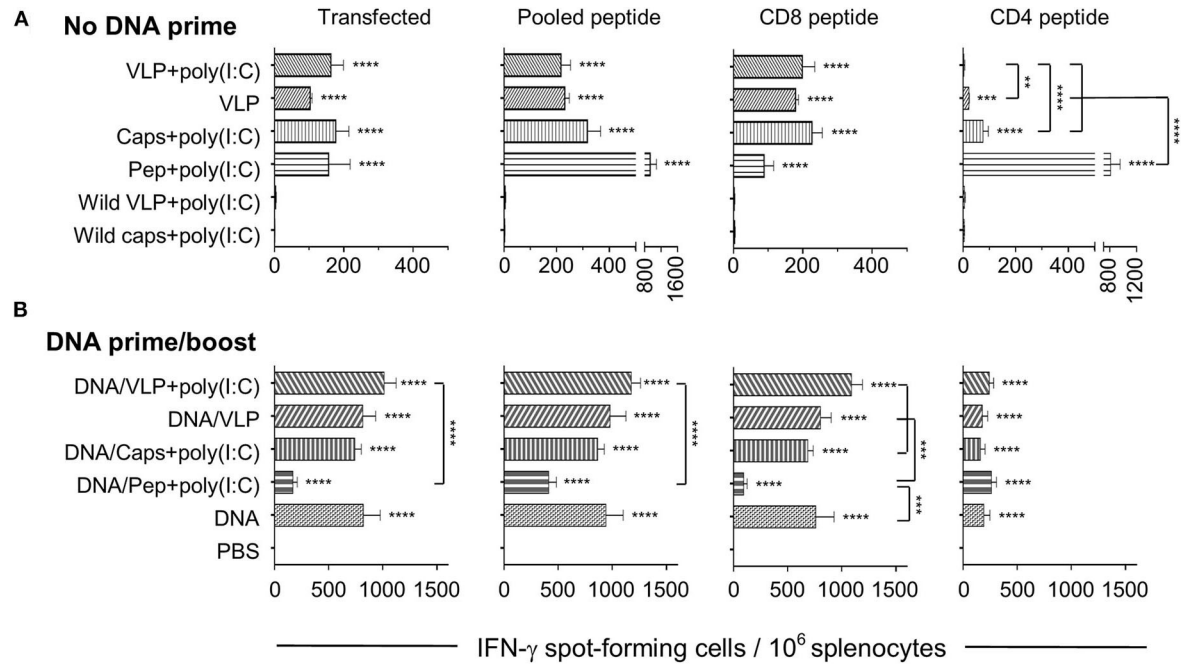

FIGURE 1 | IFN-γ ELISpot responses induced by chimeric VLP or capsomere constructs. BALB/c mice ($n = 5$/group) received three immunizations with either **(A)** homologous s.c. injections of VLPs ± poly(I:C), capsomeres or peptides with poly (I:C), or **(B)** a heterologous DNA prime/boost regimen with two i.m. *Py*CSP plasmid DNA primes followed by a single s.c. boost with VLPs ± poly(I:C), capsomeres or peptides with poly (I:C). Seven days after the final immunization splenocytes were harvested and single-cell suspensions were stimulated *in vitro* for 40 h with irradiated A20 cells transfected with *Py*CSP plasmid DNA, or with irradiated A20 cells pulsed with either $CD8_{(280-288)}$, or $CD4_{(58-79)}$ peptides or a pool of both peptides plus the B cell repeat peptide. IFN-γ spot forming cells (SFCs) were quantified with data displayed as mean SFCs per 10^6 splenocytes plus SEM. Statistical comparisons made to the PBS control group and between immunization groups with significance determined using one-way ANOVA followed by Bonferroni's post-test. $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

induced a robust IFN-γ responses which was dominated by the CD4 peptide stimulation.

Heterologous prime/boost immunization resulted in an ∼5-fold increase in IFN-γ responses over the homologous regimens for both capsomeres and VLPs (**Figure 1B**). When compared to their homologous respective counterparts, the numbers of IFN-γ spot forming cells in the heterologous DNA prime/boost immunized mice were increased significantly for capsomeres ($p < 0.001$), VLPs and VLPs with poly(I:C) ($p < 0.0001$) when stimulated with the whole *Py*CSP antigen transfected into A20 cells, and capsomeres ($p < 0.01$), VLPs and VLPs with poly(I:C) ($p < 0.0001$) when stimulated *in vitro* with pooled peptides. This increase in IFN-γ responses was driven by the DNA prime as evidenced by comparison of the responses to those of DNA only immunized mice. Consistent with results from the homologous immunization regimens, IFN-γ responses induced by capsomeres and VLPs in the heterologous DNA prime/boost regimen were not significantly different.

To differentiate the responses observed with the pooled peptide stimulation *in vitro*, we also stimulated with either *Py*CSP $CD8^+$ or $CD4^+$ T cell peptides separately. That study showed that both the capsomere and VLP induced IFN-γ responses were predominantly associated with the $CD8^+$ T cell peptide, with no significant difference between the capsomere and VLP immunized groups (**Figures 1**, **2**). ICS was used to determine whether the $CD8^+$ or $CD4^+$ T cells were responsible for the production of IFN-γ (**Figure 3**). For both *Py*CSP capsomere and VLP immunized mice, IFN-γ was predominantly produced by $CD8^+$ T cells. Similarly, in the heterologous prime/boost regimen, responses were directed against the $CD8^+$ T cell epitope and apparently driven by the DNA component with responses similar to that induced by homologous DNA only immunization.

Capsomeres Induce More Robust $CD4^+$ T Cell IFN-γ Responses Than VLPs

Although capsomere and VLP platforms induced a very similar profile of $CD8^+$ T cell responses, unexpectedly, they differed in ability to induce antigen-specific $CD4^+$ T cell responses. Specifically, homologous capsomeres induced significantly higher IFN-γ responses than VLPs with poly(I:C) ($p < 0.0001$) when stimulated with the $CD4^+$ T cell peptide (**Figure 1A**). This trend was also evident in the CBA analysis (**Figure 2**) and in the $CD4^+$ T cell response detected by ICS (**Figure 3**), although the responses did not reach the level of significance. As observed for the $CD8^+$ T cell response, the heterologous prime/boost was more effective than homologous immunization in inducing robust responses to the $CD4^+$ T cell epitope (**Figures 1B**, **2**). With this heterologous regimen, which included two priming doses of DNA, the vaccine-induced $CD4^+$ T cell response for capsomeres and VLPs were comparable

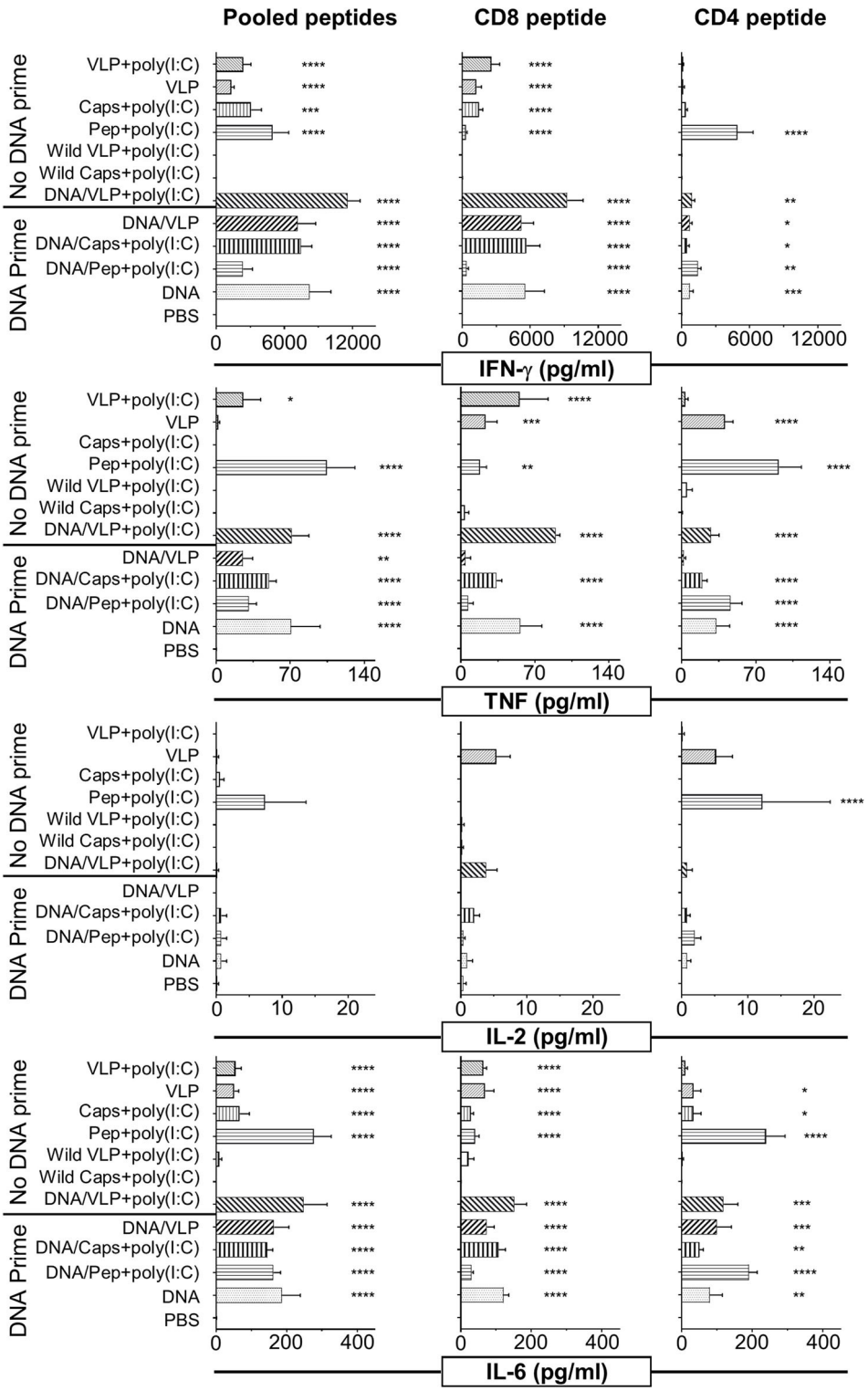

FIGURE 2 | Cytometric bead array analysis of antigen-specific cytokine responses induced by immunization with chimeric VLPs or capsomeres. BALB/c mice (n = 5/group) were immunized with either a homologous (no DNA prime) or heterologous (DNA prime-boost) regimen as described in the legend to **Figure 1**. Seven days after the third immunization splenocytes were harvested and single cell suspensions stimulated with either the $CD8^+_{(280-288)}$, or $CD4^+_{(58-79)}$ T cell peptides or with pooled peptides including both peptides plus the B cell repeat peptide and incubated for 72 h at 37°C and 5% CO_2. Culture supernatant was assayed using a cytometric bead array to quantify IFN-γ, TNF, IL-2, and IL-6 cytokine levels. Data are displayed as mean pg/ml + SEM for each cytokine with statistical comparisons made to the PBS control group with significance determined using one-way ANOVA followed by Bonferroni's post-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

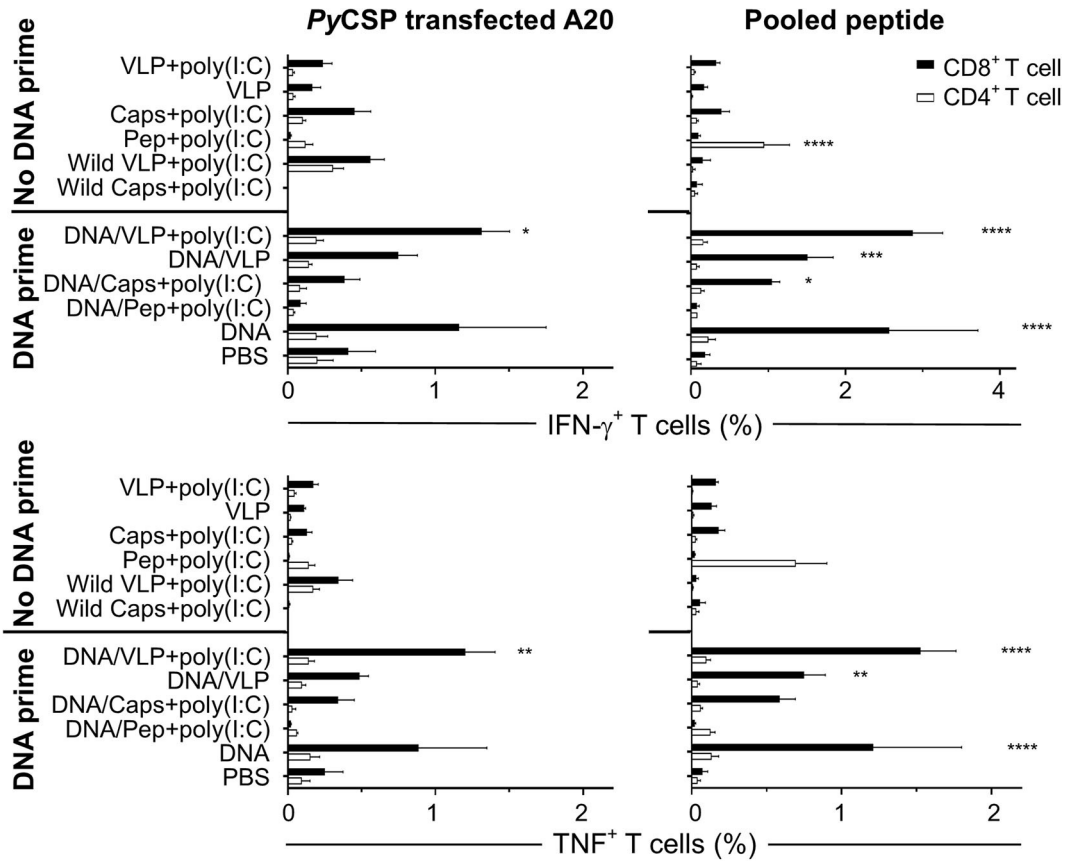

FIGURE 3 | Flow cytometry frequency of stimulated splenocyte $CD8^+$ and $CD4^+$ T cells expressing IFN-γ and TNF post-immunization. BALB/c mice ($n = 5$/group) received three immunizations with either homologous (No DNA prime) or a heterologous DNA prime-boost regimen (DNA prime) as described in **Figure 1**. Seven days after the final immunization splenocytes were harvested and single-cell suspensions were stimulated *in vitro* by culturing them with irradiated A20 cells either transfected with *PyCSP* plasmid DNA or pulsed with *Py*CSP $CD8^+$ and $CD4^+$ T cell and B cell repeat peptides and incubated in for 1 h before adding Golgi Plug followed by a further 5 h incubation. Cells were stained for CD8 and CD4 receptors, then permeabilized and stained for IFN-γ and TNF cytokines then assessed by flow cytometry. The frequency of CD8 or CD4 positive cytokine-expressing cells are shown as the group mean + SEM ($n = 5$ mice/group) with $CD8^+$ and $CD4^+$ T cells represented by solid black bars or open bars, respectively. Statistical comparisons made to the PBS control group with significance determined using one-way ANOVA with Bonferroni's post-test. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

and did not differ significantly from that of three doses of *Py*CSP plasmid DNA alone. Nonetheless, the highest IFN-γ responses to the $CD4^+$ T cell peptide were seen with homologous immunization with pooled peptides with poly(I:C) showing beneficial effects of the repeat peptide doses.

Multiplexed Cytokine Responses

A broader spectrum of cytokine responses, including TNF, IL-2, and IL-6 as well as IFN-γ, were quantified in culture supernatant from *in vitro* immune assays using cytometric bead array (CBA) (**Figure 2**). The IFN-γ response profile was consistent with that detected by ELISpot (**Figure 1**). IFN-γ responses induced by capsomeres and VLPs were comparable and directed predominantly against the $CD8^+$ T cell peptide, whereas the $CD4^+$ T cell peptide stimulated cytokine profile observed by CBA was higher for capsomeres than for VLPs although these differences were not significant.

Capsomeres did not induce a TNF response for either the $CD8^+$ or $CD4^+$ peptide, but significant TNF responses were induced by VLP immunization and this was further increased by poly(I:C) adjuvant; these responses were preferentially directed against the $CD8^+$ T cell peptide but were also significant for the $CD4^+$ T cell peptide epitopes (**Figure 2**).

Significant IL-6 responses were induced by both capsomere and VLP groups with or without DNA priming, with a similar profile for both $CD8^+$ and $CD4^+$ T cell peptide epitopes.

In the peptide-immunized mice, responses for robust for IFN-γ, TNF, and IL-6 cytokines were preferentially directed to the $CD4^+$ T cell peptide.

Negligible IL-2 levels were induced by any of the vaccine platforms tested (capsomeres, VLPs, or peptide) as assessed by ICS (data not shown) and CBA (**Figure 2**). The IL-1β, IL-4, IL-5, IL-10, IL-12p70, and IL-13 cytokine responses detected by CBA were also very low and no differences were identified between the two platforms (data not shown).

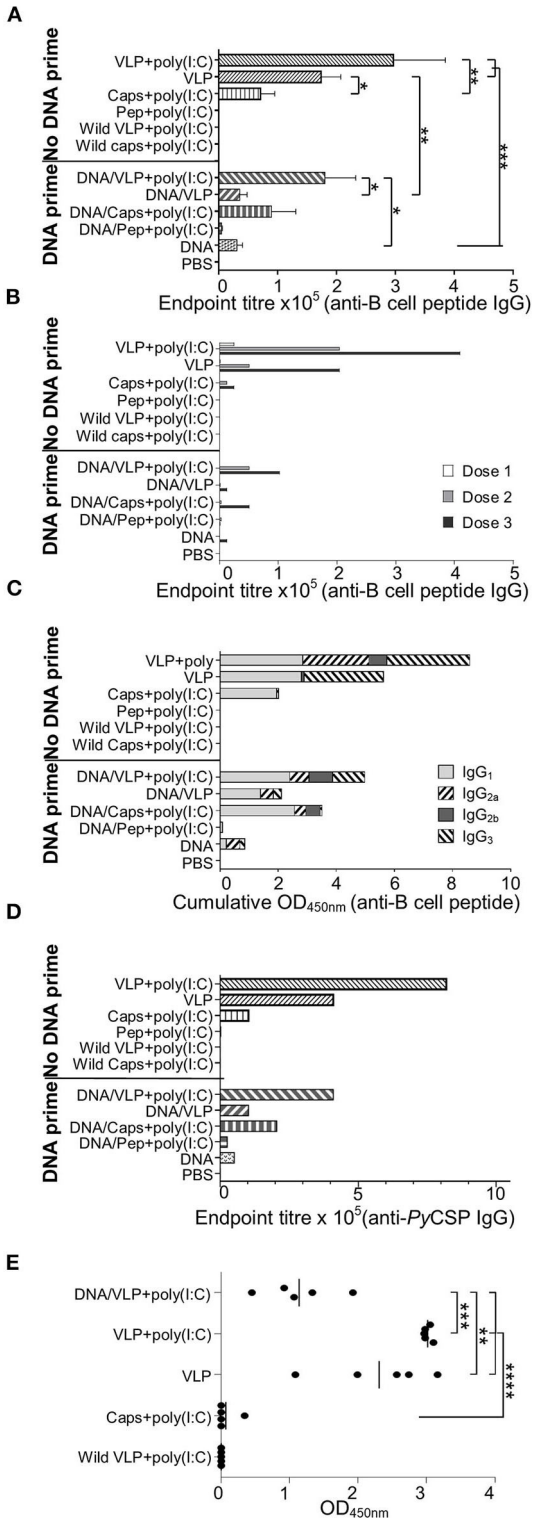

FIGURE 4 | *Py*CSP-specific antibody responses induced immunization with chimeric VLPs or capsomeres. BALB/c mice ($n = 5$/group) were immunized with DNA, VLP, capsomeres or peptides in a three-dose homologous regimen or a heterologous DNA prime boost regimen as described in **Figure 1**. Sera collected 14 days after each immunization and 5 days after the final immunization were analyzed either individually or pooled, by ELISA using the *(Continued)*

FIGURE 4 | *Py*CSP B cell repeat epitope linked to a polystyrene binding tag **(A–C,E)** or *Py*CSP protein **(D)** as capture antigen. **(A)** B cell epitope-specific endpoint total IgG antibody titres for individual mice with sera collected after the final immunization. Data is shown as mean ± SEM for each group. **(B)** B cell epitope-specific endpoint total IgG antibody titres for pooled sera collected following each immunization. **(C)** B cell epitope-specific IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3 antibody isotype responses for pooled sera collected after the final immunization. Data is shown as a cumulative OD_{450nm} readout. **(D)** *Py*CSP antigen-specific endpoint total IgG antibody titer for pooled sera collected after the final immunization. **(E)** B cell epitope-specific IgG3 antibody responses for individual mice with sera after the final immunization. Data are for individual mice are shown as OD_{450} values with bars representing the group mean. Inter-group significance was determined using one-way ANOVA followed by Bonferroni's post-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

Capsomeres Were Less Efficient Than VLPs at Inducing Antibody Responses

ELISA assays against the PST-*Py*CSP B cell repeat peptide and *Py*CSP recombinant protein were performed using individual and pooled mouse sera collected 14 days after doses 1 and 2; and 5 days after final immunization (**Figure 4**). Consistent with previous studies, VLPs induced high anti-*Py*CSP B cell repeat IgG titres which were increased with the co-administration of poly(I:C) adjuvant (**Figure 4A**). Capsomeres induced moderate titres but, importantly, these were significantly less than VLPs with and without poly(I:C) ($p < 0.01$ and $p < 0.05$, respectively). There was a trend to increased antibody response with VLP administered with adjuvant compared to the non-adjuvanted VLPs but this was not statistically significant. However, the adjuvant effect was significant when VLPs were administered with a DNA prime ($p < 0.05$) indicating that with only a single VLP dose, the inclusion of adjuvant is beneficial and this benefit becomes less important after multiple doses (**Figure 4B**). A priming dose of DNA appeared to adversely affect the antibody induction, since responses in the heterologous prime-boost regimen tended to have lower titres than their homologous counterparts especially in the absence of poly(I:C) ($p < 0.01$).

We assessed the anti-*Py*CSP B cell peptide total IgG responses using pooled sera collected after each immunization to determine the effect of the number of immunizations within each regimen (**Figure 4B**). After each dose of VLPs, there was a trend to increased antibody titres when poly(I:C) adjuvant was co-administered.

Antibody isotypes contributing to the total IgG response reported above were also delineated, using pooled sera collected after the final immunization (**Figure 4C**). Immunizations with capsomeres, VLPs and VLPs with poly(I:C) each induced robust IgG_1 responses which were similar for all groups, and the IgG2 response in the VLP-immunized mice was increased when administered with poly (I:C). VLP immunizations induced an IgG_3 response which was absent in the capsomere-immunized mice. Compared to other immunization regimens, the homologous VLP immunization groups had higher levels of IgG_3 but similar levels of IgG_1 indicating that the second and third doses caused a shift in isotype responses toward an

IgG_3 bias. IgG_{2a} responses were increased with the inclusion of poly(I:C) with the VLP but this was not present in the capsomere with poly(I:C) immunized mice, indicating the difference was platform specific. Moderate IgG_{2a} responses were also present in the DNA group and the heterologous DNA prime groups.

To confirm that the vaccine-induced antibodies had affinity to the *Py*CSP protein, pooled sera collected after the final immunization was assayed using ELISA against recombinant protein (**Figure 4D**). The endpoint titres and profile were similar to that reported above for the B cell peptide (**Figure 4A**).

To confirm that the IgG_3 response was consistent within groups, an ELISA against the *Py*CSP B cell repeat peptide was done using individual sera (1:400) collected after the final immunization (**Figure 4E**). Each VLP immunization group tested had significant amounts of IgG_3 which was absent in chimeric capsomeres. The inclusion of poly(I:C) resulted in increased levels of IgG_3 responses, and the homologous three-dose regimen significantly increased levels as compared to the heterologous prime/boost regimen ($p < 0.001$).

Antibody Recognition of Native Antigens

Importantly, antibodies induced by immunized with capsomeres or VLPs with or without poly(I:C) adjuvant were able to recognize *P. yoelii* 17XNL sporozoites, as evidenced by surface staining in the homologous and heterologous immunization regimens (**Figure 5**).

DISCUSSION

The *Plasmodium* sp. CSP is the leading antigen target for sub-unit vaccines against malaria, and is the antigenic component of the virus-like particle RTS,S which is the most advanced human vaccine candidate against *Plasmodium falciparum* malaria. It was, therefore, a logical choice for evaluation of novel vaccine delivery platforms. We know that liver-stage protection from sporozoite challenge can be achieved by either $CD8^+$ T cells (27, 36, 37), $CD4^+$ T cell (38, 39) or antibody responses (29, 40). Thus, herein, we constructed chimeric VLPs and capsomeres incorporating a $CD8^+$ T cell epitope, $CD4^+$ T cell epitope, or B cell epitope derived from *Py*CSP in order to evaluate and compare the ability of capsomeres and VLP vaccine platforms to induce epitope-specific cellular and antibody responses.

Although the antibody-inducing capacity of chimeric murine polyomavirus VLPs and capsomeres has been well-established (7–9, 23), the ability of this vaccine platform to induce epitope-specific T cell responses has not previously been comprehensively evaluated. We set out to accomplish this. Our study established that capsomeres and VLPs induced similar and significant levels of antigen-specific IFN-γ responses and these responses were primarily directed against the immunodominant *Py*CSP $CD8^+$ T cell epitope and produced by $CD8^+$ T cells. This $CD8^+$ T cell IFN-γ immune response is considered essential for protection (39, 41). Moreover, responses were increased when DNA priming was included as a heterologous regimen, as expected based on previous studies (18, 42–44). Interestingly, however, while both platforms were similar in capacity to induce an IFN-γ response, it was only the VLPs which evoked a TNF response, albeit at low levels, suggesting that VLPs may be better than capsomeres at inducing polyfunctional T cell responses.

In contrast to the comparability of the $CD8^+$ T cell responses, VLPs and their subunit capsomeres differed significantly in capacity to induce $CD4^+$ T cell responses and antibody responses. Unexpectedly, capsomeres proved to be the best platform at inducing responses to the $CD4^+$ T cell epitope. The target *Py*CSP $CD4^+$ T cell epitope $_{(59-79)}$ was selected for study because of its the ability to induce functional $CD4^+$ T cell populations of either T_H1 or T_H2 subsets associated with T cell proliferation responses or help for antibody responses (28). In our previous study, we could not detect any synergistic $CD4^+$ T cell helper effect on antibody titres by co-administering a chimeric CD4 VLP (in a VLP pool) with B cell VLPs (18). In the current study, we were unable to show any $CD4^+$ T cell helper responses as both capsomeres and VLPs were only administered in pools. We found that immunization with pooled peptides adjuvanted with poly(I:C) induced the most robust responses, however this is likely related to the dose of the target peptide epitope, as the positive control peptide pools incorporated 30 μg of the CD4 peptide whereas the amount of epitope presented in the chimeric capsomeres and VLPs was $\sim$1.77 and 0.52 μg, respectively, per dose. This may also explain why the capsomeres were more effective than VLPs at inducing responses against this peptide epitope as one of the benefits of capsomeres over VLPs as a vaccine platform is the ability to present a higher antigen load, and in our study capsomeres achieved a 3-fold higher antigen dose than VLPs. It should be noted that the antigen dose difference was the same for the $CD8^+$ T cell peptide epitope and we did not see similar increased immune responses against that antigen target. The ability of capsomeres to induce $CD4^+$ T cell responses is an important advantage for vaccinology since vaccine platforms that preferentially induce $CD8^+$ T cell responses rather than antibody responses are often poor inducers of $CD4^+$ T cell responses (45). The essential role of $CD4^+$ T cells for vaccine-induced protection against malaria has been demonstrated in $CD4^+$ T cell-depleted sporozoite-immunized mice where an absence of $CD4^+$ T cells resulted in reduced anti-sporozoite antibodies, a reduced effector capacity of $CD8^+$ T cells, loss of protective efficacy (46).

It is well-established that VLPs are very effective at inducing antibody responses, perhaps due to their ability to cross-link B cell surface receptors (25) acting in a T cell independent manner (47, 48). Indeed, for all licensed VLP-based vaccines, protection is thought to be mediated through neutralizing antibodies (10, 11). Furthermore, studies using VLPs as well as their capsomere components with adjuvants showed them to be strong inducers of antibodies to inserted antigens (7–9, 23). Here, we have shown that VLPs with and without adjuvant were significantly better than capsomeres at inducing antigen-specific antibody responses, and that inclusion of poly(I:C) adjuvant increased antibody titres (49) with a skewed T_H1 isotype profile. Importantly, these vaccine-induced antibodies were capable of recognizing the B cell repeat peptide, as well as recombinant *PyCSP* protein, and the parasite and IFAT showed antibody affinity to the surface of sporozoites establishing that they could recognize the whole parasite as well as the protein. The durability of the induced

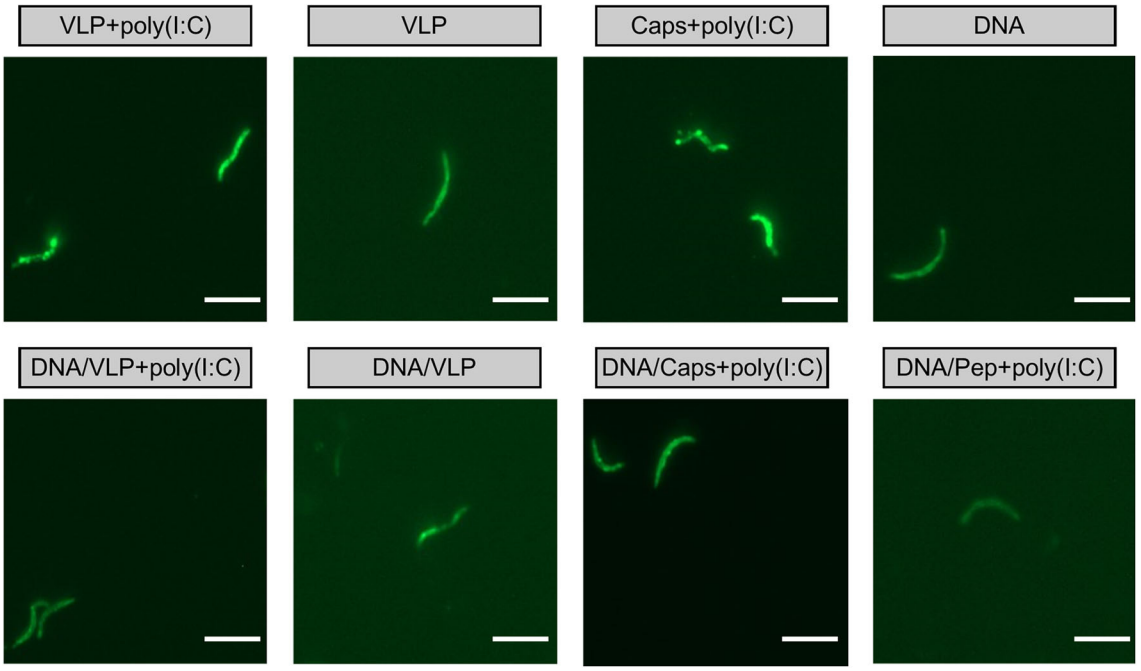

FIGURE 5 | IFAT detection of anti-sporozoite antibodies induced by immunization with chimeric VLPs or capsomeres. Mice ($n = 10$/group) were immunized using a three-dose regimen with either homologous VLPs, capsomeres or *Py*CSP plasmid DNA; or a heterologous regimen with two *Py*CSP plasmid DNA doses followed by a single VLP or capsomere boost as described in **Figure 1**. Sera collected from mice 5 days after final immunization was pooled and assayed against *P. yoelii* 17XNL sporozoite-coated slides (1:400 dilution). Slides were stained using FITC-conjugated anti-mouse total IgG and viewed on an EVOS fluorescence microscope at x1000 magnification. Scale bar represents 10 μm.

antibodies and their protective capacity has yet to be established. The value of anti-CSP antibodies has been shown by their ability to sterilely protect mice against sporozoite challenge (29), and by observations that anti-CSP IgG concentration and avidity contribute to RTS,S/AS01E-mediated protection (50) with anti-CSP antibodies estimated to prevent 32% of infections following RTS,S immunizations (51).

An interesting observation from our study is that the IgG_3 antibody isotype observed with VLP immunizations contrasts with results obtained using the same platform incorporating the Group A Streptococcus J8 peptide, where IgG_1 was the dominant IgG isotype and no IgG_3 was detected (7). It appears, therefore, that the development of IgG_3 is associated with the target epitope presented in the VLP structure rather than with the VLP platform itself. We had previously observed that an IgG_3 response was induced by immunization with three doses of chimeric B cell epitope VLPs alone (our unpublished data), establishing that the $CD8^+$ or $CD4^+$ T cell chimeric VLPs were not responsible for the subclass, as seen in other platforms (52). It is curious that the IgG_3 response was induced by immunization with VLPs but not capsomeres. IgG_3 antibodies have previously been identified as important for protection against various pathogens (53, 54) including *Plasmodium* spp. parasites (35, 40), and IgG_3 monoclonal antibodies raised against the *Py*CSP repeat (QGPGAP) protected BALB/c mice from a *P. yoelii* 17XNL sporozoite challenge (29).

There are many examples of enhanced immunogenicity and protection with DNA prime-boost regimens using various antigen delivery systems encoding the same antigen or epitope (44, 55–58) including VLPs (59) as a boost immunogen. Consistent with those reports, our data shows that the heterologous prime-boost regimen was better at inducing cellular responses than a homologous immunization regimen, for both capsomere and VLP platforms. The gain in cellular responses observed by including a plasmid DNA prime was, however, countered by a decrease in antibody titres. This is consistent with the known ability of plasmid DNA to preferentially prime a $CD8^+$ T cell response and its poor ability to prime an antibody response (45).

Chimeric capsomeres are a promising vaccine platform which build on the established vaccine potential of VLPs, but are easier and cheaper to produce than VLPs (22), and can include more antigenic insertion sites because there is no reliance on structural VLP formation. Here, we show that capsomere and VLP platforms induced similar levels of $CD8^+$ T cell responses but the capsomeres were significantly better at inducing epitope-specific $CD4^+$ T cell responses than VLPs. This enhanced $CD4^+$ T cell response may be of particular importance in the control of chronic viral infections (60), the maintenance of $CD8^+$ T cells during prolonged viral infections (61) where $CD4^+$ T cell help may be required for optimal $CD8^+$ T cell activity (62) or for $CD8^+$ T cell memory (63), or indeed for optimal responses

against any pathogens where a $CD4^+$ T cell vaccine induced response is required. On the other hand, since capsomeres have a limited capacity to induce antibody responses, VLPs would be the preferred platform for antibody mediated immunity. Given that most licensed vaccines target the induction of antibody response and the increasing interest at identifying vaccine platforms capable of inducing robust T cell responses, our data have important implications for the development of vaccines against those pathogens that have thus far proved challenging.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be obtained from the corresponding author upon reasonable request.

ETHICS STATEMENT

The animal study was reviewed and approved by QIMR Berghofer Medical Research Institute Animal Ethics Committee.

AUTHOR CONTRIBUTIONS

DP, SA, DD, and AM contributed conception and design of the study. DP, NW, TR-H, and AM contributed to construction of virus-like particles and capsomeres. DP, SA, and PG conducted the mouse experiments. DP performed the statistical analysis. DP and DD wrote the manuscript. All authors contributed to manuscript revision, have read, and approved the submitted version.

FUNDING

This research was funded by the National Health and Medical Research Council (NHMRC) Program grant #1037304 and by an Australian Infectious Diseases UQ-QIMR Seed funding grant. DD was supported by a NHMRC Principal Research Fellowship #1023636. DP was supported by an Australian Postgraduate Award.

ACKNOWLEDGMENTS

The authors thank Professor Linda Lua from the Protein Expression Facility (University of Queensland, Australia) for the plasmid containing the MuPyV-VP1 protein and Dr. Stephen Hoffman and colleagues (Sanaria Inc, Rockville, MD, USA) for providing cryopreserved sporozoites.

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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.

frontiers in Immunology

REVIEW
published: 30 September 2020
doi: 10.3389/fimmu.2020.583077

Emerging Concepts and Technologies in Vaccine Development

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Despite the success of vaccination to greatly mitigate or eliminate threat of diseases caused by pathogens, there are still known diseases and emerging pathogens for which the development of successful vaccines against them is inherently difficult. In addition, vaccine development for people with compromised immunity and other pre-existing medical conditions has remained a major challenge. Besides the traditional inactivated or live attenuated, virus-vectored and subunit vaccines, emerging non-viral vaccine technologies, such as viral-like particle and nanoparticle vaccines, DNA/RNA vaccines, and rational vaccine design, offer innovative approaches to address existing challenges of vaccine development. They have also significantly advanced our understanding of vaccine immunology and can guide future vaccine development for many diseases, including rapidly emerging infectious diseases, such as COVID-19, and diseases that have not traditionally been addressed by vaccination, such as cancers and substance abuse. This review provides an integrative discussion of new non-viral vaccine development technologies and their use to address the most fundamental and ongoing challenges of vaccine development.

Keywords: non-viral DNA-RNA vaccines, nanoparticle vaccines, virus-like particle vaccines, cancer vaccines, substance abuse, noncommunicable disease, infectious disease, COVID19

OPEN ACCESS

Edited by:
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Coalition for Epidemic Preparedness Innovations (CEPI), Norway
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Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: *14 July 2020*
Accepted: *14 September 2020*
Published: *30 September 2020*

Citation:
Brisse M, Vrba SM, Kirk N, Liang Y and Ly H (2020) Emerging Concepts and Technologies in Vaccine Development. Front. Immunol. 11:583077. doi: 10.3389/fimmu.2020.583077

INTRODUCTION

Beginning with the discovery of cowpox inoculation that can protect humans against smallpox infection by Edward Jenner in the late 18th century, vaccination has become an important means to prevent disease. Despite the success of vaccination in the eradication or control of some major pathogens, several challenges remain in vaccine development and administration. Several widespread infectious diseases such as HIV, tuberculosis, and influenza continue to pose great challenges for fully protective vaccination. Emerging and reemerging pathogens present a pressing need for expediting vaccine development and approval as a rapid response to epidemics, such as the current COVID-19 global pandemic caused by the SARS-CoV-2 virus. The advantages and disadvantages of the various vaccine platforms can make the choice for preferred platform(s) to use for vaccine development during a pandemic complicated. The traditional methods to produce a vaccine, such as live attenuated and inactivated vaccines or protein subunit vaccines have their

advantages and disadvantages, which have been extensively reviewed elsewhere (1–3). Briefly, live attenuated vaccines present the risk of reversion to a highly pathogenic form while inactivated vaccines may not be sufficiently immunogenic or in some cases can lead to an enhanced disease pathology (3). Additionally, most pandemic vaccines have to be clinically tested during an active outbreak in order to obtain sufficient safety and efficacy data, thereby limiting the number of vaccine candidates that can be deployed to save life during an emergency situation.

Less conventional approaches to vaccinology include the non-viral vaccine technologies that are the topic of this review, as well as viral vector platforms. Viral vector vaccines rely on antigen delivered on an unrelated, non-pathogenic viral backbone. This technology was developed almost forty years ago using a vaccinia virus vector expressing the hepatitis B surface antigen (HBsAg), which provided protective immunity to chimpanzees exposed to hepatitis B (4, 5). Since then, viral vectors have been used successfully in many veterinary species (6–12), although only a single viral vector has been licensed for human vaccination (rVSV-ZEBOV for Ebola virus) (13). A number of viruses have been developed as vectors for vaccine development, including poxviruses, adenoviruses, herpesviruses, arenaviruses, retroviruses, paramyxoviruses, and flaviviruses, among others (14–16). The main advantage of viral vectors over traditional vaccines is their ability to evoke a robust adaptive immune response in the absence of an adjuvant (17). However, the tradeoff for enhanced immunogenicity is the concern for potential reversion of the attenuated viral vector to virulence, especially when using a replication-competent vector (18). Replication-defective and single-cycle viral vectors are attractive alternatives that have an increased safety profile and, in some cases, are still able to elicit a strong immune response (19, 20). More details about the known viral vectors and their recent advances in vaccine development will be discussed in our forthcoming review article (Vrba, S.M., et al., in preparation).

Other fundamental challenges toward successful vaccination include the ever-changing and highly divergent nature of some viruses that allow for the potential to escape vaccine coverage, pre-existing immunity of the vaccinated populations, and pre-existing medical conditions that can prevent vaccines from being fully effective and safe. Vaccination could also provide an enticing alternative therapy against diseases such as cancers and substance abuse. However, the efficacy of these vaccines is limited by the disease complexity and the lack of a more complete understanding of protective immunity in these medical conditions. The relative contribution and balance of the different arms of host immunity, i.e., antibodies and cell-mediated responses, toward protection without adverse effects remains a challenging issue that needs to be addressed for individual disease (21). Furthermore, the immune response to vaccination can be influenced by numerous factors such as gender, age, co-existing medical conditions, genetic variations, and lifestyle (22). While vaccines have traditionally been delivered as inactivated or attenuated preparations, recent developments of non-viral vaccine systems offer potential additional solutions to meet the new challenges of vaccine development, especially during epidemic or pandemic situations. This article focuses on new non-viral vaccine development technologies and their implications for combating on-going and emerging communicable and non-communicable diseases.

EMERGING TECHNOLOGIES IN NON-VIRAL VACCINE DEVELOPMENT

Virus-Like Particle and Nanoparticle Subunit Vaccines

Subunit vaccines deliver antigens as purified proteins, which confer the advantage of enhanced safety and scalability compared to whole-pathogen vaccines due to the lack of the requirement for the expression of all viral components and the ability to express and purify any particular antigens of interest in large quantity. A disadvantage of subunit vaccines is that they are generally less immunogenic in nature and therefore require adjuvants and repeated vaccination doses (2). Several approaches have been used to increase the immunogenicity and stability of subunit vaccines, such as virus-like particle (VLP) vaccines and nanoparticle (NP) vaccines.

VLP vaccines use platforms capable of producing particles that mimic the structure of authentic viruses. VLP vaccines can be produced by expressing antigenic proteins in a eukaryotic or prokaryotic system, resulting in the formation of particles with an inherent ability of the antigenic proteins to self-assemble (23) (**Figure 1**). Alternatively, VLP vaccines can also be made by producing blank VLP templates and then chemically linking antigenic peptides onto the pre-formed particles (23). Because these VLPs do not contain a viral genome, they are unable to replicate in cells and therefore have an improved safety profile compared to live viral vaccines (24). Yet, VLP vaccines can often fully activate immune systems of the vaccinated individual. VLPs are taken up by dendritic cells, where they are processed and presented on MHC class I and II molecules to activate the adaptive immune response. Subsequent stimulation of $CD8^+$ T cells and $CD4^+$ T helper cells leads to activation of cell mediated responses and B cells (and antibody production), respectively (23, 25–29). As a result, VLP vaccines are considered highly immunogenic and can stimulate robust cellular and humoral immune responses due to their highly repetitive display of antigenic epitopes (30, 31). A number of VLP vaccine candidates are now clinically applicable with some notable examples including the hepatitis B vaccine (HBV) Engerix (32), the human papillomavirus vaccine (HPV) Cervarix (33) from GlaxoSmithKline (GSK), the HBV vaccine Recombivax® (34), and the human papillomavirus (HPV) vaccine Gardasil® (35) from Merck & Co, Inc. VLP vaccines that are currently in clinical trials include vaccines for malaria (36, 37), influenza (38), rotavirus (39, 40), tuberculosis (41), Zika virus (42), and HIV (43, 44). Efforts to further increase the immunogenicity of VLP vaccines include optimizing antigen design and production platforms (of primarily bacterial origin) (45).

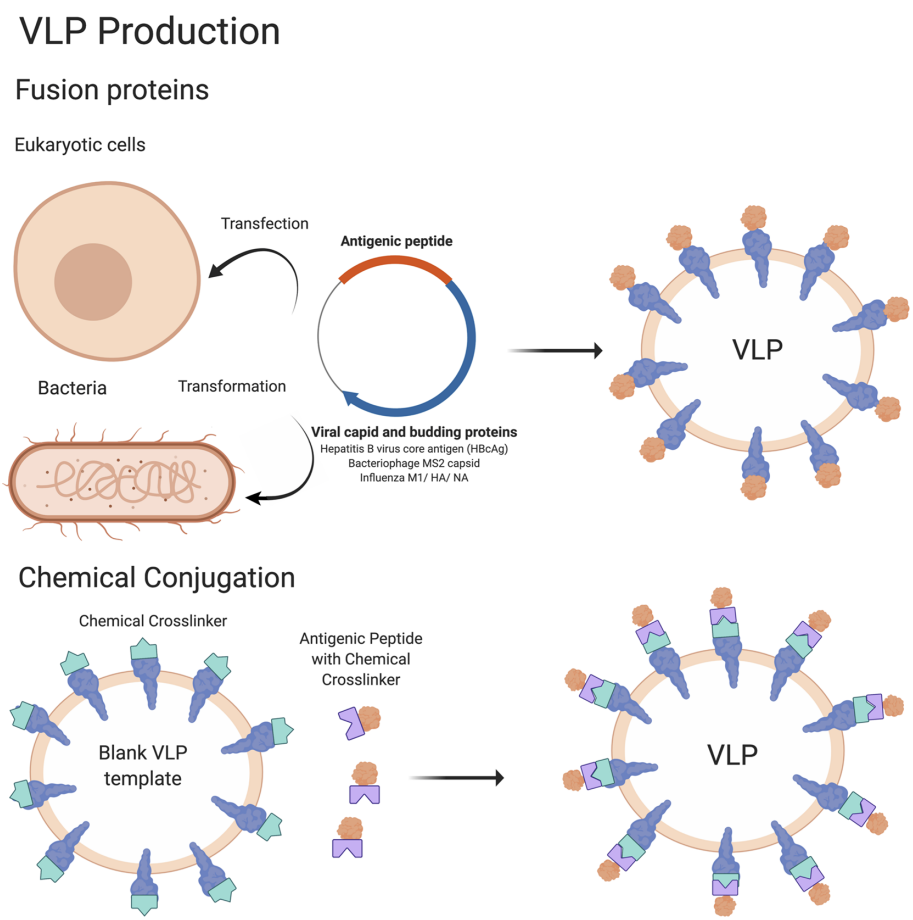


FIGURE 1 | Schematic of VLP vaccine production. The methodology to produce VLP vaccines is summarized in this cartoon. In brief, VLP vaccines are produced by transfecting eukaryotic cells or transforming bacterial cells with a DNA plasmid encoding an antigenic peptide attached to a viral capsid and/or other protein that is sufficient to form VLPs. The antigenic peptide is present on the outside of the VLP which becomes available for interaction with the immune system. Antigens conjugated with a chemical crosslinker can also be attached to VLPs containing external proteins conjugated to a complementary chemical crosslinker, which will result in antigens being linked to the VLP and being presented on the outside edges. Figure created using BioRender software.

NP vaccines are produced by chemically crosslinking protein antigens and carrier molecules to increase immunogenicity and decrease degradation of the antigens (45). These carriers can be organic (primarily lipid-based) or in-organic (primarily polymeric or metal based) (**Figure 2**) (46–49). More recently, self-assembling protein NPs, which consist of oligomers of monomeric protein, have been found in some cases to also provide the benefit normally afforded by a carrier (47, 50). NPs have similarly high rates of stability as VLPs, but they do not stimulate the innate immune response to the same extent as VLPs. However, NPs are simpler in design than VLPs by lacking the multiple protein components of VLPs, which further decrease their cost of production and increase their reproducibility and safety. The challenges associated with decreased immunogenicity of NP vaccines as compared to VLP vaccines can be partly addressed by adjusting the carrier to the desired antigen, based on factors such as size, surface charge, shape and hydrophobicity (46, 47, 50). Additionally, carriers can be used to directly target NPs to immune cells and to increase cross-presentation by antigen-presenting cells (APCs) (46, 47).

DNA and RNA Vaccines

Another promising area of vaccine development includes vaccines that are based on nucleic acids: DNA or RNA vaccines. These vaccines have gained popularity due to their cost-effectiveness, ease of design and production, attractive biosafety profile, and, in the case of DNA, stability. Nucleic acid vaccines have gained particular attention for their potential to rapidly produce vaccines against emerging infectious diseases such as those currently being tested against SARS-CoV-2, the causative agent of COVID-19, which will be discussed in some detail below.

The immunogenic and protective efficacy of DNA vaccines have been demonstrated repeatedly *in vitro* and in small animal models, and a limited number of DNA vaccines have been approved for veterinary use (51). However, DNA vaccines tend

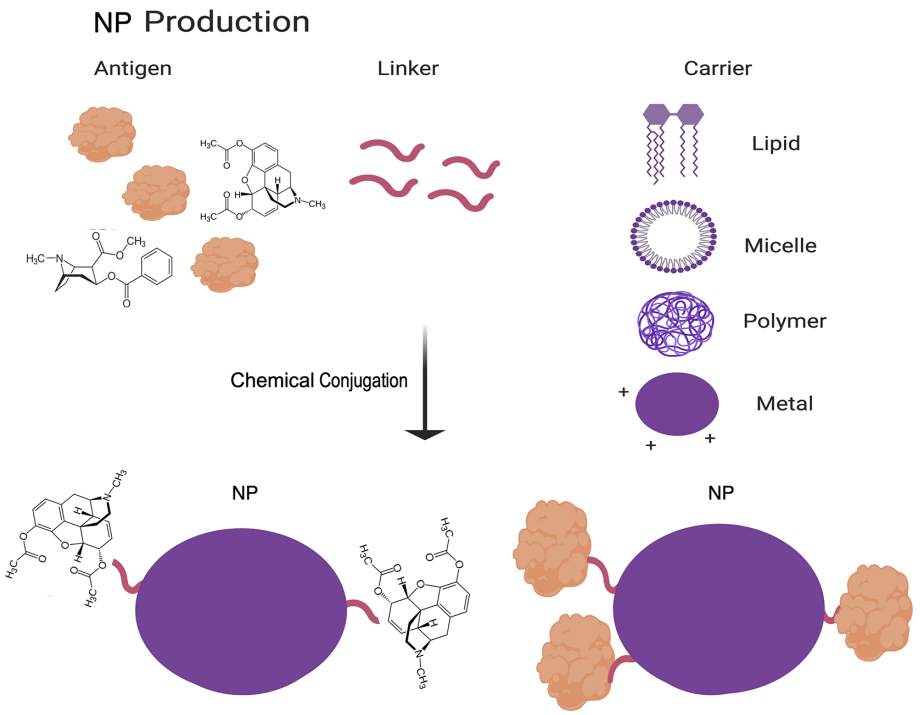


FIGURE 2 | Schematic of NP vaccine production. The methodology to produce NP vaccines is summarized in this cartoon. In brief, NP vaccines are produced by assembling a complex of antigens, a linker molecule, and a carrier molecule by chemical conjugation. Figure created using BioRender software.

to induce poor immune responses in humans and other large animal models (52). One possible explanation may be that intramuscular injection, which has been the most studied route of DNA vaccine administration in humans, tends to elicit mostly cell-mediated immune responses (53), which is likely due to significantly lower APC populations residing in muscles and antigen presentation dominated by MHC I (51). Alternatively, DNA vaccines can be coated with gold NPs and administered intradermally by a gene gun (**Figure 3**). While preliminary data suggest that this method may increase humoral responses to DNA-based vaccines (51), it is limited by its low dose per administration (54). In vivo electroporation (permeabilization of the skin by an electric current to allow plasmid DNA uptake) has thus far been shown to have the highest immunogenicity in multiple small animal models (51, 54) and has been tested in two phase I clinical trials for HIV vaccination with some promising results. In the first clinical trial, the immune system was primed with a DNA vaccine encoding the IL12 gene followed by a boost dose with the recombinant VSV-based HIV vaccine (55). The second trial evaluated the cellular immunity induced by HIV DNA vaccines through intramuscular injection administered by electroporation (56). Other efforts are being undertaken to increase the immunogenicity of DNA vaccines such as codon optimization, optimal promoter usage and epigenetic design, generating nanocarrier plasmids to increase stability and plasmids fused to proteins that specifically target APCs, adjuvant use (which will be discussed in some detail below) and short hairpin RNA (shRNA) targeting of host cells that decrease immunogenicity to DNA vaccines. These approaches have been extensively reviewed elsewhere (51, 54).

A recent development involves the successful use of mRNA as a protective vaccine. While mRNA was originally found to be viable for *in vivo* gene transfer in the early 1990's, the development of mRNA vaccines was initiated much later due to the inherent instability of mRNA compared to DNA (57). The efficacy of mRNA vaccines can be increased by several factors, such as ensuring mRNA purity, adding 5' Kozak and cap sequences, 3' poly-A sequences and modified nucleosides to increase mRNA stability and decrease detection by the receptors of innate immune cells, codon optimization, introduction by intramuscular, and intradermal injection to reduce RNA degradation, and by generating thermostable mRNA (57–59) (**Figures 4**, **5**). Methods to encapsulate RNA have also been explored to increase the stability and immunogenicity of RNA vaccines, as has been used with exosome encapsulated RNA (60) and RNA-transfected dendritic cells (61, 62). When fully optimized, RNA vaccines may have an immunogenic advantage over DNA vaccines due to the presence of multiple cellular pathways that activate innate immunity in response to foreign RNA such as the toll-like receptors (TLRs) and RIG-I-like receptors (RLRs) (63, 64).

In addition to the aforementioned non-replicating RNA, RNA vaccines can include self-replicating or self-amplifying RNA molecules that are normally based on positive-strand RNA viruses of which the structural genes are replaced by antigens (57, 58) (**Figure 4**). One study comparing the efficacy of conventional mRNA versus self-amplifying RNA found that both were effective

DNA vaccines

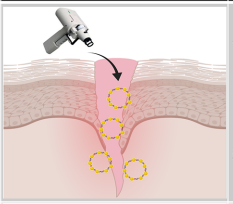
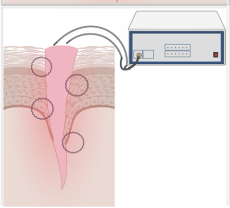
Method Name	Method Diagram	Description of Method	Results
Gene Gun		DNA plasmid is coated with gold particles and is taken up by cells via a blast of pressurized helium gas from a gene gun.	May increase humoral responses but is limited in allowable dosage
Electroporation		Plasmid is taken up by permeabilized skin followed by an electric current.	Maximizes DNA vaccine delivery efficiency. Currently in clinical trials

FIGURE 3 | Methods of improving DNA vaccines. The various methods that have been developed to improve the stability and immunogenicity of DNA vaccines are summarized in this chart. A number of design and delivery mechanisms have contributed to improving the performance of nucleic acid vaccines, such as methods of clinical delivery, genetic engineering, and linking nucleic acid vaccines to cells or biomolecules. Figure created using BioRender software.

in protecting mice against influenza infection, but that self-amplifying RNA elicited protection at a much lower RNA dose and induced a delayed yet longer-lasting antigen expression (65). Self-replicating RNA transfected into dendritic cells (66) has been shown to induce an immune response *in vivo* (67). RNA vaccines have been used in a number of studies in animal models (68) and have recently completed phase 1 clinical trials for rabies (57, 69) and influenza (68, 70). Both trials had similar safety profiles with most patients experiencing mild to moderate reactions to administration and a few patients experiencing more severe reactions. Both vaccines also demonstrated immunogenicity through neutralizing antibody levels, though antibody levels with the rabies vaccine were more highly dependent on dose and route, with needle-free intradermal dose able to sustain neutralizing antibody levels in half of the number of vaccinated individuals one year after injection but not with those receiving intramuscular or intradermal injections (57, 69). Additionally, phase 1 clinical trials are currently underway to test the self-replicating RNA vaccines for HIV and Zika virus (68).

Several challenges face the development of DNA/RNA vaccines. First, while DNA and RNA vaccines may avoid the safety concerns due to microorganism-based vaccine formulations, they have safety concerns of their own. While an early study suggested that DNA vaccination might result in some instances of random chromosomal integration, it was determined that this occurred with a significantly lower frequency than random genetic mutations (71). However, a subsequent study did not observe chromosomal integration to occur following DNA vaccination (51). The possibility of introducing unwanted bacterial DNA elements (such as antibiotic resistance genes to the gut microbiome) has been raised as a safety concern for DNA vaccination, but as of yet it has not been proven (51). As such, regulatory guidelines have been put in place for new DNA vaccine clinical trials in the United States and Europe (72). Vaccine formulation based on mRNA has the advantage of being produced in cell-free systems that can eliminate the concern of bacterial contamination and also lack the potential for chromosomal integration and long-lasting expression (57). While the World Health Organization (WHO) has recently classified mRNA vaccines as its own therapeutic class (73), similar regulations have not yet been developed due to the more limited testing of mRNA vaccines in humans.

It has been found that DNA vaccination primarily induces antigen expression at the site of administration with significantly lower levels being observed elsewhere (51), which may partly explain its poor immunogenicity. While less is known about the levels of on- and/or off-target expressions seen with RNA vaccination, they are presumed to be generally lower than DNA vaccine due to the decreased stability of RNA. However, off-target antigen expression may be relatively minor in shaping the immune response, as the route and mode of DNA/RNA vaccine delivery can markedly alter vaccine immunity, but the mechanism is yet to be fully understood. Generally, intramuscular or intradermal injection is used in animal models and human volunteers to elicit protection against infectious disease to maximize delivery to APCs, while intraperitoneal or intravenous injection has been used in selected animal models to induce systemic expression in therapeutic models, such as cancer vaccination (57). These findings implicating localized dosage routes as most effective for eliciting immunity from nucleic acid vaccines may help explain why gene gun and electroporation have been found to be the most effective routes for DNA vaccine administration. The most effective dosage routes may also be similar for RNA vaccines, as intradermal and intramuscular injection have repeatedly been found to be the most effective delivery routes for RNA vaccines, and needle-free delivery systems may also be more effective than injections (57). Interestingly, immunity can still result from injection of naked RNA in certain models, but it has been found

RNA Vaccines

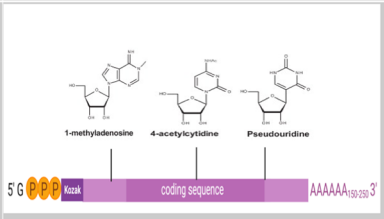
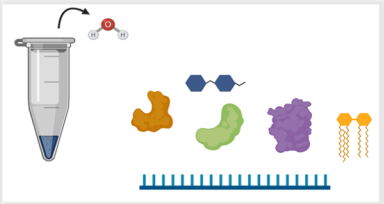
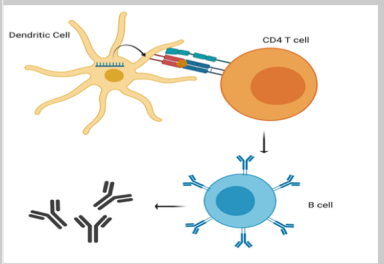
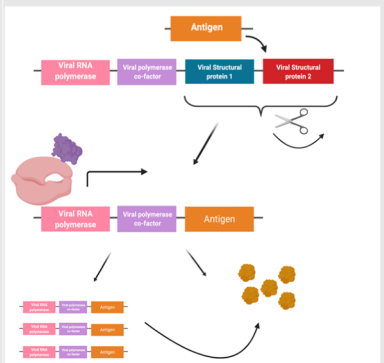
Method Name	Method Diagram	Description of Method	Results
RNA Engineering		Adding 5' cap and Kozak sequences and 3' poly-A sequence to mimic structure of endogenous mRNA to avoid detection by innate immune sensors, and adding modified nucleosides to increase RNA stability	Widely used in vaccine design
Thermostable RNA		Freeze-drying RNA and incubation with various biomolecules increase RNA stability even at high temperature	Widely used in vaccine design
Dendritic Cell Vaccines		RNA is transfected into dendritic cells in-vitro, which can then activate CD4 T cells and stimulates antibody production in vivo.	Most explored for use as cancer vaccines- several in clinical trials.
Self Replicating RNA		The structural proteins in the genome of an RNA virus are replaced with the coding sequence for the antigen- the viral polymerase can keep producing RNA which amplifies and maintains the amount of the antigenic protein.	Pre-clinical testing has shown effectiveness in inducing protection.

FIGURE 4 | Methods of improving RNA vaccines. The various methods that have been developed to improve the stability and immunogenicity of RNA vaccines are summarized in this chart. A number of design and delivery mechanisms have contributed to improving the performance of nucleic acid vaccines, such as methods of clinical delivery, genetic engineering, and linking nucleic acid vaccines to cells or biomolecules. Figure created using BioRender software.

in particular that IV administration of naked RNA results in rapid RNA degradation (57).

Rationally Designed Vaccines

A key aspect of non-viral vaccine development involves the selection of antigens that can effectively elicit a protective immune response. Whereas traditional vaccines are generally developed through attenuation or inactivation of pathogens and through the incorporation of few selected antigens as vaccine components, new technologies have recently been applied toward antigen discovery and design. For example, "reverse vaccinology" refers to the ability to screen the complete antigen sets based on whole-genome sequencing of pathogens for the ability to induce protective antibody responses. Combinations of reverse vaccinology and traditional vaccine approaches allow for an efficient development of immunogenic vaccine candidates (74, 75).

Bioinformatic tools have contributed greatly to vaccine design and evaluation in recent years. Computational approaches are continually improving in their ability to predict T and B-cell epitopes from the complete antigen pools and to rationally design antigens with potential long-lasting protective immunity. Such algorithms calculate antigen-antibody interaction energies and structures (76) that increasingly bridge modeling based on

DNA and RNA Vaccines

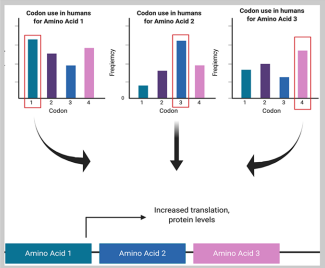
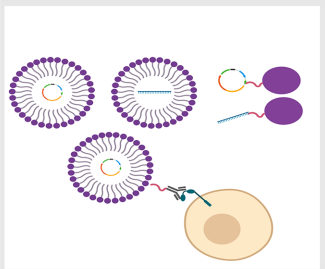
Method Name	Method Diagram	Description of Method	Results
Codon Optimization		The codons with the most usage in the species of the vaccine target are used in the sequence of the antigen.	Widely used in vaccine design
Nanoparticle Vaccines		Nucleic acids are attached to or encapsulated by a carrier, which can also be attached to an antibody for specific cell targeting.	Wide variety of techniques undergoing preclinical and clinical testing

FIGURE 5 | Methods of improving DNA and RNA vaccines. The various methods that have been developed to improve the stability and immunogenicity of both DNA and RNA vaccines are summarized in this chart. A number of design and delivery mechanisms have contributed to improving the performance of nucleic acid vaccines, such as methods of clinical delivery, genetic engineering, and linking nucleic acid vaccines to cells or biomolecules. Figure created using BioRender software.

existing templates and free-modeling based on heterologous structures and database consensus design (77). Deep sequencing combined with computational analysis allows for thoroughly characterizing the B cell repertoire in survivors of disease to identify the protective immunity (78).

A computationally designed antigen found to be protective in animal models was first reported for the respiratory syncytial virus (RSV) F antigen in 2013 (79). Many challenging vaccine targets have since been developed rationally for HIV (80–82), hepatitis C (83, 84), herpes (85), Zika (86, 87), RSV (82), HPV (88), as well as for bacteria (82, 89), fungi (90), and cancers (91). Rational vaccine design has also been utilized to improve VLP and NP vaccines by selecting a repetitive and predictive protein backbone structure for enhanced antigen presentation (92). Finally, rational antigen design is being explored for activation of dendritic cells (93) such as targeting C-type lectin receptors to activate antigen presentation in the context of the pathogens (e.g., Ebola and HIV) (94). The first rationally-designed vaccine to undergo human clinical trials is the anti-malarial vaccine Mosquirix, which was approved for use by the European Medicines Agency in 2015 (95). Human clinical trials have not yet begun for other rationally designed vaccines, however. A key point to note is that rationally designed vaccines require a comprehensive knowledge of the biology and immune response to a pathogen (95), and rational design is therefore more difficult to implement for rapidly emerging diseases.

A major challenge to rational vaccine antigen design is the lack of knowledge of T cell epitopes compared to B cell epitopes. Most successful antigens are expected to elicit both B and T cell responses. Quantitative databases have been developed more for predicting B cell receptor (BCR) epitopes than T cell receptor (TCR) epitopes. BCR epitopes can be predicted in part by the structural and chemical properties of the epitopes due to BCRs recognizing primary and tertiary antigen structures, while TCR epitopes have to be predicted based on known TCR epitopes because they only recognize the primary structures. Increasing capacity for identification of TCR epitopes by machine learning from known epitopes will likely help to mitigate this inequity (74, 75, 96).

NON-VIRAL VACCINE SYSTEMS TO ADDRESS ONGOING CHALLENGES OF VACCINE DEVELOPMENT

Vaccines for Immunosuppressed Individuals

A fundamental challenge to vaccination is the limited immunogenic response to vaccines seen in immunosuppressed individuals, namely, young children, the elderly, and those who are immunocompromised for medical reasons. The underlying causes of immunosuppression in each of these populations vary, and their underlying mechanisms should be taken into account when creating the best vaccine approach.

Young children, in particular infants and neonates, are considered to be immunosuppressed due to age-specific immune

system developments that result in children being particularly susceptible to infection (97). The specific mechanisms for the immunosuppression in this population vary, but one prominent example is the decreased expression of Th17 supporting cytokines by TLR receptors and increased expression of anti-inflammatory cytokines in neonates and particularly in premature newborns (98). On the other hand, the immunosuppressive phenomenon observed in the elderly has been referred to as immunosenescence (99) and it is caused by a number of complex changes resulting in impaired innate and adaptive immune responses (100–104), degradation of lymphoid architecture (105), and increasing proinflammatory cytokines and chemokines (106, 107). To highlight a few important changes, dendritic cells have reduced uptake of antigens (108, 109), macrophages are unable to phagocytose apoptotic cells (110), the number of naïve T cells decreases (111, 112), and B cell repertoire decreases (113). These age-related changes in the ability of the immune system to respond to infection differ from the challenges to vaccination presented by conditions or medications that result in immunosuppression. One such example of a medication that results in immunosuppression is steroids, which have been reviewed extensively elsewhere (114). Steroids exert many effects on immune cells, such as the reprogramming of dendritic cells to tolerogenic dendritic cells (115–117). These cells induce the formation of regulatory T cells (118).

The development of vaccines that can potently activate the innate immune response without using live attenuated vaccines is a central focus of vaccine development for immunocompromised individuals. This is particularly relevant for subunit vaccines, as they do not contain potential viral genomic elements that can act as pattern-associated molecular patterns (PAMPs) to activate innate immune responses. A major approach toward increasing the immunogenic response to a subunit vaccine is through the use of adjuvants. Originally discovered by including food products in equine vaccines and inducing sites of localized sterile inflammation and abscesses, the adjuvant repertoire has since been greatly expanded (119). The so-called "first generation adjuvants", which remain the most common adjuvants in clinical use, include aluminum salts (alum) and mineral oil-in-water emulsions, which function by promoting the migration of APCs to the sites of intramuscular injection (120). However, the use of these adjuvants is limited greatly by the recruitment of only a comparatively small population of immune cells that are made up of APCs (120) and a markedly Th2 response with little to no cellular immune response (120). This has become the aim of current research to design new adjuvants that can increase the breadth of the innate immune responses to the vaccine.

Much effort has been focused on enhancing the usable adjuvant repertoire to further customize the immune response and to avoid the Th2-dominated immune response seen with some adjuvants (e.g., alum) and instead support a Th1 response in certain circumstances. Specifically, a response skewed toward Th2 response is desired for antibody production and antiparasitic immune response, while a Th1 response is desired for intracellular or viral pathogens. Skewing toward Th1 or Th2 is thought to occur after APCs stimulate certain cytokine gene expression profiles (121). For example, LPS-derivative–based AS04 is being used in hepatitis B and HPV vaccines (122) and has been found to increase cell-mediated immune responses in patients with end-stage renal disease (123). Other adjuvants that have been developed to induce a Th1 immune response include IC31® (124, 125), GLA-SE (125, 126), and CAF01 (125, 127). In addition to the Th1 skewed immune response that these adjuvants displayed, GLA-SE induced antibodies and CAF01 showed a Th1/Th17 response (125). Increasing the breadth of the immune response to vaccines thus can enhance the safety and effectiveness of vaccines for both immunosuppressed populations as well as the general population.

There has been an increasing effort toward developing new vaccines that may induce a safe and immunogenic response in immunocompromised individuals. As an example, DNA vaccines could be used to encode for antibodies that could be safely and temporarily expressed in immunocompromised patients, such as throughout the course of an influenza season. Recent studies that tested the efficacy of influenza neutralizing antibodies found that protection against lethal disease could be conferred by plasmids expressing antibodies given intramuscularly by electroporation (128). However, several major considerations need to be fully addressed before these techniques can be developed for human use. Specifically, the duration and the stability of plasmid vaccination have not yet been fully characterized in humans. Additionally, it has been shown that anti-dsDNA antibodies can be produced by primary B cells isolated from mice treated with plasmid DNA (129), which appear similar to anti-dsDNA antibodies that have been shown to be expressed during systemic lupus erythematosus (130, 131). The anti-dsDNA antibodies may prefer to bind to certain CpG-rich sequences on bacterial DNA of the plasmid (129), which might serve as a means for DNA vaccine optimization. Finally, the purity of plasmid DNA stocks needs to be thoroughly confirmed in order to avoid possible stimulation of unwanted immune reactions.

Other novel adjuvant approaches include the surfactant and emulsifier-based AS03 that are currently being used in influenza-pandemic vaccines (132). LPS-derivative–based AS04 is being used in hepatitis B and HPV vaccines (122) and has been found to increase cell-mediated immune responses in patients with end-stage renal disease (123). Lipid products that form micelles in solution and act as solid particle carriers are another form of adjuvants that can activate innate immunity, as seen with CAF01 (133, 134) and AS01B/E formulation from GlaxoSmithKline (135), which are used in the only currently available vaccine for malaria (136, 137). Several other adjuvants currently in use primarily function as TLR agonists (138–140). TLR agonists have shown promise in aged and young mice (141) as various TLR agonists [e.g., CpG (TLR9), poly(I:C) (TLR3), and pam3CSK4 (TLR1 and TLR2)] can induce expression of co-stimulatory molecules on APCs (141). Another adjuvant approach taken to overcome the immune challenges presented by young children is the use of ß-glucan. These sugars, found in the cell walls of some pathogens, activate dendritic cells through the CLEC71-SYK-CARD9 pathway, and it was shown to provide protection against

tuberculosis infection (142). Recently, defective-interfering (DI) viral particle vaccines have also been explored for use as adjuvants to increase the innate immune response (143–152). These are VLPs with aberrant and defective genomes, which have been found in some cases to increase the innate immune response when compared to the replicating virion. Taken together, several innovative strategies are currently being developed to increase the immunogenicity and safety of vaccines for immunocompromised populations.

Vaccines With Non-Traditional Antigens

Because of their increased safety and versatility, such as the ability to deliver a diverse range of molecules as antigens, VLP and NP vaccines have the potential for use to provide immunity against non-protein antigens. A prime example is the development of NP vaccines to treat substance abuse disorders by attaching a drug molecule to a hapten carrier (**Figure 2).** Vaccines against drugs of abuse aim to elicit a humoral (antibody) response that can neutralize the drug target before it crosses the blood-brain barrier to induce psychotropic effects, thereby decreasing positive associations with and hopefully dependency on the addictive drug. Such vaccines have an advantage for long-term therapeutic use over pharmaceuticals targeting neural receptors by eliminating the medical complications and safety concerns of directly modulating neural signaling network. They also differ from other vaccines in that they are given to active users of drugs of abuse to prevent escalation of use or relapse and do not depend on herd immunity for effectiveness, so their efficacy is determined by individual responses to the vaccines (153).

A hapten carrier, which is a potent B cell antigen, is used to stimulate B cell responses and thereby also activates B cell responses to the attached drug. Therefore, hapten and linker design are of particular interest to ensure structural integrity and to maximize B cell responses (153–170). The vaccine can also be linked to a protein carrier designed to activate T cell responses (and particularly CD4 T cell response to then activate B cells) (153, 160, 171–181), though there has not been a clear determination of whether an increased CD4 Th2:Th1 ratio correlates with efficacy for vaccines against drugs of abuse (153). Finally, an additional consideration in designing vaccines against drugs of abuse is determining whether to target the drug itself or its possibly more psychotropic metabolites that can provide a greater level of protection. A prime example of this is heroin vaccines seemingly being most effective when they can structurally mimic the psychotropic heroin metabolite 6-acetylmorphine (6AM) (166, 172, 182). It should be noted that clinical trial results have only been reported for vaccines against nicotine and cocaine addictions, with the vaccines demonstrating efficacy only in a subset of patients that were able to achieve high neutralizing antibody titers (153, 183–186). The recent vaccine developments to ameliorate drug abuse have been reviewed more extensively elsewhere (153, 160, 187).

Additionally, VLP and NP vaccines are being used in toxoid vaccine formulations, which provide quick neutralization against a cytotoxic molecule (primarily bacterial toxins) that cannot be expressed in its full and activated form. The most well-known example is the diphtheria, tetanus and pertussis (DTaP) inactivated subunit vaccine which has been in use for decades and can elicit effective immune responses against the toxin produced by any of these bacteria if/when the vaccinated individual happens to be exposed to them. The pertussis component of DTaP has a demonstrated high level of safety that it is one of only two known vaccines (besides influenza) that is given to pregnant women in several countries (188). Current clinical trials are focusing on testing potentially more effective toxoid vaccines for pertussis (189) as well as *Haemophilius influenzae* type b, polio virus, and hepatitis B virus (190). It has also been found that using bacterial membrane or red blood cell (RBC) membrane micelles as a carrier can significantly increase the immunogenicity of the vaccines, and pre-clinical testing is currently underway to use these carriers in vaccine development against the multi-drug resistant bacteria *Staphylococcus aureus* (MRSA) (191).

Therapeutic Vaccines for Noncommunicable Diseases

As our capabilities for vaccine development and production have expanded, a paradigm shift has recently taken place to use vaccines for disease treatment in addition to disease prevention. These therapeutic vaccine designs rely on the identification of protein markers unique to a disease phenotype which may evade the development of an immune response due to the markers not being recognized by the APCs. For example, cancer vaccines to elicit immune responses against cancer-specific antigens are one of the most widely studied therapeutic vaccines to date due to the inherent challenges of developing effective cancer therapeutics and a need for targeted treatment. The immunosuppressive environment present in cancers has made developing cancer vaccines a significant challenge, especially for vaccines that rely on viral vectors. Therefore, the improved safety profile of non-viral vaccines offers an attractive potential for cancer vaccine development. Non-viral vaccines also confer an additional advantage for developing cancer vaccines in that cancer vaccines may be most effective when an antigen specific to the mutational profile of the individual cancer is used (192–194), particularly in combination to overcome immune tolerance (195). The time needed to make a vaccine against an individual antigen or against a combination of individual antigens is greatly reduced with non-viral vaccines due to the ease of encoding an antigen on a nucleic acid vaccine or purifying protein for a subunit vaccine in comparison to incorporating a personalized antigen into a viral vaccine, growing viral stocks and verifying its expression (196).

Nucleic acid vaccines have been a key area in recent developments for cancer treatment. While a number of DNA cancer vaccine candidates have entered into early phase clinical trials (197), RNA vaccines are thought to have particularly encouraging potential due to their increased immunogenicity compared to DNA vaccines (68, 198). Preliminary results indicate that intranodal injection of naked tumor antigen-encoding mRNA can control tumor growth in mouse cancer models (199–202). Additionally, naked mRNA was found to be

immunogenic *via* intradermal injection in a phase I/II clinical trial for prostate cancer (203). However, a key challenge in developing RNA cancer vaccines has been the need to further ensure the stability of the RNA and to increase its targeting to APCs (204, 205) in order to overcome the immunosuppressive environment of cancers. Developing a delivery vehicle for RNA cancer vaccines has therefore been a central focus of research and development in this area.

Loading RNA into liposomes is one method that has shown some success in controlling cancer growth in mouse models (206–208) and has demonstrated some preliminary efficacy in early stage clinical trials for use as a delivery system for anti-cancer genes (209, 210) and siRNAs (211), with different liposome constructs targeting RNA localization to the spleen. RNA-loaded liposomes can also be targeted directly to T cells by using RNA that encodes for anti-CD3 along with the cancer antigens, bypassing the need for recognition by APCs. This concept has notably been tested in conjunction with chimeric antigen receptor (CAR) T cell therapy (198, 212, 213). Another method bypasses targeting RNA to APCs by directly transfecting dendritic cells (DCs) with RNA extracted from tumors or RNA encoding tumor antigens (214–217) **(Figure 4)** and then introducing the engineered DCs into patients with a combination of cytokines and/or checkpoint blockades (218, 219). However, DC-directed RNA vaccines are currently limited by the restrained immune environment present in cancers, which can limit the activity of DCs and increases the activity of regulatory T cells (216, 220, 221). It is thought that these challenges could be mitigated by optimizing the use of cytokines and other factors that would act as adjuvants in combination with cancer RNA vaccines (195, 222–225) and by optimizing DC isolation and culturing conditions (226). A few DC-directed RNA vaccine candidates are currently in clinical trials, including those in phase III (196, 221).

Subunit vaccines have also been developed for use as cancer vaccines (227), which are being tested with many of the same delivery systems as nucleic acid-based cancer vaccines to maximize vaccine targeting to immune cells (228). NP-based vaccines in particular have been developed and tested for use as cancer treatments (228–235), the most notable of which are several HPV vaccines for prevention of cervical cancer (236). While less development has been done on VLP-based cancer vaccines, one notable target that has been used is the widely expressed cancer antigen human epidermal growth factor receptor-2/neu (HER2), which has shown to be immunogenic in mouse models (237–244) and in early clinical testing in humans (245) and dogs (246), but as a whole these vaccines have had to undergo additional design in order to overcome B cell tolerance (227, 247) and to fully characterize their anti-tumor activity.

Because subunit vaccines require antigen presentation in order to elicit an immune response, a primary challenge in VLP- and NP-based cancer vaccine developments has been optimizing their uptake by APCs (228). Vaccine uptake by APCs can be optimized by engineering VLP- and NP-based cancer vaccines to resemble the structure of viral particles as closely as possible, such as by using certain types of carriers (liposomes, polymers and ferritin cages), sizes (20–45 nm) and a spherical shape **(Figure 4).** Subsequently, these vaccines may be most successful when combined with checkpoint blockade treatment by encouraging clonal expansion of lymphocytes (248). VLPs and NPs can also be used as immuno-enhancers, e.g., to deliver cytokines and TLR agonists to target sites, which has been found to boost localized immune responses while avoiding immunopathogenic and possibly systemic inflammation (46).

Vaccines for Rapidly Emerging Viral Diseases

Emerging and reemerging pathogens, such as West Nile virus, pandemic influenza virus, Ebola virus, dengue virus, Zika virus, and the on-going global pandemic SARS-CoV-2 pose great challenges to the public health system. Rapid development and deployment of vaccines are critical to quickly build up resistance against these and other disease "X", which is a term used by the WHO to refer to future unknown disease pandemics (249). The ideal vaccine platform in a pandemic situation must be cost-effective and can be rapidly developed and produced on a large scale to meet global demands. Temperature sensitivity is also a consideration, as cold chain storage can be particularly difficult to maintain in developing countries. Development of heat stable vaccines like the oral bovine rotavirus pentavalent vaccine (BRV-PV, Rotasil® by the Serum Institute of India), which was prequalified by the WHO in 2018, can provide protection against serious diseases in regions where transportation and refrigeration are unreliable (250). In comparison, the only FDA approved Ebola virus vaccine (rVSV-ZEBOV, ERVEBO® by Merck and Co., Inc.) must be stored at −80°C or −60°C (251), which presents a major obstacle for affected countries. Rapid production of low cost, scalable, and temperature stable vaccines is an ongoing challenge in the face of emerged and emerging global disease pandemics.

Currently, rapid development of vaccines is greatly limited by the resources and regulatory policies needed to bring a vaccine from its conceptualization stage to the clinic, which has been estimated to cost between $200 and $500 million dollars and to take 5–18 years (252). Vaccines also tend to be manufactured in countries with larger economic and technical prowess and more robust disease surveillance systems than developing countries and therefore can unfairly influence the equity of vaccine distribution and usage. This was seen in the 2009–2010 influenza pandemic, where 80% of the vaccines were manufactured and used in seven industrialized regions (United States, Canada, Australia, western Europe, Russia, China, and Japan), while the majority of developing regions in the world did not receive any pandemic influenza vaccines until January 2010, 9 months after the WHO declared the influenza pandemic (253). In addition, as mentioned previously, most pandemic vaccines have to be clinically tested during an active outbreak in order to obtain sufficient safety and efficacy data, thereby limiting the number of vaccine candidates that can be deployed to save lives. This was seen during the Ebola outbreak of 2013–2015, when two vaccines were fully developed in

advance of clinical trials but only one (the simian adenovirus-based Ebola vaccine ChAd3-EBO-Z) was tested early enough in the outbreak to obtain sufficient clinical data (254). Similar challenges are also seen in selecting vaccine candidates for the large sample sizes needed for phase III clinical trials for HIV vaccine candidates. Statistical ranking systems to prioritize candidates are being developed to aid in this selection process (255). Zoonotic diseases present additional considerations, as it is economical to vaccinate the multiple species that may act as reservoirs of the pathogen(s) in order to control the spread of the disease. The first vaccine to provide protection in multiple species is the simian adenovirus-based vaccine candidate ChAdOx1 RVF, which has been shown to provide effective protection against Rift Valley Fever virus in sheep, goats, and cattle and is currently undergoing testing in larger livestock field trials and in humans (254, 256).

Other measures have been undertaken to expedite the process of vaccine development and reduce the cost of vaccine production. International institutions allow for collaborative groups to rapidly co-operate on vaccine development and shorten the vaccine manufacturing process. The Coalition for Epidemic Preparedness and Innovations (CEPI) provides funds for clinical trial and stockpiling of vaccines that would not have market incentive in a traditional funding mechanism of vaccine development and manufacturing (257). Such international collaborations will help to bridge the differing vaccine development policies and investitures across countries and use these combined resources to develop vaccines to primarily benefit those living in either underdeveloped or developing nations (258). In a recent example of this, CEPI, Gavi, and the WHO have come together to form COVAX, the vaccines pillar of the Access to COVID-19 Tools (ACT) Accelerator, with the mission to expedite the production of a COVID-19 vaccine to be equitably distributed throughout the world (259).

Technical challenges in vaccine production process can be an impediment. For example, the use of fertilized chicken eggs in vaccine production can pose challenges such as the restricted capacity of egg production, egg allergies, and the emergence of viruses with egg-culture-adapted mutations that can reduce vaccine efficacy (260). The use of animal cells for certain vaccines can also present significant challenges of cost, slow production rates, and potential high risk of contaminations. Other vaccine production systems, such as VLP vaccines produced in yeasts, insect cells and bacterial systems, as well as DNA/RNA vaccines, can benefit from increased robustness of antigen production, decreased risk of contaminations, and quicker time of response (252). This may especially be the case for DNA vaccines, where the increasing capacity of next-generation DNA sequencing, for example, has lowered the time for development of a DNA vaccine from 20 months following the 2003 SARS outbreak to 3.25 months following the 2016 Zika outbreak (261).

COVID-19 Pandemic as a Case Study to Rapidly Develop Non-Viral Vaccines

The ongoing COVID-19 pandemic has presented unique opportunities as well as challenges for vaccine development. Unlike the influenza vaccines, no coronavirus vaccines existed prior to the COVID-19 pandemic. Such a rapid and widespread need for a completely novel vaccine for COVID-19 has resulted in a drive to significantly reduce the length of time required to produce a new vaccine. It has also highlighted the necessity to use non-viral vaccine platforms with overlapping stages of vaccine development, including preclinical and clinical testing and manufacturing that would otherwise be required to happen in a stepwise process for a traditional vaccine development effort (262–264). However, a rapid progression of clinical testing will need to be balanced by the need for obtaining quality data on vaccine safety and efficacy (265), especially considering previous reports of pathological antibody-dependent enhancement responses in some patients immunized with the 2003 SARS vaccine candidates (266, 267). As with previous pandemics, widespread global availability and resource management will be another key consideration for vaccine selection, especially considering the near ubiquitous presence of COVID-19 around the globe and its disproportionate impact on populations of low socio-economic status (268, 269). It is also likely that the approval of multiple vaccine candidates will be most optimal to controlling and ending the pandemic should more than one vaccine prove to be effective in preventing COVID-19 disease. Multiple COVID-19 vaccines would allow for more clinical and regulatory choices to accommodate differences in patient responses (particularly in more vulnerable patient populations) and manufacturing and distribution capabilities (270, 271). Perhaps, with these considerations in mind, non-viral COVID-19 vaccine platforms (e.g., DNA and mRNA) have been selected among the first candidates to enter clinical testing, partly for their aforementioned reasons of safety profiles and relative ease of manufacturing **(Table 1).** Some of the RNA-based COVID-19 vaccines (all of which are currently in various stages of clinical trials) include but are not necessarily limited to:

1. The mRNA-1273 vaccine developed by the U.S. biotech company Moderna (272).
2. The mRNA CVnCoV vaccine developed by the German company CureVac (273).
3. A group of 4 RNA vaccines under the name BNT162 developed by the German company Biontech that consists of two nucleoside-modified mRNAs, a uridine-containing mRNA and a self-amplifying mRNA (274), which in an early phase I/II trial, the nucleoside-modified mRNA BNT1621b has been shown to elicit neutralizing antibodies (275) and is better tolerated particularly in older adults than BNT1621a (276, 277).
4. The self-amplifying mRNA LNP-nCoVsaRNA (COVAC1) vaccine from the Imperial College London (278).
5. The mRNA vaccine LUNAR-COV19 (ARCT-021) from US company Arcturus Therapeutics (279).
6. An unnamed mRNA vaccine candidate from Chinese company Yunnan Walvax Biotechnology (280).

Some of the COVID-19 DNA vaccines (all of which are also in various stages of clinical trials) include but are not necessarily limited to:

TABLE 1 | Non-viral vaccines currently in development for SARS-CoV-2*.

Vaccine Name	Vaccine type	Company and Country	Preliminary results
mRNA-1273	mRNA	Moderna, USA	• Self-reported preliminary data indicating all patients developed neutralizing antibody response. Patients developed moderate side effects with highest dose (250 ug) were eliminated from future study. • Entered phase III clinical trials in July 2020 with targeted enrollment of 30,000 people
CVnCoV	mRNA	CureVac, Germany	• Entered phase II clinical trials in August 2020
BNT162	mRNA (4 candidates)	Biontech, Germany	• Early phase I/II trial data showed that patients who received nucleoside-modified mRNA BNT1621b produced neutralizing antibodies. • Further phase I/II clinical trial data showed that BNT1621a and BNT1621b produced similar neutralizing antibody titers but that BNT1621b was associated with less systemic responses particularly in older adults. • BNT162b was selected to continue in phase II/III clinical trials.
LNP-nCoVsaRNA (COVAC1)	mRNA (self-amplifying)	Imperial College London, UK	• Entered phase I/II clinical trials in June 2020 • Transitioned to phase II clinical trials in July 2020
LUNAR-COV19 (ARCT-021)	mRNA	Arcturus Therapeutics, USA	• Entered phase I/II clinical trials in July 2020
Unnamed mRNA vaccine	mRNA	Yunnan Walvax Biotechnology co, China	
INO-4800	DNA	Inovio, USA	• Preliminary phase I data suggest that 94% of participants developed an immune response against the vaccine. • Preprint suggests that a single dose seroconverted vaccinated rhesus macaques. Neutralizing antibodies were produced against the D614 and G614 strains and memory responses lasted at least 4 months after vaccination.
GX-19	DNA	Genexine, South Korea	• Entered phase I/II clinical trials in June 2020
AG0301-COVID19	DNA	AnGes Inc, Japan	• Entered phase I/II clinical trials in July 2020
ZyCoV-D	DNA	Cadila Healthcare Ltd, India	• Entered phase I/II clinical trials in July 2020
bacTRL-Spike	DNA (live bacteria delivery)	Symvivo, Canada	
LV-SMENP-DC	APC (lentiviral)	Shenzhen Geno-Immune Medical Institute, China	
Covid-19/aAPC	APC (lentiviral)	Shenzhen Geno-Immune Medical Institute, China	
AV-COVID-19	APC (antigen-loaded)	Aivita Biomedical, USA	• Entered phase I/II clinical trials in May 2020
NVX-CoV2373	NP	Novavax, USA	• Entered phase I/II clinical trials in May 2020 • Self-reported data from phase I indicate that the vaccine was well tolerated and induced neutralizing antibody responses in all patients after two doses.
SCB-2019	NP	Clover Biopharmaceuticals, China	• Entered phase I/II clinical trials in May 2020
COVAX-19	NP	GeneCure Biotechnologies, USA	• Entered phase I clinical trials in June 2020
MVC-COV1901	NP	Medigen Vaccine Biologics corp, Taiwan	• Entered phase I clinical trials in July 2020
AdmirSC-2f	NP	Adimmune corp, Taiwan	• Entered phase I clinical trials in August 2020
Unnamed spike protein vaccine	NP	University of Queensland, Australia	• Entered phase I clinical trials in June 2020
Unnamed VLP vaccine	VLP	Medicago, Canada	• Entered phase I clinical trials in June 2020

**COVID19 vaccine data compiled with the aid of the BioRender COVID-19 Vaccine and Drug tracker: https://biorender.com/covid-vaccine-tracker.*
This chart summarizes the name, type of vaccine, company and country of origin and preliminary data on existing non-viral vaccines for SARS-CoV-2 that are currently undergoing clinical testing.

1. The INO-4800 vaccine developed by the U.S. pharmaceutical company Inovio (281) with preliminary phase I data suggesting that 94% of participants might have developed an immune response against it following vaccine administration by electroporation (282) and that vaccination in rhesus macaques elicited neutralizing antibodies against both the D614 and G614 SARS-CoV-2 strains (283).
2. The GX-19 vaccine developed by the South Korean company Genexine (284).
3. The AG0301-COVID19 vaccine developed by the Japanese company AnGes, Inc. (285, 286).
4. The ZyCoV-D vaccine developed by the Indian company Cadila Healthcare Ltd (287).
5. The live bacteria-mediated plasmid delivery system bacTRL-Spike developed by the Canadian company Symvivo (288).

Genetically engineered APCs are also being pursued as potential COVID-19 vaccine candidates, with DCs transfected with lentiviral vectors expressing COVID-19 antigens currently being tested in China (289, 290) and in the United States (291). Meanwhile, COVID-19 VLP- and NP-based vaccines have also advanced into clinical trials, including the NP NVX-CoV2373

vaccine from the U.S. company Novavax (292), the NP SCB-2019 vaccine from the Chinese company Clover Biopharmaceuticals (293), the NP COVAX-19 vaccine from the U.S. company GeneCure Biotechnologies (294), the NP vaccine from the Taiwanese company Medigen Vaccine Biologics (295), the NP vaccine AdmirSC-2f from Taiwanese company Adimmune corp (296), an unnamed NP vaccine from the University of Queensland (297, 298) and an unnamed VLP vaccine from the Canadian company Medicago (299). CEPI has collaborated in the development and testing of a selected number of these vaccine candidates (273, 281, 298).

It should also be noted that several viral vectored vaccine candidates for COVID-19 have also entered in clinical testing **(Table 2).** Several adenovirus vectored vaccines are currently the furthest along in clinical testing. One example is the vaccine candidate AZD1222 (formerly known as ChAdOx1 nCov-19), a replication-defective chimpanzee adenovirus developed by Oxford University which entered phase III clinical trials in August 2020. This viral vector was chosen due to its previous application as a vaccine vector for Middle East respiratory syndrome coronavirus (MERS-CoV). The ChAdOx1 vector encoding the spike (S) protein provided protection against six different strains of MERS-CoV in rhesus macaques (25), demonstrating its ability to be an effective vaccine for coronaviruses. Specific to COVID-19, AZ1222 was found to induce humoral and cell mediated immune repsonses in phase I/II cliical trial and did not result in any instances of severe side effects (300). It has recently found that AZD1222 could induce a robust humoral, CD8 and Th1 dominant CD4 response in mice and rhesus macaques and that both a prime and a prime-boost regimen protected rhesus macaques against COVID-19 related pneumonia. However, it should be noted that there was no difference in the amount of nasal virus shedding in vaccinated vs unvaccinated animals challenged with SARS-CoV-2 (301).

Two other replication-incompetent adenoviral vectored vaccines for COVID-19 have also entered clinical trials. The Ad5-nCoV candidate from Chinese company CanSino biologics was shown in early clinical trial data to induce significant antibody and T cell responses after a single dose and to have only rare instances of severe side effects that were more prevalent among the higher dose groups (302, 303). The Chinese government has recently approved the vaccine for use among its members of the armed forces (304). Additionally, the Ad26.COV2.S from Johnson & Johnson induced antibody and T cell responses in rhesus macaques after a single dose, and antibody titers negatively correlated with viral titers during viral challenge (305). Finally, the Gam-COVID-Vac candidate from the Gamaleya Research Institute of Epidemiology and Microbiology in Russia is another adenovirus-based vaccine that is the first COVID-19 vaccine to gain government approval for widespread use after a phase I trial. Phase III trials for this vaccine began in August 2020 (306).

Finally, two COVID-19 vaccine candidates based on live-attenuated measles platforms have also entered into early clinical trials. The TMV-083 candidate from the Institut Pasteur and with collaboration with CEPI is a measles vectored vaccine expressing a modified SARS-CoV-2 surface glycoprotein that entered phase I clinical trials in August 2020 (307), while the V591 candidate from Merck also entered phase I clinical trials in August 2020 (308). Many other viral vectored vaccine candidates for COVID-19 are also in preclinical stages of development.

TABLE 2 | Viral vaccines currently in development for SARS-CoV-2*.

Vaccine Name	Vaccine vector	Company and Country	Preliminary results
AZD1222 (ChAdOx1 nCoV-19)	Adenovirus	Oxford University, UK	• Vector was shown to protect rhesus macaques against six strains of MERS-CoV. • Early phase I/II clinical trial data show that vaccine was well tolerated and induced humoral and cell-mediated responses. • Vaccine was found to induce robust humoral, CD8 and Th1 dominated CD4 responses in mice and rhesus macaques, and that both a prime and prime-boost regimen protected rhesus macaques against COVID-19 related pneumonia. • Entered phase III clinical trials in August 2020
Ad5-nCoV	Adenovirus	CanSino biologics, China	• Early phase I/II clinical trial data show that vaccine induced antibody and cell-mediated responses after a single dose and was well tolerated. • Entered phase III clinical trials in August 2020 • Approved by the Chinese government for use by its members of the armed forces
Ad26.COV2.S	Adenovirus	Johnson and Johnson, USA	• Vaccine was found to induce antibody and T cell responses in rhesus macaques after a single dose, and antibody titers negatively correlated with viral titers during viral challenge. • Entered phase III clinical trials in August 2020
Gam-COVID-Vac	Adenovirus	Gamaleya Research Institute of Epidemiology and Microbiology, Russia	• Approved for widespread use by the Russian government before the release of clinical trial data • Entered phase III clinical trials in August 2020
TMV-083	Measles	Institut Pasteur, France	• Entered phase I clinical trials in August 2020
V591	Measles	Merck, USA	• Entered phase I clinical trials in August 2020

**COVID19 vaccine data compiled with the aid of the BioRender COVID-19 Vaccine and Drug tracker: https://biorender.com/covid-vaccine-tracker.*
This chart summarizes the name, viral vector used, company and country of origin, and preliminary data on existing viral vaccines for SARS-CoV-2 that are currently undergoing clinical testing.

SUMMARY

The emergence of new non-viral vaccine technologies has significantly advanced the scope and efficacy of traditional vaccine formulations that are generally based on single protein subunit vaccines or attenuated or killed vaccines. Non-viral vaccine technologies have allowed for new applications to address ongoing challenges of vaccination with customization in the areas of safety, immunogenicity, breadth of protection, scalability, and ease of production. These new technologies have also expanded the notion of what is possible with vaccination by extending their reach to once untenable areas, such as cancer treatment and neutralization of drugs of abuse. It is clear that continued development and optimization of vaccines will require multi-faceted approaches that can only be implemented with extensive cross-field collaboration and periodic review of the current state of vaccinology. These challenges as well as opportunities ensure that vaccine development will remain on the cutting edge of science for decades to come to combat new and emerging pathogens as well as other noncommunicable diseases.

AUTHOR CONTRIBUTIONS

MB, SV, NK, and HL contributed to the literature review and writing of the manuscript. MB prepared all figures and tables. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported in parts by NIH NIAID grant R01 AI131586, USDA-NIFA-Capacity Funds (Hatch and Animal Health), and the University of Minnesota School of Medicine Academic Investment Research Program (AIRP) and COVID-19 Rapid Response Funds to HL and YL, USDA-NIFA AFRI grant #2019-05384 and Minnesota Agricultural Experiment Station Rapid Agricultural Response Fund to HL, and by a pre-doctoral NIH fellowship T32 DA007097 to MB. NIH T32 training grant in Comparative Medicine and Pathology (5T32 OD010993-17) for NK.

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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.

frontiers in Immunology

REVIEW
published: 07 October 2020
doi: 10.3389/fimmu.2020.576622

The SARS-CoV-2 Spike Glycoprotein Biosynthesis, Structure, Function, and Antigenicity: Implications for the Design of Spike-Based Vaccine Immunogens

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Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: *26 June 2020*
Accepted: *16 September 2020*
Published: *07 October 2020*

Citation:
Duan L, Zheng Q, Zhang H, Niu Y, Lou Y and Wang H (2020) The SARS-CoV-2 Spike Glycoprotein Biosynthesis, Structure, Function, and Antigenicity: Implications for the Design of Spike-Based Vaccine Immunogens. Front. Immunol. 11:576622. doi: 10.3389/fimmu.2020.576622

The ongoing pandemic of coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), poses a grave threat to global public health and imposes a severe burden on the entire human society. Like other coronaviruses, the SARS-CoV-2 genome encodes spike (S) glycoproteins, which protrude from the surface of mature virions. The S glycoprotein plays essential roles in virus attachment, fusion and entry into the host cell. Surface location of the S glycoprotein renders it a direct target for host immune responses, making it the main target of neutralizing antibodies. In the light of its crucial roles in viral infection and adaptive immunity, the S protein is the focus of most vaccine strategies as well as therapeutic interventions. In this review, we highlight and describe the recent progress that has been made in the biosynthesis, structure, function, and antigenicity of the SARS-CoV-2 S glycoprotein, aiming to provide valuable insights into the design and development of the S protein-based vaccines as well as therapeutics.

Keywords: SARS-CoV-2, spike glycoprotein, receptor-binding domain, synthesis, structure, membrane fusion, neutralizing antibodies, immunogen design

INTRODUCTION

The coronavirus disease 2019 (COVID-19) global pandemic represents an unprecedented public health, social and economic challenge (1, 2). The etiological agent of COVID-19 is a new member of the Coronaviridae family that is closely related to severe acute respiratory syndrome coronavirus (SARS-CoV) and was recently referred to as SARS-CoV-2 by the Coronavirus Study Group of the International Committee on Taxonomy of Viruses (3). The virus has spread rapidly and sustainably around the global resulting in over twenty-one million cases and more than 750,000 deaths as of August 15, 2020 (4).

Coronaviruses (CoVs) are enveloped positive-sense RNA viruses (5). Enveloped CoVs entering host cells and initiating infection is achieved through the fusion of viral and cellular membranes

(6, 7). Membrane fusion is mediated by the large type I transmembrane S glycoprotein on the viral envelope and the cognate receptor on the surface of host cells (8–10). The surface-exposed location of the S glycoprotein not only allows it to carry out membrane fusion but also renders it a direct target for host immune responses, making it the major target of neutralizing antibodies (11). Because of its central roles in viral infection and eliciting protective humoral and cell-mediated immune responses in hosts during infection (10), the S protein is the primary target for vaccine design as well as antiviral therapeutics (12).

Here, we provide a comprehensive overview of the wealth of research related to the SARS-CoV-2 S glycoprotein biosynthesis, structure, function, and antigenicity, aiming to provide useful insights into the design and development of the S protein-based vaccines as well as therapeutics to prevent or treat the ongoing global spread of SARS-CoV-2/COVID-19.

SYNTHESIS, PROCESSING AND TRAFFICKING OF THE SARS-COV-2 S GLYCOPROTEIN

The SARS-CoV-2 S glycoprotein is synthesized as a 1273-amino acid polyprotein precursor on the rough endoplasmic reticulum (RER) (**Figure 1**) (13). The unprocessed precursor harbors an endoplasmic reticulum (ER) signal sequence located at the N terminus, which targets the S glycoprotein to the RER membrane and is removed by cellular signal peptidases in the lumen of the ER (14, 15). A single stop-transfer, membrane-spanning sequence located at the C terminus of the S protein prevents it from being fully released into the lumen of the ER and subsequent secretion from the infected cell (16, 17). Co-translationally, N-linked, high-mannose oligosaccharide side chains are added during synthesis (18, 19). Shortly after synthesis, the S glycoprotein monomers trimerize, which might be thought to facilitate the transport from the ER to the Golgi complex. Once in the Golgi complex, most of the high-mannose oligosaccharide side chains are modified to more complex forms (20, 21), and O-linked oligosaccharide side chains are also added (22, 23).

In the trans-Golgi network, the SARS-CoV-2 S glycoprotein is proteolytically cleaved by cellular furin or furin-like proteases at the S1/S2 cleavage site, comprising multiple arginine residues that are not found in the closely related SARS-CoV (24, 25). Cleavage at the S1/S2 site yields a surface subunit S1, which attaches the virus to the host cell surface receptor, and a transmembrane subunit S2, which mediates the fusion of viral and host cell membranes (10). The S1 and S2 subunits remain associated through noncovalent interactions in a metastable prefusion state (11). Furin-like cleavage is essential for the S-protein mediated cell-cell fusion and viral infectivity, and is required for efficient SARS-CoV-2 infection of human lung cells (24) and airway epithelial cells (26).

Following cleavage, an ER retrieval signal (ERRS) consisting of a conserved KxHxx motif (27) located at the extreme C terminus ensures that the mature SARS-CoV-2 S protein accumulates near the ER-Golgi intermediate compartment (ERGIC) (27, 28), where driven by interactions with another structural protein, the membrane (M) protein, the S protein participates in virus particle assembly and is incorporated into virus envelope (**Figure 1**) (29, 30). Besides, a fraction of mature SARS-CoV-2 S proteins travel through the secretory pathway to the plasma membrane, where they can mediate fusion of infected with uninfected cells to form multinucleated giant cells (syncytia) (24, 31). This may allow direct spreading of the virus between cells and potentially alter the virulence of SARS-CoV-2 (24).

Notably, a deletion of ~20 amino acid containing the ERRS from the cytoplasmic tail of the SARS-CoV-2 S protein has been shown to increase the infectivity of single-cycle vesicular stomatitis virus (VSV)-S pseudotypes (9) and replication-competent recombinant VSVs bearing the S glycoprotein (32, 33), which likely could be translated to single-cycle human immunodeficiency virus (HIV)-S or other retrovirus-S pseudotypes straightforward (33). Presumably, this deletion may enhance the cell surface expression of the SARS-CoV-2 S glycoprotein (32), thereby facilitating the S protein incorporation into pseudovirions and replication-competent virions.

SARS-COV-2 S PROTEIN STRUCTURE AND FUNCTION

As mentioned above, the SARS-CoV-2 S glycoprotein plays pivotal roles in viral infection and pathogenesis. Mature S glycoprotein on the viral surface is a heavily glycosylated trimer, each protomer of which is composed of 1260 amino acids (residues 14-1273) (**Figure 2A**). The surface subunit S1 is composed of 672 amino acids (residues 14–685) and organized into four domains: an N-terminal domain (NTD), a C-terminal domain (CTD, also known as the receptor-binding domain, RBD), and two subdomains (SD1 and SD2) (**Figure 2A**) (34). The transmembrane S2 subunit is composed of 588 amino acids (residues 686-1273) and contains an N-terminal hydrophobic fusion peptide (FP), two heptad repeats (HR1 and HR2), a transmembrane domain (TM), and a cytoplasmic tail (CT), arranged as FP-HR1-HR2-TM-CT (**Figure 2A**) (34).

As a typical class I viral fusion protein (35), the SARS-CoV-2 S glycoprotein shares common structural, topological and mechanistic features with other class I fusion proteins, including HIV envelope (Env) glycoprotein and influenza virus haemagglutinin (HA) (36–38). Like other class I viral fusion proteins, the SARS-CoV-2 S glycoprotein is also a conformational machine that mediates viral entry by rearranging from a metastable unliganded state, through a pre-hairpin intermediate state, to a stable postfusion state (38, 39). Since the first genome sequence of SARS-CoV-2 became publicly available (40), a number of structures have been determined for the SARS-CoV-2 S glycoprotein trimer fragments in both the prefusion and postfusion states (**Figures 2B–D**) (11, 34, 41).

The overall architecture of the prefusion SARS-CoV-2 S ectodomain stabilized by two consecutive proline mutations in

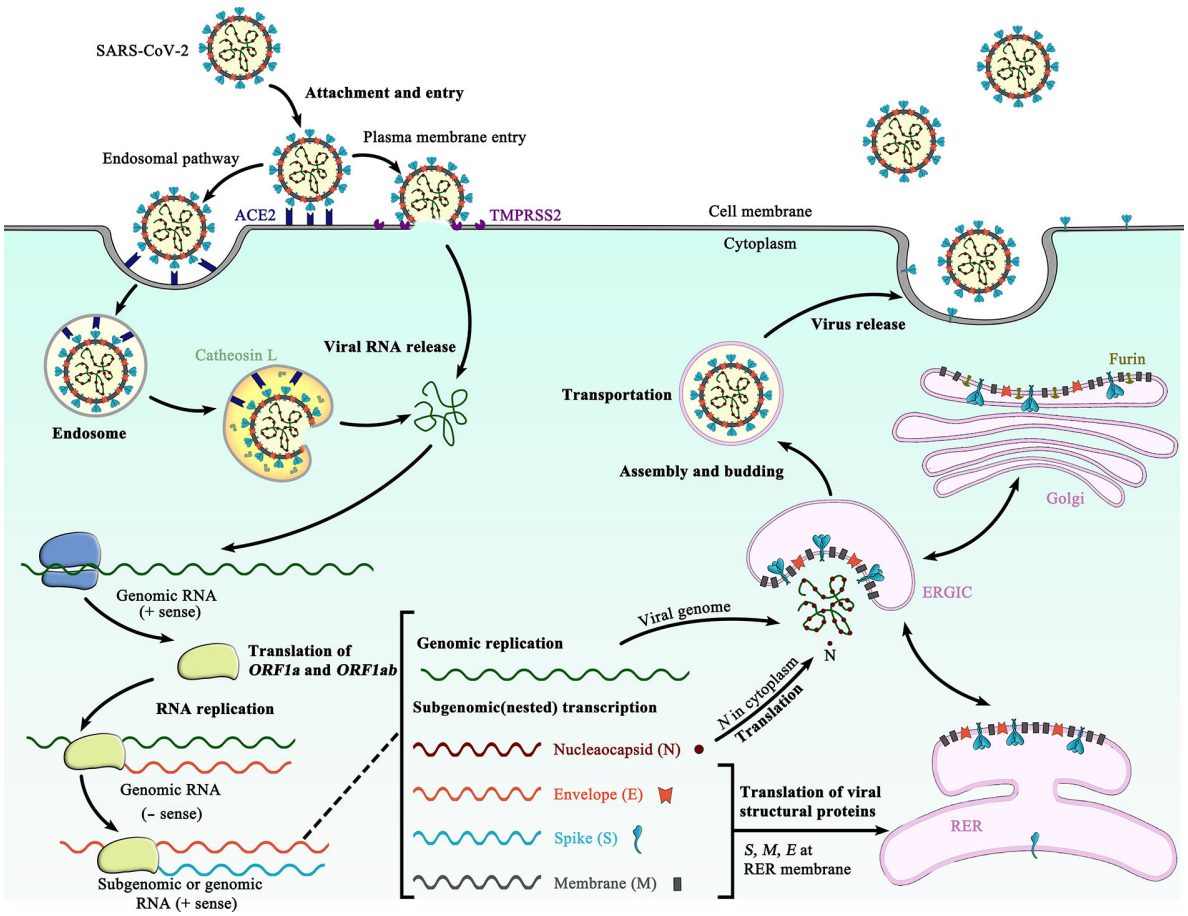

FIGURE 1 | Schematic representation of the life cycle of SARS-CoV-2. The life cycle of SARS-CoV-2 begins with membrane fusion occurring at the plasma membrane or within acidified endosomes after endocytosis, which is mediated by conformational changes in the S glycoprotein triggered by angiotensin-converting enzyme 2 (ACE2) binding. Following viral entry, SARS-CoV-2 releases its genomic RNA into the host cell cytoplasm. Genome RNA is first translated into viral replicase polyproteins (pp1a and 1ab), which are further cleaved by viral proteases into a total of 16 nonstructural proteins. A replication-transcription complex (RTC) is formed based on many of these nonstructural proteins. In the process of genome replication and transcription mediated by RTC, the negative-sense (– sense) genomic RNA is synthesized and used as a template to produce positive-sense (+ sense) genomic RNA and subgenomic RNAs. The nucleocapsid (N) structural protein and viral RNA are replicated, transcribed, and synthesized in the cytoplasm, whereas other viral structural proteins, including the S protein, membrane (M) protein and envelope (E) protein, are transcribed and then translated in the rough endoplasmic reticulum (RER) and transported to the Golgi complex. In the RER and Golgi complex, the SARS-CoV-2 glycoprotein is subjected to co-translational and post-translational processing, including signal peptide removal, trimerization, extensive glycosylation and subunit cleavage. The N protein is subsequently associated with the positive sense genomic RNA to become a nucleoprotein complex (nucleocapsid), which together with S, M, and E proteins as well as other viral proteins, is further assembled and followed by budding into the lumen of the ER-Golgi intermediate compartment (ERGIC) to form mature virions. Finally, the mature virions are released from the host cell, waiting for a new life cycle to start. This figure is adapted from the template in BioRender (https://biorender.com/).

two conformations determined by single particle cryo-electron microscopy (cryo-EM) is a ~160 Å long trimer with a triangular cross-section, with the S1 subunit adopting a "V" shape contributing to the overall triangular appearance and the S2 subunit forming the stalk (**Figures 2B, C**) (11, 34). The structural difference between these two conformations only lies in the position of one of the three S1 RBDs (**Figures 2B, C**) (11). When all three RBDs are in the "down" position, the resulting S ectodomain trimer assumes a closed conformation, in which the receptor-binding surface of the S1 RBD is buried at the interface between protomers and cannot be accessible by its receptor (**Figure 2B**) (11). The S ectodomain trimer with one single RBD in the "up" position assumes a partially open conformation and represents the functional state, as the receptor-binding surface of the "up" RBD can be fully exposed (**Figure 2C**) (11, 34). The structural information provides a blueprint for structure-based design of vaccine immunogens and entry inhibitors of SARS-CoV-2.

In the closed SARS-CoV-2 S ectodomain trimer, inter-protomer interactions occur through the S1 CTD packed against the other two S1 CTDs and one NTD from an adjacent protomer because of domain swapping and through S2, primarily between helical interactions formed by the upstream and central helices from each subunit around the trimer axis (**Figure 2B**) (11). The S1 subunits rest above the S2 trimer,

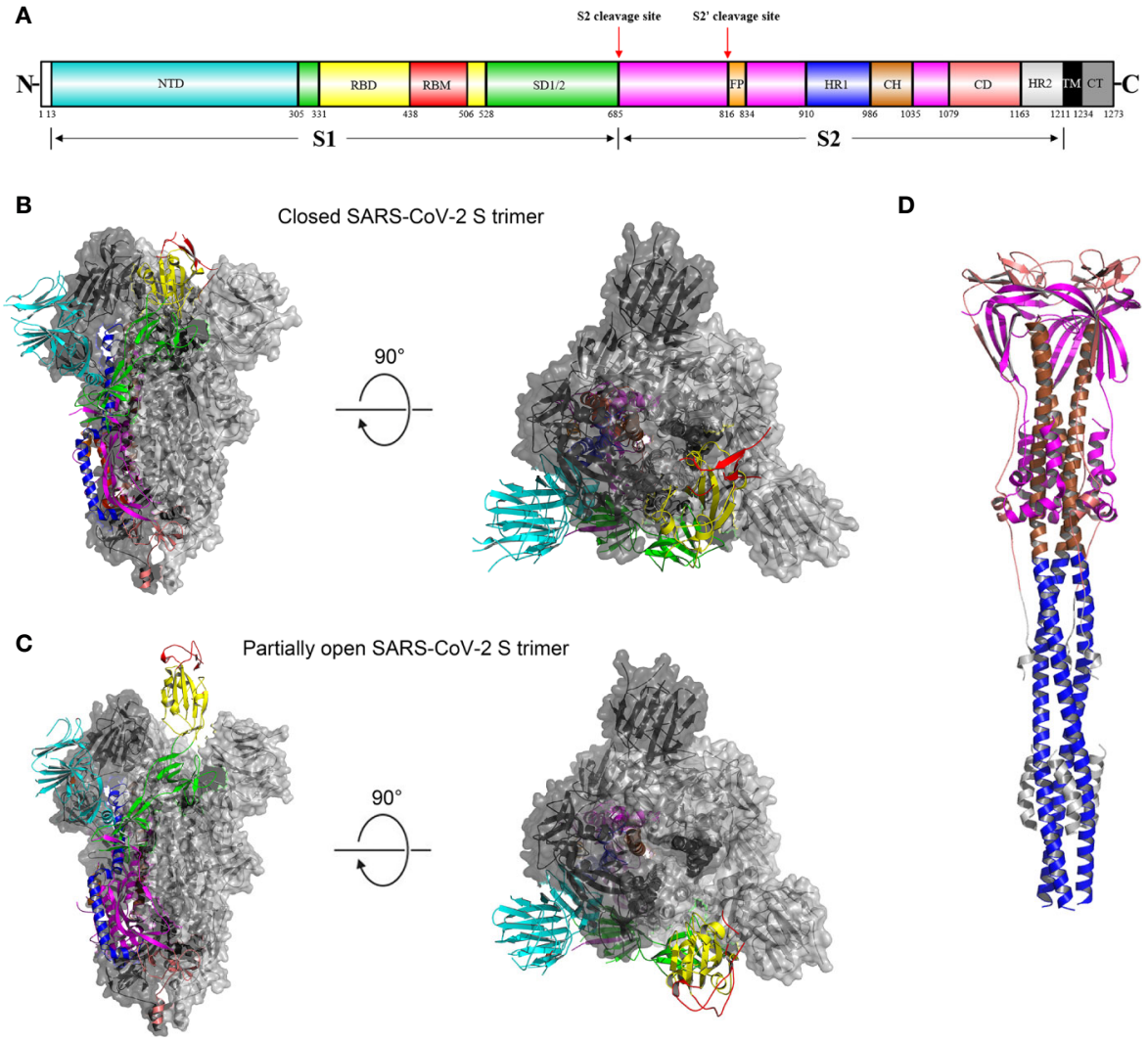

FIGURE 2 | Overall structures of the SARS-CoV-2 S glycoprotein trimer in different conformations. **(A)** Schematic representation of the domain arrangement of the SARS-CoV-2 S protein precursor. SS, signal peptide; NTD: N-terminal domain; RBD: receptor-binding domain; RBM: receptor-binding motif; SD1/2: subdomain 1 and 2; FP, fusion peptide; HR1, heptad repeat 1; CH, central helix; CD, connector domain; HR2, heptad repeat 2; TM, transmembrane domain; CT, cytoplasmic tail. Arrows denote protease cleavage sites. **(B)** Side and top views of the prefusion structure of the SARS-CoV-2 S ectodomain trimer with all three RBDs in the down conformation (PDB ID: 6VXX). One protomer is shown in ribbon representation colored corresponding to the schematic in **(A)**, a second protomer in light gray surface representation, and the third protomer in dark gray surface representation. **(C)** is identical to **(B)** except that a single RBD assumes the up conformation and is shown in ribbon representation (PDB ID: 6VYB). **(D)** Overall structure of the SARS-CoV-2 S2 trimer in the postfusion conformation is shown in ribbon representation colored corresponding to the schematic in **(A)** (PDB ID: 6XRA). The glycans were omitted for clarity.

stabilizing the later in the prefusion conformation (**Figure 2B**) (11). When the S ectodomain trimer adopts a partially open conformation, the RBD in the "up" position will abolish the contacts with the S2 subunit of an adjacent protomer, destabilizing the partially open conformation (**Figure 2C**) (11, 34). This will be beneficial to the dissociation of the S1 subunit and facilitate conformational rearrangements that the S2 trimer undergoes to mediate viral entry.

Prefusion structures of human coronavirus HKU1 (HCoV-HKU1) and mouse hepatitis virus S protein ectodomains without two consecutive proline mutations reveal only fully closed conformation (37, 42), similar to that observed for a full-length, wild-type prefusion form of the SARS-CoV-2 S glycoprotein (41). Notably, it is well established that trimeric prefusion HIV-1 Env primarily resides in a closed configuration that is conformationally masked to evade antibody-mediated neutralization (43, 44) and can spontaneously sample a transient, functional configuration (45). It can thus be speculated that native CoV S glycoproteins on mature and infectious virions share a similar conformational masking feature (46), concealing the receptor-binding surface (for those utilizing CTDs as RBDs) (**Figure 2C**), which is further discussed below.

Several lines of research have established that angiotensin-converting enzyme 2 (ACE2) is an entry receptor for SARS-CoV-2 (47–49). Detailed interactions between the SARS-CoV-2 RBD and its receptor ACE have been revealed by several structures of ACE2 in complex with RBD (50–53). Structurally, RBD consists of two subdomains: a core and an external subdomain (51, 52). An extended loop (residues 438-506), which lies on one edge of the core subdomain, presents a gently concave surface to cradle the N-terminal helix (α1) of ACE2. Analysis of the interface between the SARS-CoV-2 RBD and ACE2 reveals that a total of 17 residues in RBD are in contact with 20 amino acids in ACE2, forming a network of hydrophilic interactions that are suggested to predominate the virus-receptor engagement (51). Outside this extended loop, residue Lys417 located in helix α3 of the core subdomain, was shown to form ionic interactions with Asp30 of ACE2. As the extended loop contains almost all the amino acids of the SARS-CoV-2 RBD that contact ACE2, it is referred to as the receptor-binding motif (RBM) (51).

It has been proposed that inhibiting the interaction between RBD and ACE2 might be useful in treating SARS-CoV-2 infection. Recombinant soluble ACE2 (54) and ACE2-Fc (55, 56) have been shown to have potential applications in the prevention and treatment of SARS-CoV-2 infection *in vitro*. As the interaction between the RBD and ACE2 is extensive, small molecules probably cannot be used as entry inhibitors to effectively block the virus entry by targeting the interaction interface. However, peptides would be able to engage most of the residues belonging to RBM (57). A pioneering study demonstrated that a 23-amino acid peptide (residues 21-43), derived from the N-terminal helix (α1) of ACE2, specifically associates with the SARS-CoV-2 RBD with low nanomolar affinity and disables receptor interactions (57), representing a promising strategy for preventing the virus from invading human cells. In another study, a 65-amino acid peptide (residues 19-83), derived from the N-terminal back-to-back helices (α1 and 2) and composed of most of the residues of ACE2 that mediate interactions with the S protein, shows a similar but probably more potent inhibitory effect (58).

The formation of a trimer-of-hairpins structure (also known as six-helix bundle) comprising HR1 and HR2 in the postfusion conformation is a unifying feature of class I viral fusion proteins (37). The crystal structure of a protein construct in which SARS-CoV-2 HR1 and HR2 were connected by a six-residue hydrophilic flexible linker was determined to be a canonical six-helix bundle structure with a rod-like shape ∼115 Å in length and ∼25 Å in diameter (59). Three HR1 helices form a parallel central coiled-coil with three HR2 helices packing in an oblique, antiparallel manner against deep hydrophobic grooves on the surface of the central coiled-coil (59). Notably, when a full-length S protein construct bearing the native furin-like cleavage site was transiently expressed by Expi293F cells, the purified S proteins contained the dissociated S2 trimer in the postfusion conformation (41). The cryo-EM structure of this trimeric postfusion S2 shows that the central helix (CH) extended regular helices from the central coiled-coil, oriented toward target cells (**Figure 2D**) (41), which forms the longest central triple helical coiled-coil (~180Å) among all known class I transmembrane subunit structures.

The SARS-CoV-2 S trimer in the pre-hairpin intermediate state is very unstable and is just transiently present *in vivo* after triggering by ACE2 engagement, stymieing structural characterization of the S protein in this state (60). However, although this fusion-intermediate phase is very short, it is enough for inhibitory peptides to associate with the pre-hairpin intermediate and block the six-helix bundle formation (39). Furthermore, it has already been shown that the HR1 regions in various human CoVs are highly conserved (61), and therefore could serve as an attractive target for the design and development of potent and broad-spectrum inhibitors of pan-CoVs, including SARS-CoV-2. A highly potent pan-coronavirus fusion inhibitor, EK1C4, has been reported to have good prophylactic and therapeutic potential against SARS-CoV-2 infection (59).

GLYCAN SHIELD OF THE SARS-COV-2 S GLYCOPROTEIN

As mentioned earlier, the SARS-CoV-2 S proteins are heavily decorated by heterogeneous N-linked glycans projecting from the S trimer surface. The SARS-CoV-2 S sequence encodes up to 22 N-linked glycan sequons per protomer, which likely plays an important role in protein folding (19) and host immune evasion as a glycan shield (62). Of the 22 potential N-linked glycosylation sites on the S protein, 14 were identified to be predominantly occupied by processed, complex-type glycans (63). The remaining eight sites were found to be dominated by oligomannose-type glycans, which are divergent from those founded on host glycoproteins (63). Although glycosylation sites (N165, N234, N343) proximal to the receptor-binding sites on the SARS-CoV-2 S protein can be observed, ACE2 bound to the glycosylated and deglycosylated S ectodomains with nearly identical affinity (1.7 nM vs 1.5 nM) determined by a biolayer interferometry binding assay (64). This observation suggests that the high binding affinity between the SARS-CoV-2 S protein and ACE2 does not depend on the S protein glycosylation.

When the site-specific N-linked glycans are mapped onto the prefusion structure of the SARS-CoV-2 S ectodomain (63), the resulting model exhibited substantially higher levels of glycan-free surface than that revealed by structures of fully glycosylated, trimeric HIV-1 Env ectodomains (65, 66). This suggests that the SARS-CoV-2 S protein is covered by a less dense and less effective glycan shield compared to viral glycoproteins from HIV-1 (36, 66) and Lassa virus (67), which may be beneficial for the induction of humoral immunity and could be good news for a SARS-CoV-2 vaccine (68).

Notably, it has been shown that multiple major viral surface antigens have neutralizing epitopes that are partly or even exclusively composed of carbohydrate moieties (69, 70), exemplified by the HIV-1 Env spike, which could be recognized by a large number of carbohydrate-binding

antibodies, including 2G12, PG9, PG16, CH04, PGT121, PGT128, PGT135, and PGT145 (70, 71). In the case of SARS-CoV-2, more recently a potent neutralizing antibody against both SARS-CoV and SARS-CoV-2, S309, has been shown to recognize a highly conserved glycan-containing RBD epitope (72). These observations suggest that carbohydrate moieties could be immunogenic and highlight the need for immunogens to display the glycans important for the recognition of neutralizing antibodies (73); in support of this, specific N-linked glycans on Hemagglutinin has been shown to be essential for the elicitation of broadly neutralizing antibodies against Influenza (74). Accordingly, there has been mounting interest in exploring the potential of immunogenic glycan moieties as vaccine candidates against multiple viruses, including SARS-CoV-2 (75, 76).

SARS-COV-2 S GLYCOPROTEIN-MEDIATED MEMBRANE FUSION

Membrane fusion and viral entry of SARS-CoV-2 is initiated by binding of RBD in the viral S glycoprotein transiently sampling the functional conformation to ACE2 on the surface of target cells (**Figure 1**) (10). After receptor engagement at the plasma membrane or ensuing virus endocytosis by the host cell (8), a second cleavage (S2′ cleavage site) is generated, which is mediated by a cellular serine protease TMPRSS2 (48) or endosomal cysteine proteases cathepsins B and L (10) (**Figure 1**). Protease cleavage at S2′ site frees the fusion peptide from the new S2 N-terminal region, further destabilizes the SARS-CoV-2 S glycoprotein and may initiate S2-mediated membrane fusion cascade. Following the second cleavage, the fusion peptide at the N terminus of the S2 trimer is inserted into the host membrane (8), forming the pre-hairpin intermediate state (39). Since the pre-hairpin intermediate state is extremely unstable, the S2 fusion protein is refolded quickly and irreversibly into the stable postfusion state (39, 77). These large conformational rearrangements pull the viral and host cell membrane into close proximity, leading ultimately to the membrane fusion (8, 39).

INSIGHTS INTO THE DESIGN AND DEVELOPMENT OF S PROTEIN-BASED VACCINES

Since SARS-CoV-2 was identified as the causative agent of COVID-19, and its first genome sequence was released immediately and freely by a Chinese research group (40), SARS-CoV-2 vaccine candidates based on various vaccine platforms, such as inactivated or live attenuated vaccines, DNA and mRNA vaccines, viral vector-based vaccines, and recombinant protein-based vaccines, have been developed (12, 78). Most of these vaccine strategies are based on the full-length S glycoprotein, the major viral surface antigen (12). When a vaccine strategy requires that the SARS-CoV-2 S protein be recombinantly expressed in the human body, the ERRS should be omitted to enhance the cell surface expression level of the resulting protein.

Theoretically, the native HIV-1 Env trimer present on the surface of intact virions is thought to be a most ideal immunogen (60), as most of the neutralizing antibodies thus far described could recognize and bind to the prefusion form of trimeric HIV-1 Env, although it is with great difficulty that such neutralizing antibodies against this glycan-covered, sequence-variable native form are induced (36). For SARS-CoV-2, different lines of research have shown that convalescent sera from SARS-CoV and SARS-CoV-2 patients showed no or limited cross-neutralization activity against these two viruses by pseudotyped and authentic viral infection assays, despite significant cross-reactivity in binding to the S glycoproteins of both viruses (9, 79–81). Similar results were also observed in infected or immunized animals (48, 79, 81). Together with the finding that although the SARS-CoV-2 S protein shares a high degree of amino acid sequence identity with that of SARS-CoV (~76% overall), the RBM is less conserved (~47% identity) than any other functional region or domain (82), it can thus been surmised that the RBM has the most immunodominant neutralizing epitope(s) of the whole S protein, capable of readily eliciting strong neutralizing antibody responses. However, the native trimeric SARS-CoV-2 S protein could conceal each of its immunodominant RBMs by adopting the closed conformation (41, 83). Therefore, SARS-CoV-2 evades immune surveillance also through conformational masking, which is well-documented for HIV-1 (43, 44); while at the same time, the S protein could transiently sample the functional state to engage ACE2, consistent with the notion that the fusion glycoprotein of highly pathogenic viruses have evolved to perform its functions while evading host neutralizing antibody responses.

Another concern for vaccine candidates based on the full-length S glycoprotein of SARS-CoV-2 is raised by the observation that the S1 subunit could spontaneously dissociate from the S glycoprotein probably as a trimer that still assumes the RBD closed conformation, leaving only the postfusion S2 trimer (41). The resulting S1 and S2 subunits might expose immunodominant, nonneutralizing epitopes that are utilized by SARS-CoV-2 to serve as decoys to distract the host immune system, inducing a large proportion of ineffective antibody responses, as documented for HIV-1 (60) and respiratory syncytial virus (RSV) (84).

It should be noted that although vaccine candidates based on the full-length S protein of the closely related SARS-CoV could elicit neutralizing antibody responses against infection of SARS-CoV, they may also induce harmful immune responses, including liver damage of the vaccinated animals, infection of human immune cells by SARS-CoV, and antibody-dependent enhancement of SARS-CoV infection (85–89). Therefore, although the S proteins of both SARS-CoV and SARS-CoV-2 are thought to be promising vaccine immunogens for generating protective immunity, optimizing antigen design is critical to ensure an optimal immune response through exposing more

neutralizing epitopes and displaying fewer potentially weakly or non-neutralizing epitopes (90). Vaccines containing or expressing the full-length S protein or its soluble ectodomain form should thus be engineered to sample a RBD(s) "up" conformation while the rest is still kept in the prefusion state (91, 92).

Apart from recombinant, soluble, stabilized ectodomains that are engineered to expose the immunodominant RBD by adapting the RBD(s) "up" conformation, RBD proteins of SARS-CoV and SARS-CoV-2 have also been widely used as recombinant protein-based vaccines (85, 93–95). The RBD of SARS-CoV is highly immunogenic (96, 97) and is targeted by most of the neutralizing monoclonal antibodies that have been characterized (98). Based on the observation that a 193-amino acid fragment (residues 318-510) was previously identified to be the minimal RBD region of SARS-CoV (99), a corresponding 194-amino acid fragment (residues 331-524) can be readily selected as the minimal RBD region of SARS-CoV-2 and has already been characterized (100). This minimal form of RBDs of both viruses could serve as a vaccine candidate (100).

However, a conserved cysteine residue is located immediately upstream of the minimal RBD fragments of both viruses and always forms a disulfide bond in nearly all published structures containing this residue (101, 102); this is also the case for Middle East respiratory syndrome coronavirus (MERS-CoV) (103, 104) and HCoV-HKU1 (37), consistent with the observation that all RBDs of these viruses share a conserved structural core. The disulfide bond contributes to stabilization of the RBD structure and likely modulates the protein immunogenicity. This notion is consistent with the observation that mice immunized with a longer form of the SARS-CoV RBD (residues 318-536) produced a higher titer of neutralizing antibodies compared with mice immunized with the minimal RBD region (residues 318-510) (105). Therefore, when each of the minimal RBD fragments of SARS-CoV and SARS-CoV-2 is used as vaccine candidates, the critical cysteine residue should not be ignored and thus should be included (106).

Besides the RBD, which has been shown to a major target for human neutralizing antibody responses (107), the NTD was recently identified to be a new vulnerable site of the SARS-CoV-2 S protein for antibody neutralizing and therefore could also serve as a recombinant protein-based vaccine (108–110). As expected, NTD-specific neutralizing antibodies could target the S protein in both closed and open conformations (108). In addition, the apparent accessibility of the fusion peptide and HR1 region in published structures of the SARS-CoV-2 S ectodomain trimer as well as their high sequence conservation among CoVs suggests that they would be good immunogen candidates for epitope-focused vaccine design aimed at raising broadly CoV neutralizing antibodies (46). The epitope-focused vaccine design has proven to be successful in generating neutralizing antibodies against RSV fusion glycoprotein (111). However, neutralizing antibodies targeted against these two regions still need to be isolated in infected individuals to support this notion.

Unlike wild-type full-length S protein of SARS-CoV-2, the above monomeric fragments do not induce any infection-enhancing antibodies or harmful immune or inflammatory responses (106, 112), all of which could be potentially avoided through structure-based immunogen design to improve immunogenicity (113, 114). However, wide-type full-length or soluble ectodomain form of the SARS-CoV-2 S protein could trigger stronger cellular immune responses (115), which have been demonstrated to play an important role in controlling diseases caused by CoVs (116, 117), including SARS-CoV-2 (118), and are probably also an important determinant of effective vaccines against SARS-CoV-2 (115, 119). Additionally, when more than one RBD of the S protein trimer is engineered to be locked in the "up" conformation (120, 121), the antigenicity and immunogenicity of the resulting RBDs would be significantly enhanced compared to monomeric RBD form (97, 122). Moreover, improved protection is likely to be achieved when vaccinated with full-length or soluble ectodomain form of the SARS-CoV-2 S protein in that both forms can elicit neutralizing antibodies directed against non-RBD sites, as observed for MERS-CoV (123).

Genetic variation has been used by many viruses that have RNA genomes (124), including HIV and influenza, as a mechanism to avoid antibody-mediated immunity, and is partially responsible for the great difficulty in developing effective and durable vaccines against these viruses (36). As an RNA virus, however, SARS-CoV-2 has a very low mutation rate overall (125) likely because CoVs have a genetic proofreading mechanism (126). All reported variations occurred in the SARS-CoV-2 S glycoprotein have a prevalence of no more than 1% (127), with an exception of D614G, which has become the most prevalent genotype in the global COVID-19 pandemic (127). Fortunately, although the D614G mutation of the SARS-CoV-2 S protein has been shown to enhance viral infectivity (128–130), until now there is no evidence that infection with SARS-CoV-2 carrying the G614 mutant will be associated with disease severity (127, 131). Furthermore, assays using both monoclonal and polyclonal antibodies generated from individuals naturally infected with D614- or G614-carrying viruses demonstrated that the D614G mutation retains or even increases viral susceptibility to neutralization (127, 130, 132, 133). This suggests that the D614G mutant maintains or favors an open, functional conformational state (134).

Although at an extremely low frequency, natural variations, including L452R A475V, V483A, and F490L that render the S glycoprotein resistant to certain neutralizing antibodies targeting the RBD, emerged under no selection pressure exerted by approved vaccines or neutralizing antibodies or entry inhibitors (127, 132). However, it has been shown that SARS-CoV-2 escape mutants could be easily selected and quickly amplified under the selection pressure of single antibody treatment (135). These observations suggest that a combination of at least two neutralizing antibodies that recognize and bind to distinct and non-overlapping epitopes on the SARS-CoV-2 S glycoprotein (e.g., RBD and NTD, as well as HR and glycan) is required to restrict the possible occurrence of viral escape mutants and potential subsequent loss of single antibody-mediated neutralization (135–138). When these

observations are taken into consideration for vaccine design and development, an ideal SARS-CoV-2 immunogen should contain as many exposed neutralizing epitopes as possible, although the RBD also possesses extra epitope(s) besides the epitope in the RBM region (72, 139–141).

CONCLUDING REMARKS AND PROSPECTS

SARS-CoV-2 is a highly contagious pathogen that continues to spread quickly around the globe, causing COVID-19 to be one of the worst pandemics in recorded history. A safe and efficacious vaccine represents one of the best ways to reduce or eliminate the COVID-19 pandemic (142). Unfortunately, no vaccines for any of the known human CoVs have been licensed (143, 144), although several potential SARS-CoV and MERS-CoV vaccines have advanced into human clinical trials for years (117, 145), suggesting the development of effective vaccines against human CoVs has always been challenging. However, it has been shown that both SARS-CoV and SARS-CoV-2 could readily induce neutralizing antibodies following natural infection or immunization (146–149). Moreover, a growing number of neutralizing monoclonal antibodies targeting the SARS-CoV-2 S glycoprotein with high potency have been isolated from plenty of convalescent donors (33) as well as humanized mice (136, 141), some of which have been shown to afford protection against SARS-CoV-2 challenge in animal models. It thus seems that vaccine candidates designed to elicit such neutralizing antibodies are feasible. It is widely accepted that the S protein of SARS-CoV-2 is a most promising immunogen for producing protective immunity (150). However, it is likely that the S protein has evolved to perform its functions while evading host neutralizing antibody responses and thus should be engineered to ensure an optimal immune response (151, 152). The immunogen design strategies described in this review based on the wealth of the SARS-CoV-2 S glycoprotein research related to its biosynthesis, structure, function, antigenicity as well as immunogenicity will likely contribute to the ultimate success of safe and efficacious vaccines against SARS-CoV-2/COVID-19.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

FUNDING

Our research was supported by the Natural Science Foundation of Henan Province (Grant No.182300410327), the National Natural Science Foundation of China (Grant No. 81871312 and 81701546), and by the 111 Project (No. D20036).

ACKNOWLEDGMENTS

We would like to thank Prof. Xinqi Liu for critical reading of the manuscript; and Drs. Yanbin Feng, Mengyuan Xu, Jing Ma and Jianrong Feng for helpful comments and discussions on the manuscript.

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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.

frontiers in Immunology

ORIGINAL RESEARCH
published: 28 October 2020
doi: 10.3389/fimmu.2020.587615

Immunoinformatic Analysis of SARS-CoV-2 Nucleocapsid Protein and Identification of COVID-19 Vaccine Targets

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OPEN ACCESS

Edited by:
Katie Ewer,
University of Oxford, United Kingdom

Reviewed by:
Gunnveig Grødeland,
University of Oslo, Norway
Salvador Iborra,
Universidad Complutense de Madrid, Spain

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Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: *26 July 2020*
Accepted: *02 October 2020*
Published: *28 October 2020*

Citation:
Oliveira SC, de Magalhães MTQ and Homan EJ (2020) Immunoinformatic Analysis of SARS-CoV-2 Nucleocapsid Protein and Identification of COVID-19 Vaccine Targets. Front. Immunol. 11:587615. doi: 10.3389/fimmu.2020.587615

COVID-19 is a worldwide emergency; therefore, there is a critical need for foundational knowledge about B and T cell responses to SARS-CoV-2 essential for vaccine development. However, little information is available defining which determinants of SARS-CoV-2 other than the spike glycoprotein are recognized by the host immune system. In this study, we focus on the SARS-CoV-2 nucleocapsid protein as a suitable candidate target for vaccine formulations. Major B and T cell epitopes of the SARS-CoV-2 N protein are predicted and resulting sequences compared with the homolog immunological domains of other coronaviruses that infect human beings. The most dominant of B cell epitope is located between 176–206 amino acids in the SRGGSQASSRSSSRSRNSSRNSTPGSSRGTS sequence. Further, we identify sequences which are predicted to bind multiple common MHC I and MHC II alleles. Most notably there is a region of potential T cell cross-reactivity within the SARS-CoV-2 N protein position 102–110 amino acids that traverses multiple human alpha and betacoronaviruses. Vaccination strategies designed to target these conserved epitope regions could generate immune responses that are cross-reactive across human coronaviruses, with potential to protect or modulate disease. Finally, these predictions can facilitate effective vaccine design against this high priority virus.

Keywords: severe acute respiratory syndrome coronavirus 2, Coronavirus Disease 2019, epitopes, vaccine, T cells, B cells, nucleocapsid

INTRODUCTION

The pandemic Coronavirus Disease 2019 (COVID-19) is a worldwide threat caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (1). By July 2020, SARS-CoV-2 had infected over 16 million people worldwide and killed more than 645,000 individuals. A better understanding of the immunogenicity and pathogenesis of SARS-CoV-2 infections in humans is thus urgently needed as a basis for the development of new vaccines against SARS-CoV-2 (2).

The coronaviral genome encodes a relatively small number of proteins, classified as either structural or non-structural. Among structural proteins, the spike glycoprotein (S), and the nucleocapsid protein (N) are the major ones, while the envelope protein (E) and membrane protein (M) are smaller structural components (3, 4). The spike (S) protein is arrayed on the surface of the virus particles, giving the characteristic 'crown' appearance (5). The S protein comprises two subunits: S1 and S2. The S1 subunit consists of an amino-terminal domain and a receptor-binding domain (RBD) (5, 6). The RBD binds to ACE2 as its host cell target receptor, which allows virus entry (5, 7). Various reports related to SARS-CoV-2 suggest a correlation between neutralizing antibodies and the number of specific T cells to viral particles (8). Some vaccine candidates have been shown to protect from infection in laboratory animals models (9). Most vaccine studies so far have focused on antibody responses generated against the S protein, the most exposed protein of SARS-CoV-2 (10, 11). However, antibody responses are not detectable in all infected patients, especially those with less severe forms of COVID-19 (12). Previous studies with SARS-CoV-1 have also shown that memory B cell responses tend to be short-lived after infection (13). In contrast, memory T cell responses can persist for many years (14), and in mice, these protect against lethal challenge with SARS-CoV-1 (13). Additionally, the spike protein has several hotspots for mutations (15), whereas the nucleocapsid gene is more stable and has acquired fewer mutations to date (16).

In this study, we focus on the SARS-CoV-2 nucleocapsid protein that is involved in viral pathogenesis (4, 17). The nucleocapsid is the most abundant protein in coronaviruses, is highly immunogenic, and its amino acid sequence is largely conserved as previously reported (4). Therefore, this protein has advantages as a candidate for vaccine development (4, 18). Previous studies on SARS-CoV-1 reported N protein epitopes as capable of eliciting massive production of antibodies in infected subjects (4). T cell responses to SARS-CoV-1 are in some cases shown to last up to 11 years thus representing a valid alternative for the design of vaccines (4, 19). Monkeys vaccinated with an adenovirus vectored SARS-CoV-1 vaccine were shown to have consistent T cell responses to the N protein (20). Similarly in MERS the nucleocapsid has been examined as a potential vaccine candidate (21, 22). Recall responses of T cells reacting with peptides of SARS-COV-2 N protein have been demonstrated in both SARS-CoV-1 recovered patients, 17 years after exposure, and those with no history of SARS-CoV-1 exposure (23, 24). Preliminary studies of SARS-CoV-2 have also demonstrated antibodies directed to the N protein (2).

Studies involving computer simulations for the identification of the epitopes recognized by antibodies and T cells are central to immunological applications such as drug design and vaccine development. Bioinformatics tools offer the advantage, in addition to speed and biosafety, of being unbiased by peptide selection. Approaches which use overlapping peptides, spaced other than single amino acid displacement, may exclude the key peptides. There have been several reports of bioinformatics analyses of SARS-CoV-2 using a variety of platforms (25–29). Herein, we applied bioinformatics analysis to determine the antigenic potential of the SARS-CoV-2 N protein. Major B and T cell epitopes of the SARS-CoV-2 N protein are predicted and these peptides were compared to other coronaviruses that infect humans. As other studies have suggested that prior exposure to less virulent human coronaviruses may confer some protection (24, 30–32), we focused particularly on identifying conserved motifs which potentially could elicit cross-reacting T cell responses through shared T cell exposed peptides. The epitope mapping and comparison of potential cross-reactive epitopes presented in this study may provide an opportunity for the development of new vaccines and immunodiagnostic tools. Finally, the sudden emergence of SARS-CoV-2 apparently from bats is an indicator that similar betacoronaviruses could emerge in the future. It is therefore of interest to determine if there are potential antigens that are conserved and could cross protect against future zoonotic coronaviruses.

MATERIAL AND METHODS

Accession Numbers

Accession numbers of the nucleocapsid proteins analyzed are as follows: HKU1:YP_173242.1; 229E:NP_073556.1; MERS: YP_009047211.1; NL63:YP_003771.1; OC43: YP_009555245.1; SARS COV1:NP_828858.1; SARS-COV2: YP_009724397.2.

Determination of Predicted Epitopes for SARS-CoV-2 Nucleocapsid

B cell linear epitope probability and MHC binding affinity were determined for all sequential peptides with a single amino acid displacement, using an updated version of methods previously described (33, 34). Briefly, in lieu of representing peptides as simple alphabetic sequences, multiple physicochemical properties of each amino acid are transformed to mathematical vectors by principal component analysis. Using a training set of known MHC binding reactions, B cell epitope binding and cathepsin cleavage reactions, neural networks are used to derive predictive equations applicable to any peptide. Predictions are made for 70 MHC I alleles and 65 MHC II alleles. To estimate population behavior comprising multiple MHC alleles with varying affinities for any peptide, the LN ic50 binding data estimates were transformed and standardized to a zero mean unit variance within each protein using a Johnson Sb distribution (35). To compute a permuted average across human alleles, the highest predicted binding affinity at each peptide position was determined for every possible haplotype pairing and averaged; this was computed using predicted binding for 31 MHC IA, 31 MHC IB, and 24 DRB alleles as previously demonstrated (36). Predictions of the probability of cathepsin cleavage at each dimer were similarly derived by training on known cleavage reactions (34). These predictive methods have

been experimentally validated in proteins of multiple origins (34, 37–40).

Nucleocapsid Sequence Alignments and Structural Analysis

Several protein sequences were analyzed by using the Basic Local Alignment Search Tool specific for protein sequences (BLASTp) (41). Multiple sequence alignments were prepared with Clustal Omega (multiple sequence alignment) and manually edited in *py*BoxShade 3.21 (https://github.com/mdbaron42/pyBoxshade). We selected statistically significant matches to calculate a similarity tree for related coronaviruses. The epitopes were mapped based on the amino acid physical–chemical properties and location at possible areas of cross-reactivity and antigen-binding by using an in-house software (data not shown). Analysis of the protein secondary structure prediction and annotation was carried out with PSIPRED Protein Analysis Workbench (http://bioinf.cs.ucl.ac.uk/psipred/) (42, 43). The epitopes were identified, built in Chimera v.1.13.1. We also used Chimera to prepare images and calculate RMSD between sequences (44). Distance between residues were measured by using wizard measurement tool from PyMOL (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC.).

T Cell Exposed Motifs

All sequential T cell exposed motif patterns were extracted from each protein and ranked as previously described for each of three recognition patterns of amino acids which engage T cell receptors (33, 36, 45). These T cell exposed recognition patterns comprise the amino acids not hidden in pocket positions. These are positions ~~~4,5,6,7,8~ within a MHC I binding 9-mer and ~2,3,~5~7,8~ or −1~~,3,~5~7,8~ relative to the 9-mer core of a MHC II binding 15-mer.

RESULTS

SARS-CoV-2 Nucleocapsid B and T Cell Epitope Mapping

The nucleocapsid of SARS-Cov-2 exhibits both strong B and T cell epitopes distributed across the whole protein. **Figure 1** provides an overview map of both probable linear B cell epitopes and regions of predicted high affinity MHC binding for multiple alleles. Corresponding sequences of predicted antigenicity are shown in **Table 1**. As shown in **Figure 1**, we predicted multiple high probability B cell linear epitopes. A 9-mer peptide was scored as "high probability" if they were predicted to be in the top 25% of probability of being in a B

TABLE 1 | SARS-CoV-2 predicted antigenicity of B and T cell epitopes.

B cell epitopes	
Position	**Peptide sequence**
21–32	SDSTGSNQNGER
76–82	TNSSPDD
176–206	SRGGSQASSRSSSRSRNSSRNSTPGSSRGTS
235–243	SGKGQQQQG
249–263	KSAAEASKKPRQKRT
363–379	FPPTEPKKDKKKKADET
MHC I binding regions for multiple alleles	
Position	**Peptide sequence**
97–137	GDGKMKDLSPRWYFYYLGTGPEAGLPYGANKDGIIWVATEG
209–232	RMAGNGGDAALALLLLDRLNQLES
261–279	KRTATKAYNVTQAFGRRGP
306–335	QFAPSASAFFGMSRIGMEVTPSGTWLTYTG
MHC II binding regions for multiple alleles	
Position	**Peptide sequence**
97–127	GGDGKMKDLSPRWYFYYLGTGPEAGLPYGANK
213–238	NGGDAALALLLLDRLNQLESKMSGKG
293–320	RQGTDYKHWPQIAQFAPSASAFFGMSRI

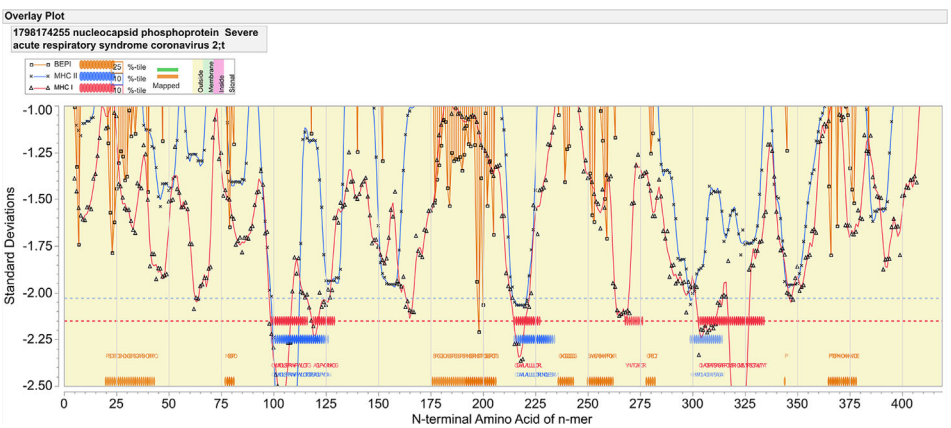

FIGURE 1 | Epitope mapping of nucleocapsid protein of SARS-CoV-2. The X axis indicates the index position of sequential peptides with single amino acid displacement. The Y axis indicates predicted binding affinity in standard deviation units for the protein. The red line shows the permuted average predicted MHC-IA and B (62 alleles) binding affinity by index position of sequential 9-mer peptides with single amino acid displacement. The blue line shows the permuted average predicted MHC-II DRB allele (24 most common human alleles) binding affinity of sequential 15-mer peptides. Orange lines show the predicted probability of B-cell receptor binding for an amino acid centered in each sequential 9-mer peptide. Low numbers for MHC data represent high binding affinity, whereas low numbers equate to high B cell receptor contact probability. Ribbons (red: MHC-I, blue: MHC-II) indicate the 10% highest predicted MHC affinity binding. Orange ribbons indicate the top 25% predicted probability B-cell binding. Horizontal dotted lines demarcate the top 5% of binding affinity for the protein (red MHC I, blue MHC II).

cell epitope for the protein as a whole. The most dominant of these lies between 176 and 206 in the sequence SRGGSQASSRS SSRSRNSSRNSTPGSSRGTS. Additional high probability B cell epitopes are indicated in **Table 1**. When analyzed by the same immunoinformatic approach alongside all structural proteins in the virion, the nucleocapsid B cell epitope at 176–206 stands out as dominant with respect to the epitopes in the spike glycoprotein (data not shown).

Figure 1, in which consideration is given to the predicted binding of multiple common human MHC I and MHC II alleles, indicates three regions of predicted high MHC II binding and four regions of high affinity MHC I binding for multiple alleles, which comprise the top 10% highest predicted affinity for the protein. These are shown in **Table 1**. However, as the examples shown in **Figure 2** underscore, there are differences in MHC allele-specific binding. The differences are more marked for MHC I, where binding is often restricted to one or two sequential 9-mers, whereas the broader sequences identified for MHC II, tend to span more alleles. For example, adjacent to the dominant B cell epitope we see that a DRB1_1501 has a stronger

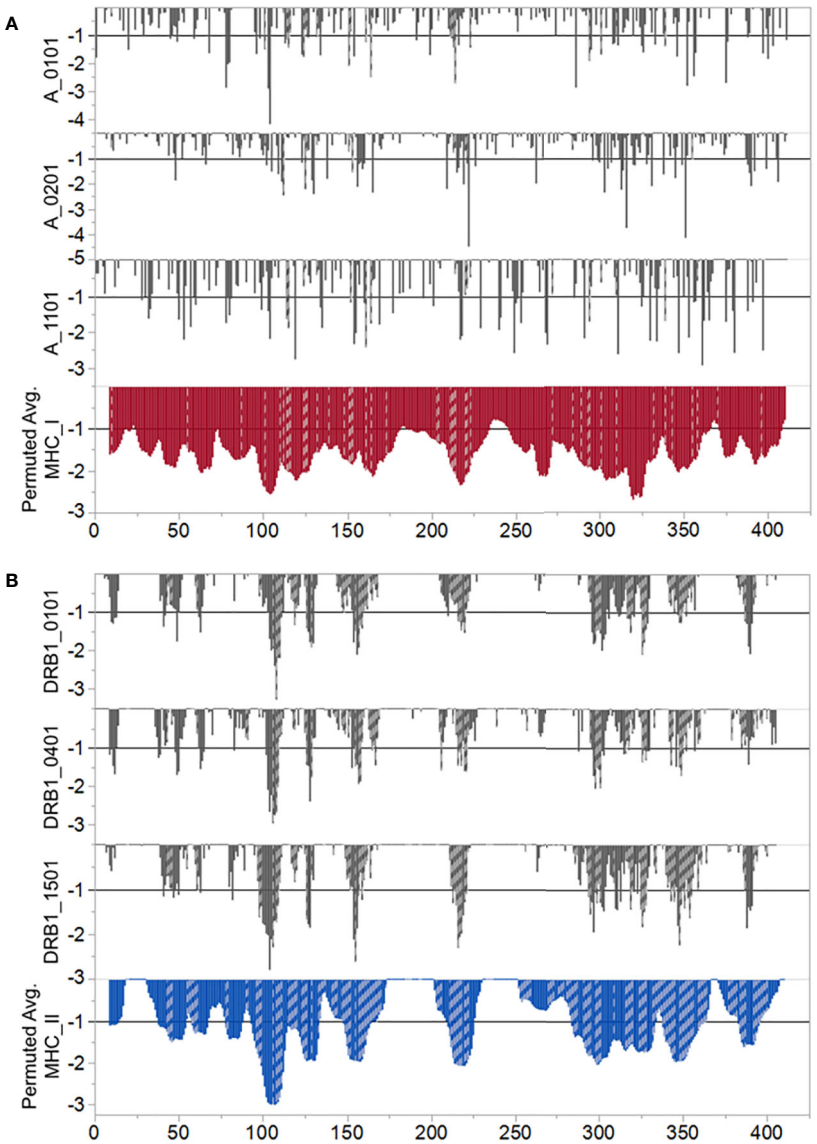

FIGURE 2 | Predicted differential binding of example alleles. **(A)** MHC I and **(B)** MHC II. In both panels the Y axis indicates predicted binding affinity of sequential peptides. The X axis indicates the index position of each 9 mer (MHC (I) or 15-mer (MHC II) represented by a vertical bar. Bars which are cross hatched are those peptides predicted to be excised for binding and presentation by either cathepsin S or cathepsin L. For MHC I the cathepsin predictions are those which excise a 9 mer. For MHC II a predicted excision of a 12–18 mer is shown. The lower tier of each panel shows the population permuted average predicted binding affinity as described for **Figure 1**. The top three tiers contrast the responses of selected example alleles. For MHC I we show predicted responses of A_0101, A0201, and A1101. For MHC II we show predicted responses of DRB1_0101, DRB1_0401, and DRB1_1501. Other alleles evaluated show a similar diversity of predicted response.

predicted MHC II binding which could indicate more T cell help than is the case for an individual of DRB1_0101. Furthermore, when consideration is given to probable cathepsin cleavage, not all peptides may actually be presented. However, we appreciate that cathepsins play a major role in generating peptides to be presented for the vacuolar pathway (endolysosomes and phagosomes) as demonstrate by Shen et al. (46). Therefore, cathepsins are primarily involved in TAP-independent MHC class I crosspresentation. Nevertheless, this analysis suggests that individuals of different immunogenetics would be expected to show differing responses. The proximity of MHC binding sequences to the B cell epitopes at 76–82 and 176–206 amino acids indicates these epitopes may also receive strong epitope specific T cell help.

Conservation of T Cell Epitopes Among Coronaviruses

We next compared the epitope map of SARS-COV-2 N protein to that of other coronaviruses known to have infected humans. Here, we focused on the T cell exposed motifs, which indicate where potential T cell cross-reactivity may occur. A single T-cell receptor engages only with the few amino acids of a bound peptide MHC that are protruding from a MHC histotope, together with contact points within the histotope. We refer to this pentamer motif as the T cell exposed motif (36). **Figure 3** shows the patterns of T cell exposed motif sharing between human alphacoronaviruses 229E and NL63 with betacoronaviruses HKU1, OC43, MERS, SARS-CoV-1, and SARS-CoV-2. While some of the T cell exposed motifs are conserved, the flanking regions of these peptides, comprising the groove exposed motif, differ. Most notably there is a region of potential T cell cross-reactivity within the SARS-CoV-2 N protein position 102–110 that traverses the human alpha and beta coronaviruses, except for MERS. In MERS substitution of Leu>Thr at the SARS-CoV-2 position 113 (equivalent to the MERS 103 position) removes the conservation of the T cell exposed motifs with SARS-CoV-2. The region in which the conserved motifs occur is also predicted to have high affinity binding for multiple MHC I and II alleles. Here, we used 70 human MHC I and 65 MHC II alleles for our analysis of permuted binding that represents about 85% of human population. The T cell motif sharing is further extended within the betacoronaviruses. The conserved T cell exposed motifs are shown in **Supplementary Table 1**. When the N proteins of the six viruses sharing most motifs are aligned at the peptide comprising the most conserved T cell exposed MHC I motif ~~~FYYLG~ (in SARS-CoV-2 position 107), the commonality of epitope patterns is evident (**Figure 4**).

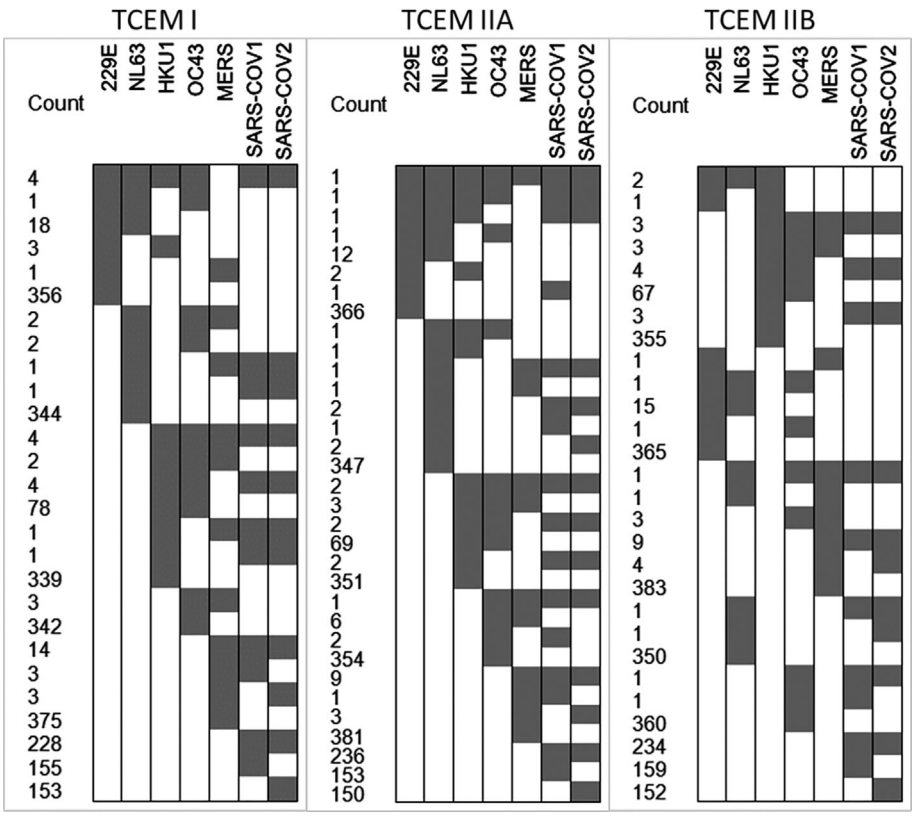

FIGURE 3 | T cell exposed motifs conserved across coronaviruses. The cell plots show in gray where there are T cell exposed motifs shared between SARS-CoV-2 and other human coronaviruses, as shown in the X axis. The number of shared motifs indicated in the Y axis counts. The most highly conserved motifs are also shown in **Supplementary Table 1**.

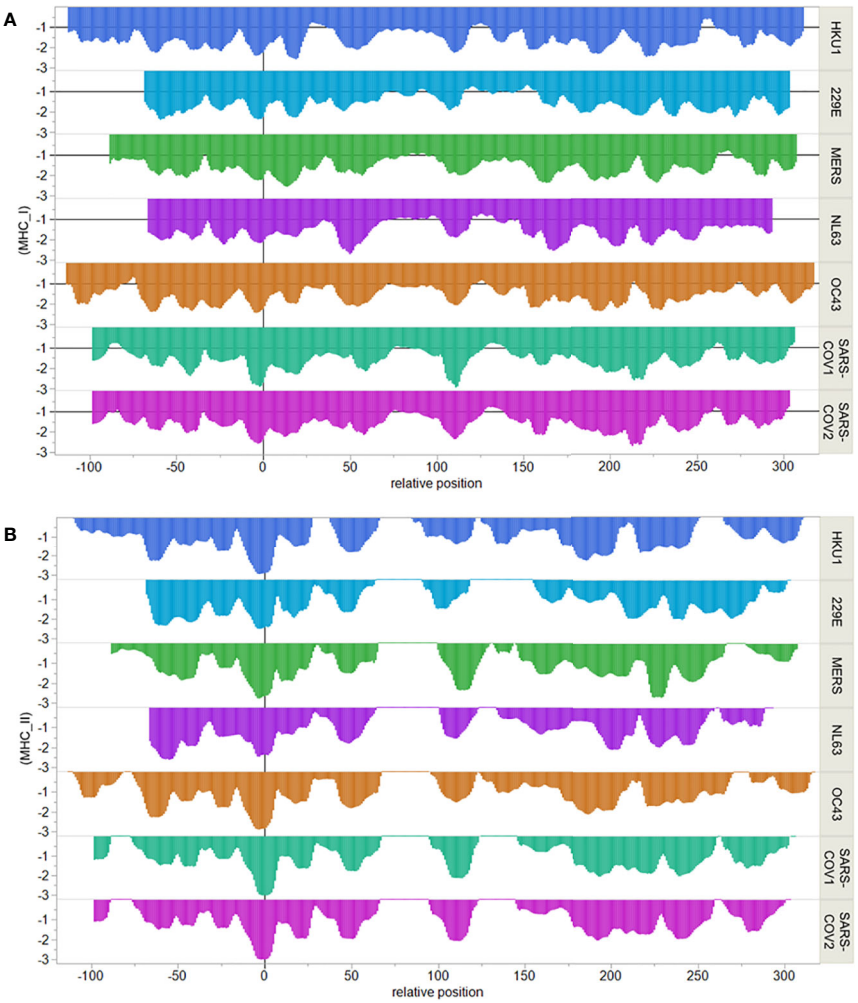

FIGURE 4 | Comparative MHC binding patterns in human coronavirus nucleocapsid protein. X axis shows sequential peptides aligned relative to the most conserved MHC I pentamer. Peptides are 9-mers for MHC I and 15-mers for MHC II and are indicated at their index positions. Y axis shows permuted predicted binding in standard deviation units below the mean for the protein. **(A)** shows MHC I alleles. **(B)** shows MHC II DRB alleles.

3-D Structure Model of Nucleocapsid From Different Coronaviruses

The coronavirus nucleocapsid protein consists of two folded domains (NTD and CTD) linked by an unstructured region (47). In more details the N protein includes the following domains: serine–glycine–arginine-rich domain (SGRD), N-terminal domain (NTD), serine-rich domain (SRD), C-terminal domain (CTD) as described in **Figure 5A** (48, 49). Our alignment has revealed that despite the conservation of some motifs, the N protein from various different coronaviruses often exhibit different properties, due primarily to their otherwise low sequence homology (~50%) (**Supplementary Figure 1**). The structural similarity appears to be at the whole folded level with its five-stranded anti-parallel β-sheet sandwiched between loops (or short 3–10 helix) on the outside (**Figure 5B**). Several nucleocapsid NTD domains are similar in topology and surface electrostatic profiles as observed. The root mean square deviation (RMSD) between the structures coordinates is 0.867 Å over superimposed C atoms. The most dramatic differences can be observed in loops L1 (between β2 and β3, residues 96 to 104) and L3 (residues 119 to 128). Other authors also observed that strands β2 and β3 are connected by a long flexible loop composed of amino acid residues 96 to 104 protruding out of the core (50, 51). We could identify and observe (**Figure 5B**) the structure of the highly conserved twelve-residue peptide corresponding to the region $_{107}$RWYFYYLGTGPY$_{118}$ (YP_009724397.2). This peptide is located at the NTD of N protein, close to the L1 loop and has a conserved and important epitope located in an exposed beta-strand, with two exposed tyrosines (**Figure 4B1**). Both tyrosines (Y111 and Y112) have been proposed to be involved in RNA recognition, stacking with consecutive nucleotide bases. The NTD of the N protein from the selected coronavirus was compared to assess the similarity level existing between the conserved protein sequences of the human

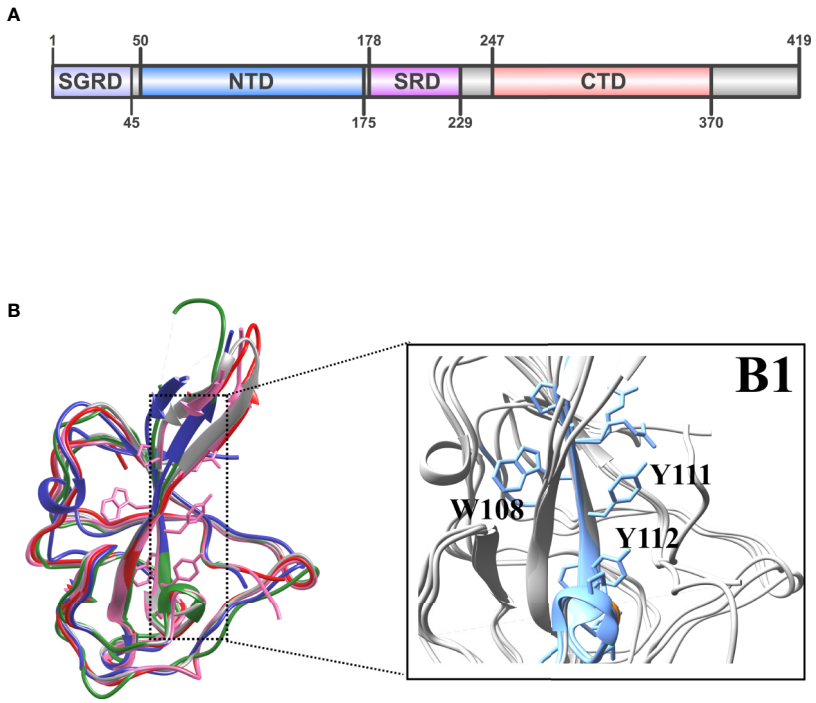

FIGURE 5 | **(A)** Domain organization of coronaviral N proteins. The four domains labeled are as follows: SGRD, serine–glycine–arginine-rich domain; NTD, N-terminal domain; SRD, serine-rich domain; and CTD, C-terminal domain. **(B)** Superimposition of the HCoV-Sars-2 NTD in pink (pdb ID: 6M3M) with NTDs from Sars-CoV-1 in red (pdb ID: 2OFZ), HCoV-OC43 in green (pdb ID: 4J3K), HCoV-NL63 in blue (pdb ID: 5NK4), MERS in gray (pdb ID: 4UDI). (B1) The beta-strand (β3) region for the major conserved epitope is highlighted in blue with the two conserved tyrosines for RNA binding.

coronaviruses (4). Structural mapping of the epitopes shown in **Figure 5B1** into 3D models of the NTD N protein (6M3M, 5NK4, 4J3K, 1SSK, 4UD1 entries of PDB database) reveals a conserved epitope predicted in a highly immunogenic peptide exposed to the extracellular environment, likely, to other host immune system components. We were also able to demonstrate that the predicted B cell epitope in SARS-CoV-2 at $_{176}$SRGGSQASSRSSSRSRNSSRNSTPGSSRGTS$_{206}$ is inside the unstructured region inside of SGRD domain of SARS-CoV-2 (**Supplementary Figure 1**, sequence colored in blue). Unfortunately, this region could not be mapped in the 3D model due to the lack of a structure model for the whole protein length.

DISCUSSION

COVID-19 pandemic challenged the world to speed up research for a vaccine against SARS-CoV-2 infection. Despite massive effort and many thousands of studies published within the first 8 months of the pandemic, our understanding of how humans respond to SARS-CoV-2 is still quite limited (2). Worldwide efforts are currently underway to map the determinants of immune protection against SARS-CoV-2. In this study, we used a bioinformatics approach to map B and T cells epitopes in the nucleocapsid protein of SARS-CoV-2. The SARS-CoV-2 S protein is being studied as the leading target antigen in vaccine development (52, 53). However, a better understanding of viral entry is required to avoid further complications with the vaccine immune response, similar to those observed with HIV type 1 (HIV-1) Env protein candidate vaccine (53, 54). Additionally, the spike protein has several hotspots for mutations (15). In contrast, the nucleocapsid gene is more conserved and stable, with fewer mutations over time (16). Nucleocapsid proteins of many coronaviruses are highly immunogenic and are expressed abundantly during infection (53, 55). High levels of IgG antibodies against nucleocapsid have been detected in sera from SARS patients (53, 56), and the N protein is a representative antigen for the T-cell response in a vaccine setting (20, 53).

In this study, our bioinformatics analysis was able to identify epitopes conserved in several human coronavirus N proteins. The results show that there are several overlapping conserved peptides. When combined, our analysis could thus predict not only high binding individual 9-mer peptides, but also highly exposed structural regions of immunological peptides, which could have potential importance as candidates for vaccines. Our findings are consistent with the strong antigenicity previously noted in SARS N protein and prior reports for SARS-CoV-2 (24). The predicted B cell epitopes we identify are consistent with the strong IgG, IgM, and IgA responses to the N protein in an acutely infected patient documented by Dahlke et al. using peptide arrays (2) and with the observations of Grifoni et al. (31). We

identified a strong immunodominant B cell epitope SRGGS QASSRSSSRSRNSSRNSTPGSSRGTS between 176 and 206 amino acids in the nucleocapsid protein sequence. With appropriate T cell help this epitope may be a good target for neutralizing antibodies and long-lived immune response.

Additionally, we performed an *in-silico* survey of the major T cell epitope sequences of the nucleocapsid protein from coronaviruses known to have infected humans (4). The demonstration of conserved T cell exposed motifs between the N protein of multiple human coronaviruses may account for the reported recall of T cell responses over decades, even in the absence of SARS-CoV-1 exposure (23, 57). We found a region of potential T cell cross-reactivity within the SARS-CoV-2 N protein positions 102–110 and equivalent positions in the human alpha and beta coronaviruses, with the exception of MERS. Comparison of the individual allele predicted binding affinities to the SARS-CoV-2 peptides shows differences in responses based on individual genetics. The conserved T cell exposed motifs shared between coronaviruses are each contextualized in different flanking regions comprising pocket positions that will bind with differing affinities. These complexities underscore the nuanced differences in individual patient's responses. As much of the pathogenesis of COVID-19 disease appears linked to the immune and inflammatory response, it is important to keep in mind that individual differences in clinical response may be rooted in the patients MHC alleles as well as in presence of the preexisting cognate T cell clones, which may have been primed by different peptides. We also address the potential T cell epitopes by a complementary structural bioinformatics method, which was able to assess the conservation of these epitopes across different human coronaviruses. We explored the fact that 89.74% of amino acid sequence of the N protein of SARS-CoV-1 is similar to SARS-CoV-2, with high similar 3D structures demonstrated by homology modeling, and biophysical feature comparison (58). The relevant amino acids are close to a highly dynamic loop, which is important for the protein primary biological function as the scaffolding agent for the viral genomic stability (59).

The role and diversity of the T cell response to SARS-CoV-2 was reviewed by Altmann and Boyton (60). There have been multiple efforts to map epitopes in the viral proteome, using both bioinformatics and *ex vivo* approaches. While most of these have prioritized the spike protein, several epitopes in the N protein have been reported. Mateus et al. identify CD4+ T cell allele-specific epitopes encompassed in the sequences we identify from positions 213–238 to 293–320 as binding multiple MHC II alleles (30). Most notably, our findings parallel those of Le Bert et al. (24) who demonstrated CD4+ and CD8+ T cell responses to peptides that overlap the multiallelic binding regions we predicted. In particular, patients who were not exposed to SARS-CoV-2 had CD4+ T cells responsive to N101–120, which comprises the most conserved T cell exposed motifs (**Supplementary Table 1**).

The existence of broadly conserved T cell exposed motifs in the N protein indicates that, even while peptide context is different, there may be potential to develop a vaccine which offers protection across multiple coronaviruses. This was addressed for MERS by Shi et al. (21). Among the epitopes they identified, there are several CD8+ T cell peptides in homologous positions to those we have predicted in SARS-CoV-2, although as noted the T cell exposed motifs conserved in SARS, SARS-CoV-2 and the other human coronaviruses do differ from MERS. Yang et al. also proposed a nucleocapsid based vaccine for SARS-CoV-1 (61).

In summary, the use of available information related to SARS-CoV-2 epitopes associated with bioinformatics predictions points to specific regions of viral nucleocapsid that are targets to human immune responses (25). We understand that lack of biological confirmation of identified peptides may limit the impact of our discovery. However, testing the antigenicity of these B and T cell epitopes will be the next step on our research program. The observation that some T cell epitopes are highly conserved between SARS-CoV-2 and other human coronaviruses is critical. Vaccines that target human immune responses toward these conserved epitopes could generate immunity that is cross-protective across alphacoronaviruses and betacoronaviruses (25). This would be an advantage given the potential of future novel coronavirus emergence.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

Conceptualization: SO, MM, EH. Methodology: SO, MM, EH. Formal analysis: SO, MM, EH. Investigation: SO, MM, EH. Writing: SO, MM, EH. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq) grant #465229/2014-0, 401209/2020-2 and 302660/2015-1 (to SO) and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) grant #2017/24832-6 (to SO) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) grant #88887.506611/2020-00 and 88887.504420/2020-00 and National Institute of Health (NIH) grant# R01 AI 116453 (to SO).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.587615/full#supplementary-material

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Conflict of Interest: EH is an employee and equity holder in ioGenetics LLC.

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.

frontiers in Immunology

ORIGINAL RESEARCH
published: 04 November 2020
doi: 10.3389/fimmu.2020.591885

Adenoviral-Vectored Mayaro and Chikungunya Virus Vaccine Candidates Afford Partial Cross-Protection From Lethal Challenge in A129 Mouse Model

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Mayaro (MAYV) and chikungunya viruses (CHIKV) are vector-borne arthritogenic alphaviruses that cause acute febrile illnesses. CHIKV is widespread and has recently caused large urban outbreaks, whereas the distribution of MAYV is restricted to tropical areas in South America with small and sporadic outbreaks. Because MAYV and CHIKV are closely related and have high amino acid similarity, we investigated whether vaccination against one could provide cross-protection against the other. We vaccinated A129 mice (IFNAR –/–) with vaccines based on chimpanzee adenoviral vectors encoding the structural proteins of either MAYV or CHIKV. ChAdOx1 May is a novel vaccine against MAYV, whereas ChAdOx1 Chik is a vaccine against CHIKV already undergoing early phase I clinical trials. We demonstrate that ChAdOx1 May was able to afford full protection against MAYV challenge in mice, with most samples yielding neutralizing $PRNT_{80}$ antibody titers of 1:258. ChAdOx1 May also provided partial cross-protection against CHIKV, with protection being assessed using the following parameters: survival, weight loss, foot swelling and viremia. Reciprocally, ChAdOx1 Chik vaccination reduced MAYV viral load, as well as morbidity and lethality caused by this virus, but did not protect against foot swelling. The cross-protection observed is likely to be, at least in part, secondary to cross-neutralizing antibodies induced by both vaccines. In summary, our findings suggest that ChAdOx1 Chik and ChAdOx1 May vaccines are not only efficacious against CHIKV and MAYV, respectively, but also afford partial heterologous cross-protection.

Keywords: adenovirus-vectored vaccines, alphavirus, chikungunya virus, cross-protection, arthritis, A129 mice, Mayaro virus, chimpanzee adenovirus

OPEN ACCESS

Edited by:
Neeltje van Doremalen, Rocky Mountain Laboratories (NIAID), United States

Reviewed by:
Tarek A. Ahmad, Bibliotheca Alexandrina, Egypt
Christine Wong, Charité – Universitätsmedizin Berlin, Germany

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Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: *05 August 2020*
Accepted: *07 October 2020*
Published: *04 November 2020*

Citation:
Campos RK, Preciado-Llanes L, Azar SR, Kim YC, Brandon O, López-Camacho C, Reyes-Sandoval A and Rossi SL (2020) Adenoviral-Vectored Mayaro and Chikungunya Virus Vaccine Candidates Afford Partial Cross-Protection From Lethal Challenge in A129 Mouse Model. Front. Immunol. 11:591885. doi: 10.3389/fimmu.2020.591885

INTRODUCTION

Mayaro virus (MAYV) and chikungunya virus (CHIKV) are arboviruses, members of the *Togaviridae* family, and the etiologic agents of Mayaro fever (MAYF) and chikungunya fever (CHIKF), respectively. Both illnesses are characterized by flu-like symptoms including fever, myalgia, arthralgia and/or skin rash (1–4), making their symptomatology largely indistinguishable from each other and from other common arboviral diseases (5–7). CHIKV circulates in many continents (8), whereas MAYV is thought to be restricted to areas close to forests in Central and South America, where it causes small outbreaks (9–13). However, since MAYV is present in regions where many arboviruses co-circulate, the number of human infections is likely underreported. There is co-incidence of both diseases in the Americas, especially in South America, where it is estimated that 1% of all febrile cases with symptoms and clinical signs of arboviral disease may be caused by MAYV (7). Although most outbreaks have been small, its potential to produce large outbreaks became evident in 1978, when MAYV was responsible for infecting approximately 20% of the 4,000 inhabitants in Belterra, Brazil, most living near the forest (12). Although MAYV is able to cause disease in humans and produce high viremia, mosquitoes of the *Haemagogus* genus, which are the primary vectors of MAYV, are absent in urban settings (6, 14). Vector competency studies in laboratory settings have reported that MAYV may be transmitted by urban and peri-urban mosquitoes of the *Aedes* genus (15–17). Although MAYV has been isolated from *Aedes aegypti* in nature (18), transmission from these mosquitoes to humans has not been reported to date. MAYV could adapt to emerge into an urban transmission cycle, just as was determined to have happened for its close relative CHIKV, which adapted to *Aedes albopictus* after acquiring a mutation in the amino acid in the position 226 of the E1 viral protein (19, 20). Due to the presence of both viruses in the same regions, and the possibility of MAYV adaptation to the urban cycle (13, 21), there is significant interest in developing vaccines which could simultaneously protect against both diseases. Therefore, it is important to understand the impact that vaccination for CHIKV may have on MAYF and reciprocally, the effect that vaccination for MAYV may have on CHIKF. The similarities between MAYV and CHIKV are vast, not only in their mode of transmission and disease profile, but also in their viral structure and antigenic relationship. Thus, it is not surprising that several studies have investigated the possibility of cross-protection between MAYV, CHIKV and other alphaviruses (22–25). Webb and colleagues (25) reported different degrees of protection with two CHIKV candidate vaccines. The live-attenuated vaccine CHIKV-IRES, protected partially against MAYV challenge, whereas the chimeric host-restricted vaccine EILV-CHIKV did not protect against MAYV disease.

As CHIKV has a noteworthy health and economic burdens and MAYV may emerge to pose serious threats, it is imperative that countermeasures are developed to prepare against outbreaks, however, no licensed vaccine is available to date. Several strategies have been used to develop vaccines against alphaviruses, including live-attenuated virus, viral protein subunits, viral vectors and nucleic acid-derived vaccines (26, 27). Engineered adenoviral-vectored vaccines have been widely investigated (28, 29), as they are known to be potently immunogenic, inducing both antibodies and T cell responses. However, the use of human adenoviruses has been limited, mainly due to pre-existing immunity against these viruses among the general population (30). To circumvent this issue, chimpanzee adenoviruses are being used due to their negligible seroprevalence in human populations (30, 31). ChAdOx1 is a chimpanzee adenoviral vector, developed from the adenovirus isolate Y25 subgroup E (32). ChAdOx1 has deletions on the E1 and E3 genes that render it replication-deficient thereby enhancing its safety (32). We have previously reported that ChAdOx1 encoding CHIKV structural proteins (ChAdOx1 Chik) elicits long-lasting IgG antibodies against CHIKV E2 in BALB/c mice (33). ELISA measurements at two weeks, six weeks and 10 months post vaccination, showed that the levels of IgG anti-CHIKV E2 are maintained over time, suggesting long term immunity of at least 10 months. In the same work, we also found a high frequency of T cells recognizing CHIKV peptides as early as two weeks after immunization, thereby suggesting that ChAdOx1 Chik induces specific T cell responses (33). In another study, we found that ChAdOx1 Chik provides complete protection from a lethal CHIKV challenge in the highly permissive A129 mouse model (34).

We have constructed ChAdOx1 May, a chimpanzee adenoviral vectored vaccine that expresses the MAYV structural proteins. In this study, we demonstrate that ChAdOx1 May elicits rapid and robust immunity with high titers of neutralizing antibodies against MAYV, able to protect A129 mice from a lethal and reducing viremia to undetectable levels. Furthermore, we show that vaccination with ChAdOx1 May offers cross-protection against a lethal CHIKV challenge. Conversely, the equivalent chikungunya vaccine named ChAdOx1 Chik, and which is currently undergoing clinical trials (NCT03590392), appears to have a very limited effect against MAYV. Our results are particularly relevant in the setting of outbreaks, where pre-existing immunity against MAYV may lead to immunity against CHIKV, and vice versa.

MATERIALS AND METHODS

Design and Production of the ChAdOx1 May Vaccine

The structural cassette MAYV sequence derived from various MAYV lineages was codon optimized. To improve initiation of translation a Kozak consensus sequence was included before the 5' end of the transgene. Finally, the transgene design included the required enzymatic restriction sites to allow the in-frame cloning of the transgene between the CMV promoter and the PolyA sequence region contained in our shuttle and expression vector (pMono). A synthetic gene cassette was produced by GeneArt® (Fisher Scientific, Regensburg, Germany) and was named sMAYV. The plasmid containing the structural Mayaro virus (sMAYV) cassette (Capsid,

Envelope 1–3 and 6K) was digested with KpnI and NotI restriction enzymes (NEB, Ipswich, MA, U.S.) to allow in-frame ligation between the CMV promoter and the Poly(A) regions contained in the shuttle plasmid (pMono). The recombinant DNA plasmids were expanded and purified from *E. coli* using the Qiagen MIDI-prep kit. Resulting plasmids were verified by restriction analysis and 5' and 3' flanking sequencing. To generate ChAdOx1 vaccine, the shuttle plasmids containing attL regions sequences were each recombined with those attR regions contained in the destination vector ChAdOx1 using an *in vitro* Gateway reaction (LR Clonase II system, Invitrogen™). Successfully recombined ChAdOx1 May (also known as ChAdOx1 sMAYV) was verified by DNA sequencing using flanking primers (forward promoter primer and Poly-(A) reverse primer). Standard cell biology and virology techniques were performed to generate the non-replicative adenoviral vectors.

Design and Production of the ChAdOx1 Chik Vaccine

ChAdOx1 Chik (also known as ChAdOx1 sCHIKV) was designed and produced as previously reported (33). The immunogenicity and efficacy profiles of ChAdOx1 in mice has been recently demonstrated (33, 34).

Control Vaccines

ChAdOx1 Zika (also known as ChAdOx1 Zika prME ΔTM) was produced as previously described (35) and used as the off-target control vaccine in our challenge experiments. The MAYV-IRES vaccine were previously developed (36), by inserting an IRES in the genome of MAYV and passaging, respectively. Unrelated ChAdOx1 dengue NS1 (unpublished) was used as the mock vaccine in the immunogenicity studies.

Viruses and Cells Used

Vero CCL-81 cells from the American Type Culture Collection were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% of fetal bovine serum (FBS) and 1% of penicillin/streptomycin at 37˚C in a humidified incubator containing 5% of CO_2. The MAYV-IRES cDNA clone was used to produce viral stocks by electroporating Vero cells as previously reported (36, 37).

Virus Titration

Samples underwent 10-fold serial dilutions in DMEM with 2% FBS and incubated on monolayers of Vero cells as described previously. After 1 h of incubation at 37°C rocking every 15 min, an 0.4% agarose overlay was added. Cells were then incubated at 37°C with a 5% CO_2 atmosphere for 48h (for MAYV) and 36h (for CHIKV), fixed with a solution of 3.7% formaldehyde and stained with crystal violet (0.25% w/v in 30% methanol). Titers were shown as PFU/ml and had a limit of detection (LOD) of 100 PFU/ml. In the statistical analyses, values below LOD were set as 50% of the LOD (50 PFU/ml).

Plaque Neutralization Reduction Test

PRNT assays were done using the same viruses used in the challenges, MAYV CH strain (also known as IQT4235 strain) and CHIKV La Reunion (LR) strain, as previously described. Sera was heat-inactivated for 1 h at 56°C and underwent 2-fold serial dilutions in media. The virus was then incubated with the serum for 1h at 37°C and then added to monolayers of Vero cells and then treated as a virus titration from that step. A reduction of 50% or 80% in the number of virus plaques compared to virus only control was used to call PRNT50 and PRNT80 cutoffs, respectively. The LOD was 1:20.

Animals

The A129 mice were purpose-bred from a colony maintained at UTMB, which is an AALAS-approved facility. Mice were kept in sterilized cages. Cohorts with male and female mice were ear-notch identified and vaccinated at 5 weeks-old. ChAdOx1 vaccines were diluted to deliver a dose of 1 x 10^8 IU and MAYV-IRES vaccine was diluted in PBS and the inoculum was backtitered to be 1.6 x 10^4 PFU/mouse. For each mouse, 25 µl of vaccine was injected intramuscularly in each leg. All the work was done according to our approved Institutional Animal Care and Use (IACUC) protocol (1708051). Mice reaching a humane endpoint such as a loss of 20% or greater of their body weight or any evidence of neurological disease (including inability to move when stimulated, inability to eat/drink, tremors and paralysis) were euthanized by CO_2 asphyxiation. The number of animals used for each condition are indicated in **Table 1**.

Female inbred BALB/c (H-2d), (6–8 weeks) were used for the assessment of immunogenicity (n = 6 per group). Mice were

TABLE 1 | Survival of A129 mice challenged with Mayaro virus (MAYV) or chikungunya virus (CHIKV).

Vaccine	Challenge	Number of mice in cohort	% Survival (number)	MDD[1]	*p*-value[2]
PBS	MAYV	6	0% (0/6)	3 +/– 0	N/A
ChAdOx1 Zika	MAYV	5	0% (0/5)	3.6 +/– 0.5	0.0339
MAYV-IRES	MAYV	5	100% (5/5)	N/A	0.0016
ChAdOx1 May	MAYV	5	100% (5/5)	N/A	0.0016
ChAdOx1 Chik	MAYV	5	60% (3/5)	9.5 +/– 3.5	0.0016
PBS	CHIKV	4	0% (0/4)	4.4 +/– 0.5	N/A
ChAdOx1 Zika	CHIKV	5	0% (0/5)	4.4 +/– 0.5	0.1763
ChAdOx1 Chik	CHIKV	5	100% (5/5)	N/A	0.0047
ChAdOx1 May	CHIKV	5	80% (4/5)	9 +/- 0	0.0047

[1]*Mean Day of Death (MDD) +/– standard deviation. Excludes mice that survived.*
[2]*p-value, based upon Log-ranked (Mantel-Cox) test compared with PBS.*

purchased from Envigo RMS Inc. (Bicester, G.B.). The experimental design took into account the 3R reduction (Replacement, Reduction, Refinement) and procedures were approved by the Animal Care and Ethical Review Committee (PPL 30/2414). No randomization was used in this work.

Vaccination

ChAdOx1 vaccines were thawed on ice and MAYV-IRES at 37° C. All vaccines were then diluted in Dulbecco's phosphate buffered saline (DPBS). ChAdOx1 vaccines were administered at 1×10^8 infectious units (IU) per animal. MAYV-IRES was titrated after vaccination and determined to be 1.3×10^4 PFU/mouse. Animals were anesthetized using isoflurane and then injected with 25 µl of vaccine intramuscularly in each hind leg.

Challenge and Monitoring of Morbidity Readouts

Mice were challenged thirty days post-vaccination. The challenge viruses used were MAYV-CH (backtiter: 1.6×10^4 PFU/mouse) and CHIKV-LR (backtiter: 9.7×10^4 PFU/mouse). Mice were anesthetized with isoflurane and MAYV-CH or CHIKV-LR were injected intradermally in a volume of 20 µl of virus into the left foot with a 28G insulin syringe. Back titration of the challenge viruses was 1.6×10^4 and 9.7×10^4 and PFU/mouse for MAYV and CHIKV, respectively. After injection, mice health and weights were monitored daily and mice that lost more than 20% of their starting weight were euthanized. Footpad thickness was also measured daily after infection as previously reported (34) and mice were provided with soft bedding and nesting materials to reduce stress and pain. On day 25 post vaccination and day 2 post infection, blood was collected from anesthetized mice using a capillary tube on the retro-orbital sinus. Blood and sera collected from clarified blood samples were used for PRNT assays and viremia tests, respectively.

Enzyme-Linked Immunosorbent Assay

Specific antibody binding to MAYV or CHIKV envelope proteins (E2 or E1) was measured by an IgG enzyme linked immunosorbent assay (ELISA) as previously described (33). Briefly, mice sera were diluted in Nunc Maxisorp Immuno ELISA plates coated with the MAYV or CHIKV envelope proteins (E2 or E1) diluted in PBS to a final concentration of 5 µg/mL and incubated at room temperature (RT) overnight. Plates were washed 6 times with PBS/0.05% Tween (PBS/T) and blocked with 300 µL with Pierce™ protein-free (PBS) blocking buffer (Thermo Fisher Scientific, Waltham, MA, U.S.) for 2 h at RT. Mouse serum was added and serially diluted 3-fold down in PBS/T with 50 µL per well as final volume and incubated for 2 h at RT. Following washing 6 times with PBS/T, bound antibodies were detected following a 1 h incubation with 50 µL of alkaline phosphatase-conjugated antibodies specific for whole mouse IgG (A3562-5ML, Sigma Aldrich, SLM, U.S.). Following an additional 6 washes with PBS/T, development was achieved using 100 µL of 4-nitrophenylphosphate diluted in diethanolamine buffer and the absorbance values at OD405 were measured and analyzed using a CLARIOstar instrument (BMG Labtech, Aylesbury, GB). Serum antibody endpoint titers were defined by an absorbance value three standard deviations greater than the average OD_{405} of the control.

Production of MAYV Proteins for ELISA

For expression and purification of the MAYV E2 protein, the codon-optimized gene of E2 (a.a. 1–351) was cloned into the pHLsec vector. In order to improve secretion of the E2 protein, the C-terminal region of E2 (a.a. 352–422) was removed. The pHLsec MAYV E2 plasmid (500 µg) was transfected in HEK-293T cells using polyethyleneimine (PEI) in roller bottles (surface area of 2,125 cm2) under standard cell culture conditions. Five days after transfection cells were discarded and media was filtered through 0.22 µM disposable filters. The secreted protein was purified from the supernatant by Ni Sepharose affinity chromatography (HisTRAP™, GE Healthcare), using the Äkta Start chromatography system and eluted with Imidazole 500mM. Finally, the eluted protein was dialyzed using Slide-A-LyzerTM cassette against 1X PBS. The MAYV E1 and CHIKV E1 proteins were produced in a similar manner using the codon-optimized genes of CHIKV and MAYV E1 (a.a 1-410). CHIKV E2 protein was produced as previously described (33).

Statistical Analyses

Statistical analysis was performed in GraphPad Prism v8.4. Data was analyzed by one-way ANOVA, two-way ANOVA or restricted maximum likelihood (REML) mixed model as appropriate. *Post hoc* corrections were implemented with Dunnett`s against a control group or Sidak's when comparing selected groups. Virus titer data were $\log_{10}$ transformed before statistical analyses. Survival curve comparisons were made using a log-ranked (Matel-Cox) test. In all statistical tests p values below 0.05 were considered significant.

RESULTS

ChAdOx1 May Induces Antibodies Against MAYV E2 and May E1 as Early as 2 Weeks Post-Vaccination in BALB/c Mice

We constructed ChAdOx1 May, a chimpanzee adenoviral vectored-vaccine that expresses the MAYV structural proteins capsid, E1, E2, E3, and 6K (**Figure 1A**). To assess the specific immunogenicity of ChAdOx1 May vaccine, we immunized groups of BALB/c mice (n=6) with a single and unadjuvanted dose of ChAdOx1 May or ChAdOx1 Chik at 1×10^8 infectious units (IU) per animal. Specific IgG antibody responses against E1 or E2 proteins from both MAYV and CHIKV were measured by ELISA at 2 weeks and 4 weeks after immunization.

At 2 weeks post-immunization, the mean MAYV E2-specific antibody titers elicited by ChAdOx1 May was 2.34 $\log_{10}$, whereas the mean CHIKV E2-specific antibody titers elicited by ChAdOx1 Chik was 2.57 $\log_{10}$. By week 4, the anti-E2 titers for both ChAdOx1 May and ChAdOx1 Chik vaccinated animals increased to 3.45 $\log_{10}$ and 3.69 $\log_{10}$, respectively (**Figures 1B, C**). No specific IgG antibody binding to MAYV E2 or CHIKV E2

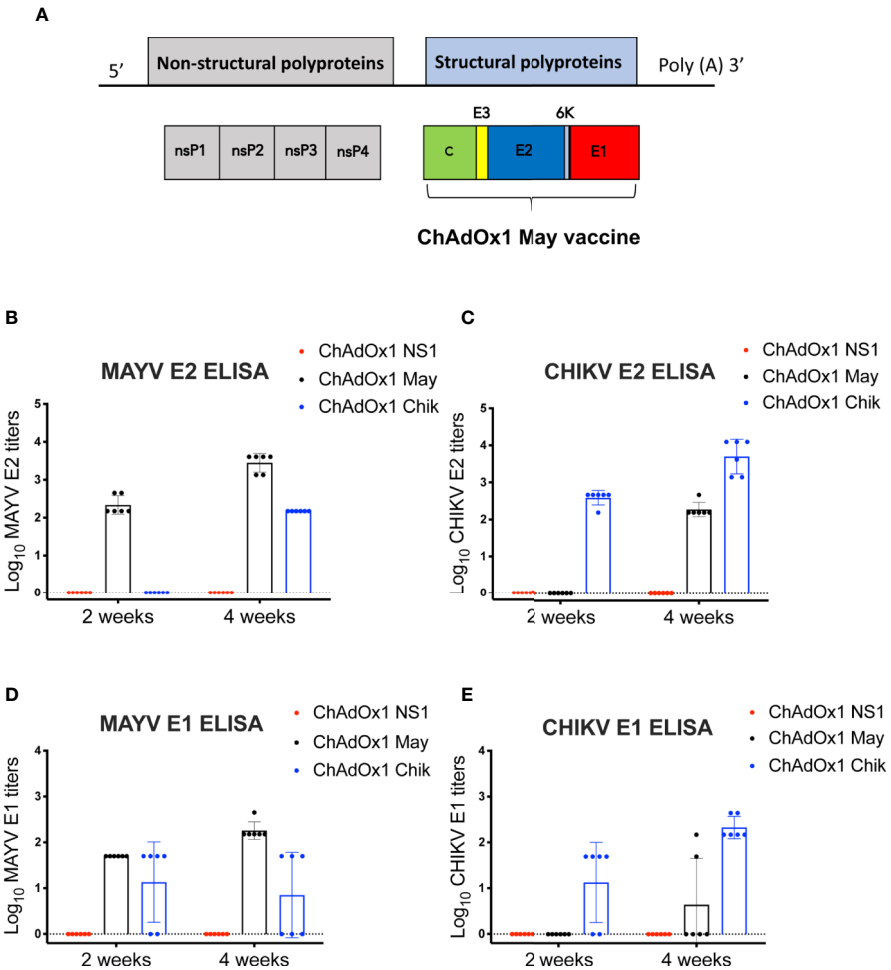

FIGURE 1 | Mayaro virus (MAYV) genome organization, design of ChAdOx1 May vaccine candidate and the humoral responses elicited. **(A)**. Genome organization of MAYV and generation of ChAdOx1 May vaccine. **(B–E)**. Humoral IgG responses against chikungunya virus (CHIKV) and MAYV E1,E2 2 and 4 weeks post-immunization measured by ELISA. The reciprocal log ELISA titers were calculated for all groups shown in the figure. Lines represent the mean with SD and error bars are shown.

was detected in the mock-vaccinated group (ChAdOx1 dengue NS1) at any time point. No cross-reactive IgG antibody binding to MAYV E2 or CHIKV E2 was detected at 2 weeks. However, at 4 weeks after vaccination, anti-MAYV E2 antibodies were detected in mice vaccinated with ChAdOx1 Chik (mean titers 2.18 $\log_{10}$). ChAdOx1 May vaccinated animals also showed cross-reactive anti-CHIKV E2 antibodies (mean titer 2.26 $\log_{10}$) by week 4. This indicates that there is some degree of cross-reactivity between anti-CHIKV E2 antibodies toward the MAYV E2 protein and vice versa.

ChAdOx1 May vaccinated mice had a mean anti-MAYV E1 antibody titer of 1.70 $\log_{10}$ at 2 weeks post-immunization and this increased to 2.26 $\log_{10}$ by 4 weeks (**Figure 1D**). Four and three mice vaccinated with ChAdOx1 Chik had detectable cross-reactive anti-MAYV E1 antibodies at 2 weeks and 4 weeks after vaccination, respectively. Vaccination with ChAdOx1 Chik induced anti-CHIKV E1 antibodies in four out of six animals at 2 weeks, and by 4 weeks all mice had detectable anti-CHIKV E1 antibodies (mean titer 2.33 $\log_{10}$) (**Figure 1E**). Mice vaccinated with ChAdOx1 May did not show any cross-reactive anti-CHIKV E1 antibodies at 2 weeks post-immunization, but two mice had detectable anti-CHIKV E1 antibodies at 4 weeks.

Taken together, we show that a single dose of ChAdOx1 May and ChAdOx1 Chik is immunogenic and induces specific anti-E1 and E2 IgG antibodies as early as two weeks post-immunization. Moreover, our results suggest that ChAdOx1 May and ChAdOx1 Chik induce cross-reactive antibodies, in particular toward the E2 protein at 4 weeks post-immunization.

ChAdOx1 Vaccination Do Not Cause Adverse Events in A129 Mice

Next, we sought to determine if the antibody response observed in vaccinated BALB/c mice would elicit protective immunity in

the A129 mouse challenge model. A129 mice that are deficient in IFN-α/β receptor signaling pathway offer a rigorous test for vaccine safety because interferon is important for an efficient antiviral response, and as such, A129 mice are highly susceptible to infections and lethal disease. In agreement with our previous publication (34), we did not observe weight loss or any adverse events in A129 mice (IFNAR −/−) vaccinated with any of the ChAdOx1 viral vectors, including ChAdOx1 Chik, ChAdOx1 May or the off-target vaccine (ChAdOx1 Zika, **Figure 2**). In contrast, the live-attenuated MAYV-IRES vaccine used as a positive control (36), caused adverse clinical signs such as mild weight loss (**Figure 2**), lethargy, ruffled fur and squinty eyes. MAYV-IRES vaccinated animals recovered completely prior to challenge, except for one mouse that continued presenting squinty eyes until the end of the experiment, a sequela likely caused by MAYV-IRES.

ChAdOx1 May and ChAdOx1 Chik Afford Homologous Protection and Partial Heterologous Cross-Protection Against MAYV and CHIKV-Induced Disease in A129 Mice

To test the effectiveness of our ChAdOx1 May and ChAdOx1 Chik candidate vaccines, and to investigate whether they could induce cross-protection, we carried out CHIKV and MAYV challenges in A129 mice. Thirty days following vaccination, mice were challenged with a lethal dose of MAYV-CH (1.6 x 10^4 PFU/mouse) or CHIKV-LR (9.7 x 10^4 PFU/mouse), *via* intradermal injection on the left rear foot. Survival, weight loss, foot swelling, and other signs associated with MAYV or CHIKV-induced disease were assessed daily and used as readouts.

We recently demonstrated that vaccination with ChAdOx1 Chik prevents lethal disease in mice when challenged with CHIKV (34). Here, we demonstrate that mice vaccinated with ChAdOx1 May and the live-attenuated MAYV-IRES control vaccine were protected and all survived MAYV challenge until the end of the experiment (**Table 1**). Remarkably, ChAdOx1 May and ChAdOx1 Chik vaccines demonstrated different degrees of cross-protection, three out of five mice vaccinated with ChAdOx1 Chik survived the MAYV challenge, whereas four out of five mice vaccinated with ChAdOx1 May survived the CHIKV challenge (**Table 1**). As expected, mice vaccinated with PBS or ChAdOx1 Zika were not protected against MAYV or CHIKV infections and had to be euthanized a few days after challenge.

In comparison with the control groups injected with PBS and the ChAdOx1 Zika vaccine, which lost about 10% of their weight by day 3 after MAYV challenge, both MAYV-IRES and ChAdOx1 May vaccines protected mice against significant weight loss (**Figure 3A**). ChAdOx1 Chik provided partial cross-protection by preventing significant weight loss in two out of five animals (40%) and by delaying weight loss in 1/5 mice (**Figure 3A**). In an equivalent CHIKV challenge model, we previously demonstrated that ChAdOx1 Chik protected mice against weight loss, viremia and foot swelling at the inoculation

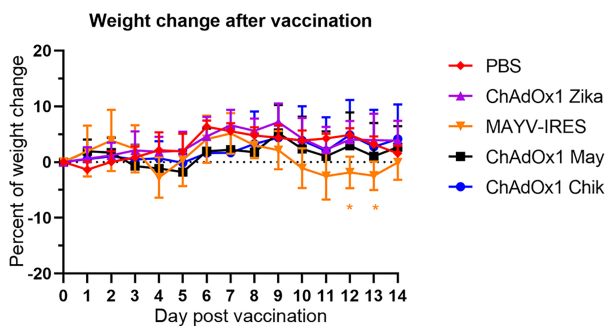

FIGURE 2 | Administration of ChAdOx1 vaccines do not cause weight loss in A129 mice. Percentages of weight change following vaccinations are shown. The weights of A129 mice in each group were compared to their weights just before vaccination (day 0). Data are represented as means and SEM. Two-way ANOVA with repeated measures with Dunnet's compared with PBS group; *p < 0.05.

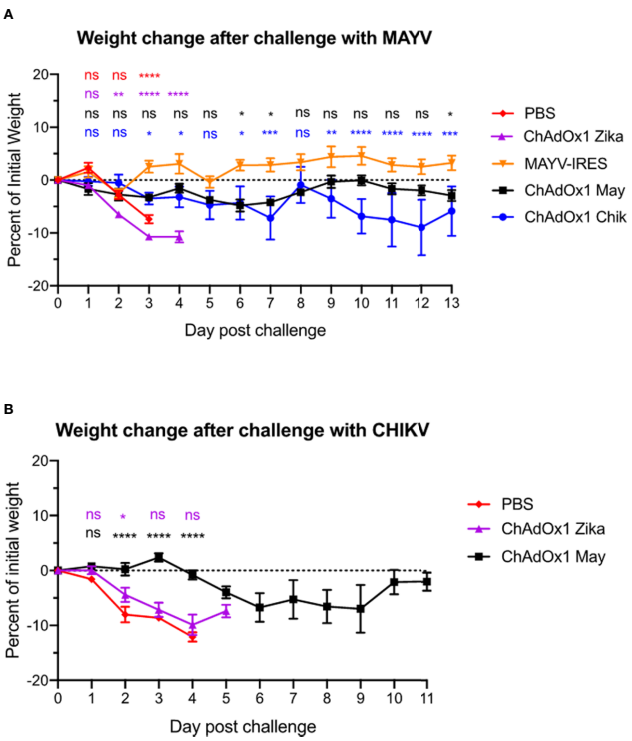

FIGURE 3 | ChAdOx1 May and ChAdOx1 Chik vaccination protect against weight loss. Animals were challenged thirty days post-vaccinations with Mayaro virus (MAYV) or chikungunya virus (CHIKV) (backtiters 1.6 and 9.7 x 10^4 PFU/mouse). **(A)** Weight change in vaccinated mice after challenge with MAYV. Data represented as mean and SEM, restricted maximum likelihood mixed model with Dunnett's (compared with MAYV-IRES group). **(B)** Weight change in vaccinated mice after challenge with CHIKV. Data represented as mean and SEM, restricted maximum likelihood mixed model with Dunnett's (compared with PBS group) *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. ns, not significant.

site (34). In this CHIKV challenge, vaccination with ChAdOx1 May resulted in a delayed and mild weight loss, with only one out of five mice (20%) in the group losing over 20% of its weight by day 9 (**Figure 3B**).

Viremia is an important hallmark of disease for alphavirus infections (38, 39). MAYV and CHIKV were measured in serum on the second day after challenge, when peak viremia is predicted (25, 40). Virus titers in the PBS-injected and ChAdOx1 Zika-vaccinated groups were of around 9 $\log_{10}$ PFU/ml for MAYV and around 6 $\log_{10}$ PFU/ml for CHIKV. Vaccination with ChAdOx1 May afforded sterile protection, with undetectable virus titers of MAYV in serum (**Figure 4A**). Consistent with the survival results, we observed that ChAdOx1 May and ChAdOx1 Chik provide effective cross-protection by significantly decreasing viremia. ChAdOx1 Chik vaccinated mice had on average over 3 $\log_{10}$-fold reduction in MAYV viremia compared with negative controls (**Figure 4A**). ChAdOx1 May had an even larger impact on cross-reactivity in mice following challenge with CHIKV, reducing titers by 4 $\log_{10}$-fold, with three out of five samples below the limit of detection of our assay (**Figure 4B**).

Swelling at the inoculation site is another hallmark of arthritogenic alphavirus infection in the A129 mouse model (41), which reproduces the joint inflammation caused by MAYV and CHIKV in humans (42). In this experiment we inoculated one foot only and used the other foot as an internal control for each animal. Mice that were vaccinated with PBS or ChAdOx1 Zika showed severe foot swelling in comparison to the uninfected foot. In most of these animals, the inoculated feet quickly doubled in thickness, as early as day 3 after CHIKV inoculation and by day 4 after MAYV inoculation (**Figure 5**). Throughout the whole duration of the experiment, we did not observe foot swelling in any of the ChAdOx1 May or MAYV-IRES vaccinated mice when challenged with MAYV (**Figures 5A, B**). While vaccination with ChAdOx1 Chik failed to afford significant cross-protection in mice challenged with MAYV (**Figures 5A, B**), vaccination with ChAdOx1 May resulted in a limited but significant reduction of foot swelling in mice that were challenged with CHIKV (**Figures 5C, D**).

In summary, these results demonstrate that ChAdOx1 May not only fully protects mice from lethal MAYV-induced disease but also cross-protects against CHIKV viremia, limits CHIKV-induced weight loss, delays foot inflammation and prompts its resolution. Conversely, ChAdOx1 Chik vaccine is not as effective in cross-protecting against MAYV viremia and fails to reduce or delay foot inflammation.

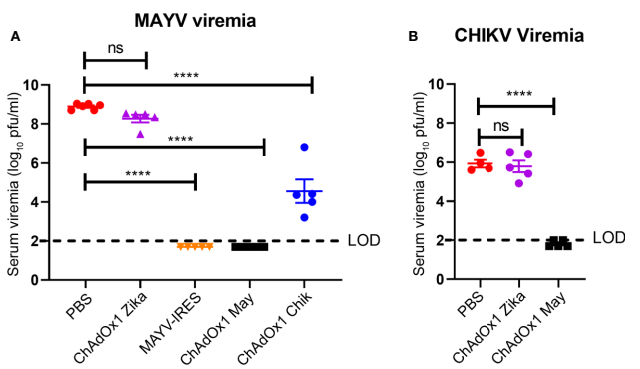

FIGURE 4 | ChAdOx1 May and ChAdOx1 Chik vaccines afford cross-protection by reducing viremia. Blood was collected 2 days post challenge and serum was titrated. **(A)** Mayaro virus (MAYV) viremia. **(B)** Chikungunya virus (CHIKV) viremia. The limit of detection (LOD) is 2 $\log_{10}$ PFU/ml. Data represented as mean and SEM, one-way ANOVA with Dunnett's, ****$p < 0.0001$. ns, not significant.

ChAdOx1 May and ChAdOx1 Chik Induce Low Levels of Cross-Neutralizing Antibodies

To determine whether the efficacy of the ChAdOx1 vaccines was correlated to the production of neutralizing antibodies, we performed plaque reduction neutralizing tests (PRNT) against both alphavirus using mouse sera obtained 25 days following vaccination (**Figures 6A–D**).

At the most stringent 80% cutoff ($PRNT_{80}$) MAYV-IRES induced neutralizing titers of between 1:320 and 1:620 against MAYV, with all animals surpassing titers of 1:640 at the 50% cutoff ($PRNT_{50}$). Sera from ChAdOx1 May vaccinated mice was also highly neutralizing against MAYV, with antibody titers ranging from 1:160 to 1:320 at $PRNT_{80}$ and from 1:160 to 1:640 at $PRNT_{50}$ at the vaccine dose used (**Figures 6A, C**). Only two mice from the ChAdOx1 Chik vaccinated group had detectable cross-neutralizing antibodies against MAYV at $PRNT_{50}$, but these titers fell below the detection limit when $PRNT_{80}$ was considered (**Figures 6A, C**).

In a separate experiment, we also measured cross-neutralizing antibodies against CHIKV in mice sera 25 days post vaccination This group had $PRNT_{80}$ titers below the detection limit (**Figure 6D**), with low $PRNT_{50}$ titers of 1:20 and 1:40 being detected in 2 mice (**Figure 6B**). As expected, mice vaccinated with PBS or ChAdOx1 Zika had antibody levels below our detection limit (at a 1:20 dilution) in all the PRNT performed.

DISCUSSION

MAYV and CHIKV are arthritogenic mosquito-borne viruses of medical importance, mainly due to the long-term polyarthritis that they can cause. CHIKV is a global threat and has caused large urban outbreaks (8), whereas MAYV has potential for emergence due to its potential to spread from a rural sylvatic cycle to an urban one (6, 12, 15). Although significant effort has been made toward vaccine development (36, 43, 44), vaccines are not yet available against these viruses. Given that MAYV and CHIKV co-circulate in Central, South America and the Caribbean and have a close phylogenic and antigenic relationship, it is imperative to evaluate not only the efficacy of candidate vaccines but also their cross-reactivity capacity.

In this study, we demonstrate that ChAdOx1 May, a novel chimpanzee adenoviral-vectored vaccine candidate, induces sterilizing immunity and high titers of neutralizing antibodies

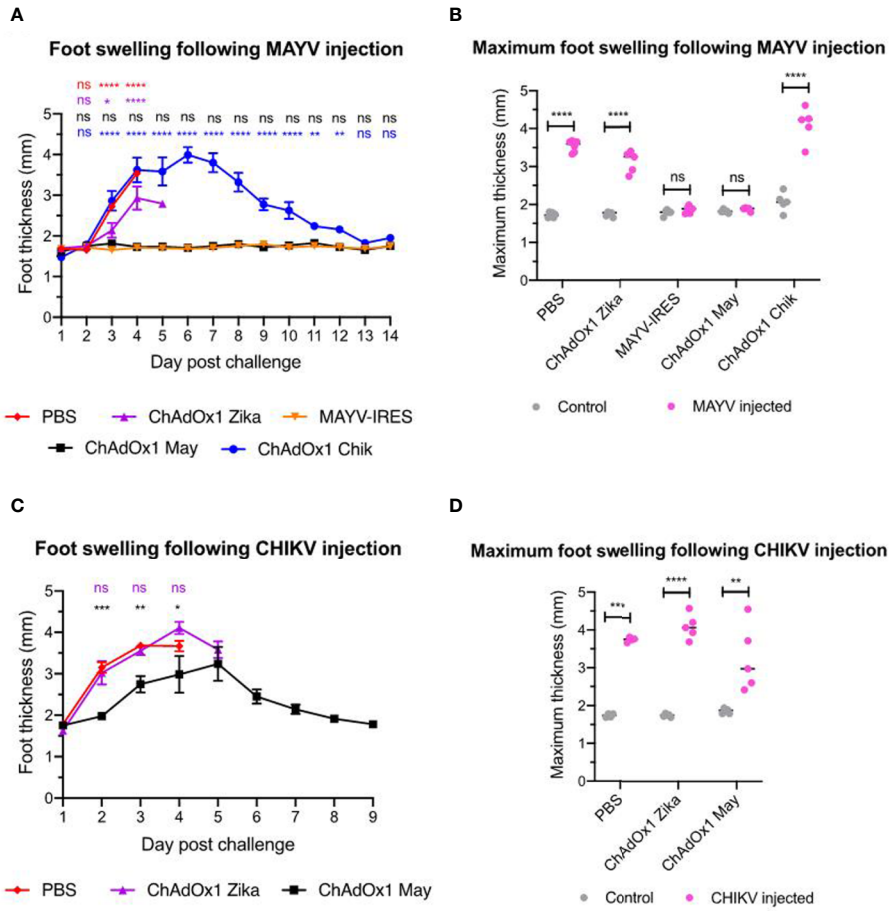

FIGURE 5 | ChAdOx1 May but not ChAdOx1 Chik provides some cross-protection against foot swelling. **(A)** Foot thickness (in mm) at the Mayaro virus (MAYV) injection site. Data represented as mean and SEM, restricted maximum likelihood mixed model with Dunnett's (compared with MAYV-IRES group). **(B)** Comparison between control foot (in grey) and the maximum foot thickness (in pink) at any given timepoint following MAYV injection. Dots represent each mouse, two-way ANOVA with Sidak's. **(C)** Data represented as mean and SEM, restricted maximum likelihood mixed model with Dunnett's (compared with PBS group). **(D)** Comparison between control foot (in grey) and the maximum footpad thickness (in pink) at any given timepoint following CHIKV injection. Dots represent each mouse, two-way ANOVA with Sidak's. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. ns, not significant.

that protect A129 mice from lethal MAYV disease. We did not detect viremia in ChAdOx1 May vaccinated mice, nor observe weight loss or foot swelling which are hallmark signs of morbidity in the A129 model. We also provide evidence that ChAdOx1 May affords a good degree of cross-protection against CHIKV, by reducing lethality, preventing viremia, as well as limiting and delaying morbidity. Although cross-reactivity induced by ChAdOx1 Chik against MAYV was also observed in our model, the magnitude of the response appears variable and transitory.

Viremia is one of the hallmarks of the acute phase in alphavirus infections (45) and is considered an important factor related to the spread of these viruses by mosquitoes. As the bloodmeals taken by mosquitoes are less than 5 µl in volume (46), high titers of virus are important for them to become infected and function as a vector the virus. The effect that ChAdOx1 May had on viremia was robust and cross-reactive, lowering both MAYV and CHIKV titers to undetectable levels in our challenge model. Although we have evidence that ChAdOx1 Chik also provides sterile immunity against CHIKV (34), ChAdOx1 Chik afforded only partial cross-protection against MAYV, as reflected by a reduction in viremia by about 4 $\log_{10}$. This could be a consequence of different viral loads because, consistent with other studies, we observed that A129 mice challenged with MAYV have higher viremia at two days post infection than the titers observed in an equivalent CHIKV challenge. Reports suggest that CHIKV viremia in humans are in the magnitude of about 7 $\log_{10}$ (44), which is more than the 5.34 $\log_{10}$ titers detected in the blood of a patient with acute febrile MAYV infection (15), although the investigation on ranges of MAYV viremia has been more limited.

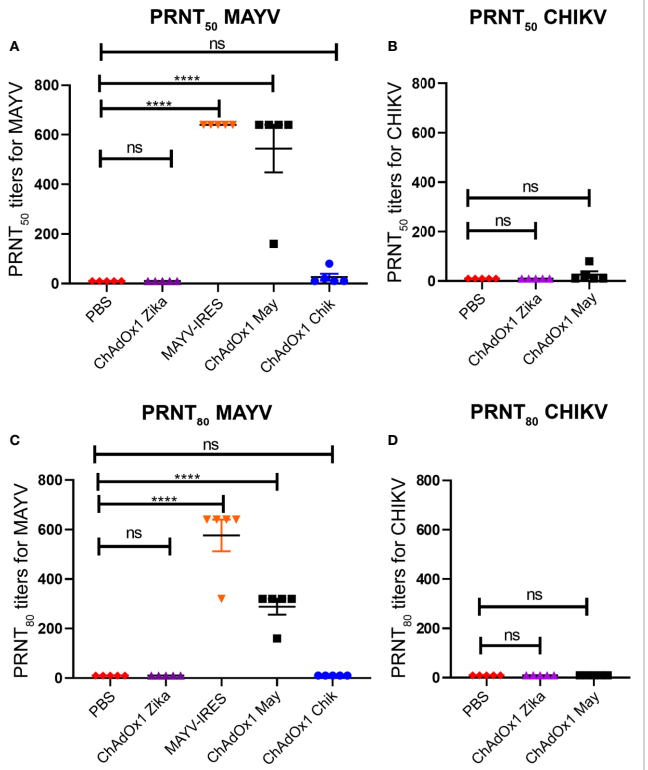

FIGURE 6 | ChAdOx1 May induces high levels of neutralizing antibodies against Mayaro virus (MAYV) but low levels of cross-neutralizing antibodies. Reciprocal neutralizing antibody titers in $PRNT_{50}$ **(A)** and $PRNT_{80}$ **(C)** against MAYV. Reciprocal neutralizing antibody titers in $PRNT_{50}$ **(B)** and $PRNT_{80}$ **(D)** against CHIKV, done in a separate experiment. The limit of detection (LOD) is 20 (1:20 dilution), data points recorded as 10 had neutralization values < LOD. Dots represent titers for each animal, bars represent mean and SEM, one-way ANOVA with Dunnett's, ****$p < 0.0001$. ns, not significant.

In mouse models used to investigate arthritogenic alphaviruses, foot swelling at the site of injection is commonly used as a readout and hallmark of morbidity (47), which reflects some of the joint inflammation that takes place in humans. Numerous immune cells will infiltrate the site of infection and pro-inflammatory cytokines will be released in an effort to eliminate the virus, but this response is largely immunopathologic; leading to swelling, tissue damage and chronic arthralgia (47, 48). After the initial infection, long-term protection against alphavirus-induced disease, including foot swelling in mice, is thought to be mediated mainly by neutralizing antibodies (41, 49). In our study, ChAdOx1 May prevented MAYV-induced foot swelling and also delayed and diminished the swelling caused by CHIKV. In contrast, ChAdOx1 Chik vaccination did not prevent the foot swelling caused by MAYV. Another study (25), reported that vaccination of A129 mice with an insect-specific virus platform containing CHIKV structural proteins did not afford protection and resulted in increased foot swelling.

Neutralizing antibodies are a key correlate of protection against alphaviruses. Cross-neutralization is likely to occur because MAYV and CHIKV are phylogenetically related, with the strains used in this study sharing approximately 71% of amino acid similarity. Webb and colleagues (25), reported cross-protection against MAYV disease in A129 mice vaccinated with a live attenuated CHIKV-IRES vaccine, with this phenomenon demonstrated to be antibody mediated and not altered by T cell responses. In support, a selected group of monoclonal antibodies generated from CHIKV-infected mice were shown to be broadly neutralizing, capable of limiting the viral lifecycle of several alphaviruses by blocking their cell entry and exit (22). Authors performing PRNT assays using convalescent human sera from CHIKV infected individuals, have also described a degree of cross-neutralizing activity against MAYV (24, 25). However, a recent publication (50) reports that equally potent monoclonal neutralizing antibodies against the same MAYV epitope, some of which were even also cross-reactive against CHIKV, did not protect mice from MAYV disease equivalently. They were able to find that the effectiveness of their antibodies was not only related to the neutralization potency, but it was also related to the antibody's F_c effector function on phagocytosis and cytolysis. Although in this work ChAdOx1 Chik and ChAdOx1 May afforded significant cross-protection against heterologous disease, we did not detect significant titers of cross-neutralizing antibodies.

Antibodies against E2 protein, in particular those binding to the β domain, appear to be strongly neutralizing (22), although there is evidence suggesting that neutralization against E1 protein may also be important (22). We detected specific IgG antibody binding to MAYV E2 and E1 and CHIKV E2 and E1 as early as 2 weeks after immunization with ChAdOx1 May and ChAdOx1 Chik, respectively. Some degree of cross-reactivty was observed in both ChAdOx1 May and ChAdOx1 Chik vaccinated groups, in particular toward CHIKV E2 and MAYV E2 proteins. This induced cross-reactive anti-E2 antibodies may partially account for degree of cross-protection observed in our A129 challenge model.

In summary, we provide evidence of the protective efficacy of ChAdOx1 May against MAYV, as well as its cross-reactive effects on CHIKV. If this cross-protection also takes place in the context of immunity secondary to natural infection, it is likely that the emergence potential of MAYV may be reduced by pre-existing CHIKV immunity. Reciprocally, immunity to MAYV may affect the breadth of the ongoing CHIKV outbreaks, modulating both geographical spread and the severity of the impact on human health. Overall, our work sheds light into the immunogenic interactions between MAYV and CHIKV and is of high relevance in the occurrence of large outbreaks in areas where CHIKV and MAYV co-circulate. Finally, it is also important for the development of Phase II/III clinical trials that aim to assess the efficacy of CHIKV and MAYV vaccine candidates in endemic areas where cross-reactive pre-existing immunity may be present. In the future, a dual vaccination approach with ChAdOx1 May and ChAdOx1 Chik should be tested, as there may be the possibility of immune synergy to occur.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Animal manipulations were done according to an approved Institutional Animal Care and Use (IACUC) protocol (1708051).

AUTHOR CONTRIBUTIONS

Conceptualization: RC, LP-L, AR-S, SR, CL-C, and SA. Formal analysis: RC, LP-L, and SR. Investigation, RC, SA, YK, and SR. Resources: LP-L, YK, CL-C, AR-S, and SR. Data curation: RC and SR. Writing—original draft preparation: RC, LP-L. Writing—review and editing: RC, LP-L, YK, SA, AR-S, and SR. Project administration: AR-S and SR. All authors contributed to the article and approved the submitted version.

FUNDING

This research was funded by Innovate UK (reference number 971557) with funds supplied by the United Kingdom Department for Health and Social Care Innovate UK.

ACKNOWLEDGMENTS

We thank the excellent technical support from the staff of the Animal Recourse Center (ARC) at UTMB and Grace Rafael for her help maintaining the A129 mouse colony.

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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.

frontiers in Immunology

ORIGINAL RESEARCH
published: 04 November 2020
doi: 10.3389/fimmu.2020.592370

Rapid Response Subunit Vaccine Design in the Absence of Structural Information

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Prior to 2020, the threat of a novel viral pandemic was omnipresent but largely ignored. Just 12 months prior to the Coronavirus disease 2019 (COVID-19) pandemic our team received funding from the Coalition for Epidemic Preparedness Innovations (CEPI) to establish and validate a rapid response pipeline for subunit vaccine development based on our proprietary Molecular Clamp platform. Throughout the course of 2019 we conducted two mock tests of our system for rapid antigen production against two potential, emerging viral pathogens, Achimota paramyxovirus and Wenzhou mammarenavirus. For each virus we expressed a small panel of recombinant variants of the membrane fusion protein and screened for expression level, product homogeneity, and the presence of the expected trimeric pre-fusion conformation. Lessons learned from this exercise paved the way for our response to COVID-19, for which our candidate antigen is currently in phase I clinical trial.

Keywords: Disease X, subunit vaccine, molecular clamp, pre-fusion conformation, membrane fusion protein, *Paramyxoviridae*, *Arenaviridae*, Coalition for Epidemic Preparedness Innovations

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Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: *06 August 2020*
Accepted: *06 October 2020*
Published: *04 November 2020*

Citation:
Wijesundara DK, Avumegah MS, Lackenby J, Modhiran N, Isaacs A, Young PR, Watterson D and Chappell KJ (2020) Rapid Response Subunit Vaccine Design in the Absence of Structural Information. Front. Immunol. 11:592370. doi: 10.3389/fimmu.2020.592370

INTRODUCTION

Viral fusion proteins, which catalyse fusion between viral and cellular membranes during viral entry, are embedded in the virion membrane and displayed on the virion surface. Hence, they are primary targets of neutralizing antibody responses. Importantly, all viral fusion proteins on the virion surface, are found on the virion surface in a metastable "pre-fusion" form that is primed and ready to undergo a major conformational change following triggering by receptor engagement and/or by the low pH environment of endosomes (1). Refolding into this highly stable "post-fusion" form is what drives the process of virus-host cell membrane fusion. Similarly, purified or isolated viral fusion proteins are naturally unstable and trigger into the post-fusion conformation. The conformational changes involved in this transition can dramatically alter the surface topography of the fusion protein itself and hence their display of antigenic epitopes. Therefore, in order for vaccination to elicit an immune response that can prevent infection, the antibodies induced must recognize viral proteins in the active, pre-fusion conformation present on the virion surface to ensure efficient virus neutralization. Recent studies of human antibody responses to naturally

acquired infections have clearly shown that the majority of neutralizing antibodies induced are those that recognize the pre-fusion form of these viral fusion proteins (2, 3).

We have developed a proprietary recombinant protein expression approach, Molecular Clamp, comprising a highly stable trimerization domain, that when incorporated into viral fusion proteins effectively constrain them in their native pre-fusion conformation (**Figure 1**). While generic trimerization motifs, such as GCN4 (an isoleucine zipper) and foldon (from the T4-phage fibritin), have previously been used for the stabilization of viral fusion proteins, additional modifying mutations and/or the inclusion of disulphide bridges have been required to achieve sufficient stability; as has been shown for Respiratory Syncytial virus (RSV) Fusion protein (F) (4), Human Immunodeficiency virus (HIV) glycoprotein 140 (gp140) (5), and Middle Eastern Respiratory Syndrome Coronavirus (MERS-CoV) Spike protein (6). Although these stabilized antigens show promise as vaccine candidates and are advancing into the clinic, such structure-based changes are not always compatible with rapid vaccine development against emerging viruses, as they require detailed knowledge of protein structure and significant time to identify and validate stabilizing mutations. In comparison, and as will be highlighted in the current study, the Molecular Clamp alone is able to facilitate pre-fusion stabilization without the need for labor-intesive, additional modifications, at least for class I virus fusion proteins. The Molecular Clamp is a trimerization motif of 80aa in length (~9.2 kDa) derived from N- and C-terminal heptad repeat (HR) regions of HIV-1 gp41 which self-assemble into a stable six-helical bundle structure that is critical for driving membrane fusion and cell entry of HIV-1 (7) (**Figure 1**). Furthermore, the enhanced stability of the Molecular Clamp facilitates rapid *in silico* antigen design based solely on a shared trimeric architecture which could ensure that immune responses to conformational epitopes that are unique to the pre-fusion form and essential for protection are elicited following vaccination of the subunit vaccine candidates developed using the Molecular Clamp.

All class I viral fusion proteins are trimeric and share a number of conserved features, including an N-terminal signal peptide, a C-terminal transmembrane domain, and a small cytoplasmic domain (**Figure 1**). Due to this shared architecture, the Molecular Clamp can be easily incorporated into recombinant viral glycoproteins in place of the C-terminal transmembrane/cytoplasmic region. While inclusion of the C-terminal Molecular Clamp trimerization domain is sufficient to achieve pre-fusion stabilization of a soluble trimeric protein, we have also observed that protein yield and homogeneity can be affected by antigen design. However our experiences with expression of recombinant viral fusion proteins from a number of different virus families have allowed us to identify a limited set of protein structural regions that can impact on yield and homogeneity, including **(i)** the N-terminal signal peptide, **(ii)** proteolytic cleavage site(s), **(iii)** the fusion peptide, and **(iv)** the membrane-proximal external region (MPER). Considering the importance of time within an emergency scenario, sequential optimization of these factors would not be possible. Instead, to ensure maximal yield downstream, we generate a panel of 20–30 antigens containing deletions and substitutions at sites based on modifications to the structural regions described above. This panel of antigens is then screened for protein expression, homogeneity and pre-fusion stabilization and an optimal design (or possibly small subset of designs) can be quickly selected for further preclinical development including immunogenicity analysis in pre-clinical models and simultaneous manufacturing process optimization.

In the current study, we present findings of two proof of concept studies where we stress tested the antigen design process described in **Figure 1** to develop subunit vaccine candidates

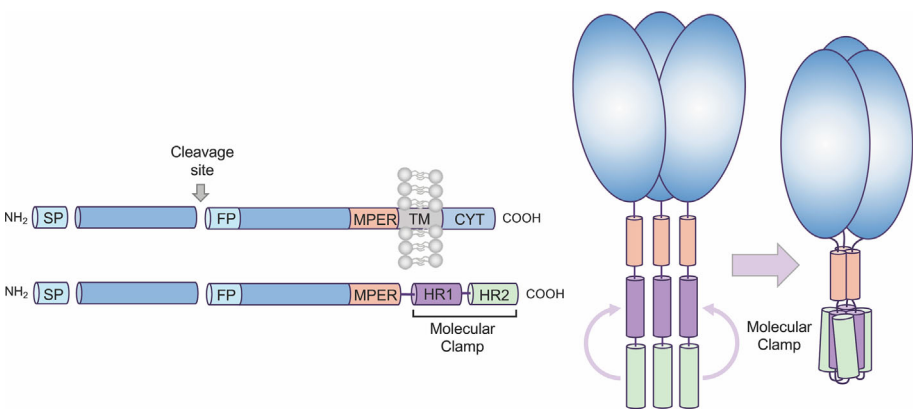

FIGURE 1 | Overview of Molecular Clamp stabilized antigen design. Class I and III viral fusion proteins share common molecular features including a C-terminal transmembrane domain. To generate a soluble, secreted antigen the Molecular Clamp, comprising of hepad repeat (HR) regions 1 and 2 of human immunodeficiency virus (HIV)-1 glycoprotein 41 (gp41), is incorporated into recombinant viral glycoproteins in place of the C-terminal transmembrane/cytoplasmic region. We have also shown that selected modifications within the signal peptide (SP, replacement with a more efficient SP such as IgK SP), cleavage site/s (ablated or enhanced), fusion peptide (deletion), and deletion of the transmembrane domain adjacent to the membrane-proximal external region (MPER) to introduce the clamp domain can increase expression levels of soluble recombinant antigens.

targeting the F or glycoprotein (GP) of 2 independent class I fusion protein bearing viruses: Achimota virus 2 (AchPV2) and Wenzhou mammarenavirus (WENV). AchPV2 has been isolated from straw-colored fruit bats in Ghana and serological analysis indicates possible spillover events into human populations in Ghana and Tanzania (8). WENV and closely related viruses have been found in rodents in China, Cambodia, and Thailand (9) and were recently found to be associated with an outbreak of influenza-like disease in humans (10). There are obviously no subunit vaccine candidates that have been developed against either of these viruses and there is limited published data outside of sequence information to assist development. Consequently, targeting AchPV2 and WENV was ideal for the purpose of this study and to simulate a Disease X type scenario. Importantly, the antigen design and analysis process of this study was used to optimize and aid the rapid development of a SARS-CoV-2 Sclamp subunit vaccine which is now being tested in Phase I clinical trials.

METHODS

Molecular Cloning

To express the prefusion GP ectodomain, codon-optimized AchPV2 (Genbank: YP_009094464.1) and WENV (GenBank: MF595889.1) gene with variations including (i) substitution at the furin cleavage site, (ii) substitution at the signal peptide (SP), and (iii) truncation at C-terminal domain was generated with primers containing overlapping sequence by PCR mutagenesis using Phusion polymerase (New England Biolabs). The Achimota virus 2 F (AchVP2-F) and Wenzhao Mammarena virus GP (WENV-GP) codon-optimized amplicons ranging from AchPV2-$F_{1\text{-}488}$ or WENV-$GP_{1\text{-}428}$ were cloned upstream of a mammalian plasmid vector containing a HIV1281 (PDB ID 3P30) trimerisation motif according to the manufacturer's protocol for in-fusion cloning and Stellar Competent Cells (Takara Bio). Integrated DNA Technologies (IDT) synthesized the codon-optimized geneblocks and primers. Plasmid DNA from transformed Stellar Cells and Agarose gel extractions were purified using NucleoSpin® Gel and PCR Clean-up kits and PureYield™ Palsmid Midiprep System (Promega) according to the manufacturer's protocol and the concentration of the purified DNA was analyzed using the NanoDrop One (Thermofisher Scientific). Sequencing reactions to verify the plasmid DNA constructs were performed at the Australian Genome Research Facility and the sequencing data was analysed using the CLC Main Workbench (version 8.1).

Recombinant Protein Expression

Soluble GP proteins were expressed using the ExpiCHO-S expression system (ThermoFisher Scientific) and in ExpiCHO-S Expression Medium (Gibco™). This expression system comprises mammalian Chinese Hamster Ovary (11) cells, the most commonly used expression system for the production of biotherapeutics (12). We hypothesize that CHO-based expression of recombinant fusion proteins will result in a glycosylation profile reflecting that of the native viral protein following infection. Transfection was conducted following the manufacturer's protocol for transient expression (ThermoFisher Scientific) using plasmid DNA at a ratio of 1 μg of DNA per 1 ml of culture volume when the culture cell density was 6×10^6 cells/ml. Five to 7 days after transfection, the cells from each culture flask was pelleted following centrifugation (3,500 g at 4°C for 10 min) and the culture supernatant (SN) was sterile-filtered (0.22 μm) to perform protein purifications.

Recombinant Protein Purification

AchVP2-Fclamp and WENV-GPclamp proteins were purified using immunoaffinity chromatography on an ÄKTA Pure Protein Purification System (Cytiva). This was peformed using an in-house immunoaffinity chromatography column embedded with the anti-clamp monoclonal antibody (mAb) HIV1281 coupled to 1 or 5 ml HiTrap-NHS activated HP columns (Cytiva).

To purify proteins, culture SN was added to an anti-clamp affinity column pre-equilibrated with high salt PBS (PBS with 400 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4, pH 7.4.) using the AKTA Pure. Bound resin was washed with 15 column volumes of high salt PBS before elution with high pH glycine buffer (100 mM glycine, 400 mM NaCl, 5 mM EDTA, pH 11.5). Eluted fractions were neutralised with a 1:1 v/v ratio of 1M Tris (pH 6.8) before concentration and buffer exchange into PBS using Merck Amicon Ultra-4 or Ultra-15 centrifugal filter units. The concentration of buffer-exchanged protein was analyzed at an absorbance of 280 nm using the NanoDrop One (Thermofisher Scientific).

Electrophoretic Separation of Proteins

NuPAGE 4 to 12%, Bis-Tris, 1.0 mm, mini protein precast gel (Life Technology, Australia) were loaded with 5–10 μg of purified proteins under denaturing condition using LDS Sample Buffer, following manufacturer's protocol (BioRad). Visualization of in-gel proteins were achieved by staining with Bio-Safe™ Coomassie stain (BioRad). Gel documentation was performed using the ChemiDoc MP Imaging system (BioRad).

Size Exclusion Chromatography (SEC) Analysis of Proteins

To assess oligomeric state of the fusion proteins, 7.5–300 μg of purified recombinant protein was loaded onto a Superdex 200 Increase 10/300 GL size-exclusion chromatography column (Cytiva) using a 300–500 μl loop. Proteins were eluted using a mobile phase of PBS, pH 7.4 at a flow rate of 0.5 ml/min.

Negative Stain Transmission Electron Microscopy (TEM)

SEC purified complexes were deposited at approximately 0.01 mg/ml onto carbon-coated copper grids and stained with 1% (w/v) uranyl acetate (13) for 2 min. Grids were imaged at 120 KeV using a Hitachi HT7700. Images were collected using AXT 2kx2k CMOS. Image processing was performed using Relion3.1 (14). Contrast transfer functions of the images were corrected using CTFFIND (15). Individual particles (3,683 particles for construct Q of

ACHPV2-Fclamp) in 30,000× images were selected manually followed by reference-free alignment and classification.

RESULTS

Workflow to Rapid Development of Subunit Vaccine Candidates

In the context of subunit vaccine development to counteract a Disease X outbreak, it is imperative that the vaccine antigen is developed as rapidly as possible. From the many thousands of viruses reported to date, we selected two viruses that possess class I membrane fusion proteins. We established a workflow to expedite the development of a pre-fusion stabilized vaccine antigen based on the Molecular Clamp technology within 3 weeks following revelation of the virus genomic sequence (**Figure 2**). The development process involves cloning a panel of 20–30 rationally designed variants of the viral membrane fusion protein ectodomain sequence with the Molecular Clamp coding sequence inserted in place of the native transmembrane domain. Rational design of the antigen panel and cloning strategy can be completed in a matter of hours. Nucleotide geneblocks and oligonucleotide primers were ordered from Integrated DNA Technologies, Singapore, and arrived within 4–7 days for the current study.

The use of in-fusion cloning (Takara Bio) can allow us to complete the cloning within 1–2 days after receipt of the geneblock and primers. We have found that by PCR screening of colonies directly from plates using Taq DNA polymerase (New England BioLabs) provided high confidence of successful cloning and allowed us to proceed directly to protein expression without waiting for sequence conformation. *E. coli* colonies positive by PCR were grown overnight and plasmid extracted by midiprep plasmid purification (Promega). ExpiCHO-S cells (ThemoFisher) were revived from banked vials and passaged in parallel with cloning to ensure they were ready for transfection as soon as DNA was available. Each plasmid was transfected into a 25 ml culture of ExpiCHO-S cells as per the manufacturer's instructions. Sequence verification was conducted in parallel with protein expression over 5–7 days.

Following expression, soluble protein was then purified from clarified CHO supernatant using an immunoaffinity column which was coupled with a monoclonal anti-clamp human Immunoglobulin G antibody produced in house. The purified proteins are then analysed for the expression yield by absorbance at 280 nm, homogeneity and purity by an SDS-PAGE, and for the formation of the desired trimeric, pre-fusion conformation using SEC and negative stain TEM. At the conclusion of this process the lead candidate(s) can then be selected to advance into further preclinical development including immunogenicity analysis in relevant pre-clinical models (e.g. mice) and simultaneous manufacturing process optimization. An overview of the process is shown in **Figure 2**.

The Use of the Molecular Clamp to Develop an AchPV2 Subunit Vaccine

Using the workflow strategy described above (**Figure 2**) we aimed to develop an AchPV2-F protein (class I) subunit vaccine candidate in a pre-fusion stabilized confirmation using the Molecular Clamp. Using the permutations and the parameters described in **Figure 1**, we devised a cloning strategy for a panel of 24 variant antigens that included (*i*) two SP variants (native and insertion of the IgK SP), (*ii*) three variants at the furin cleavage site (native, mutation to ^{103}NKKN^{106} or ^{103}GGSG^{106} to prevent Furin cleavage), and (*iii*) incorporation of the Molecular Clamp sequence at four distinct sites at the C-terminus (aa473, 479, 483, and 488). For simplicity, the resulting 24 permutations of these changes are denoted alphabetically from A–X and summarized in **Figure 3A**.

Following transient expression in ExpiCHO cell culture and purification by anti-clamp immunoaffinity chromatography, the expression yield of the purified Fclamp proteins ranged from 0.4 mg/L to as high as 15 mg/L. Protein homogeneity as determined by SDS-PAGE also differed depending on the construct with

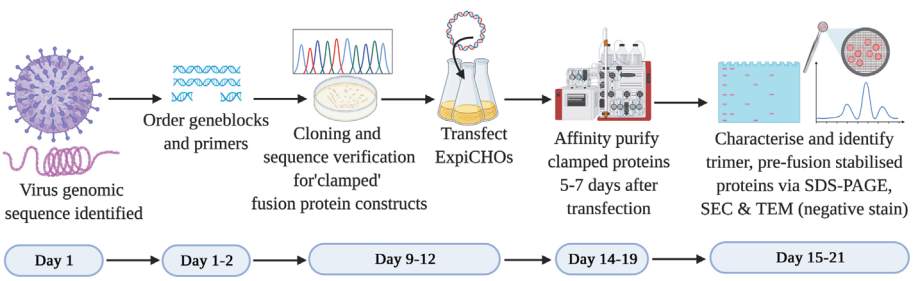

FIGURE 2 | Timeline for the production of subunit vaccine candidates using the Molecular Clamp technology. Day 1–2: Following immediate availability of the virus genome sequence information, *in silico* design of ~20–30 constructs are initiated to allow for selection of optimally expressed antigens. The geneblocks and the primers arrive within 4–7 days following order placement from IDT after which cloning and transfection of the sequence confirmed constructs can be completed within 4 days. Small-scale expression of the transiently transfected cultures is completed within 5–7 days prior to affinity purification of the expressed clamped proteins using the culture supernatants. There is no need for pathogen-specific probes for the purification owing to the availability of human anti-clamp antibody embedded columns in-house. Purified proteins are analyzed *in vitro* for the expression yield, protein homogeneity on an SDS-PAGE, and the presence of the desired pre-fusion conformation by SEC and TEM (negative stain). The protein purification and *in vitro* analysis can be completed within 1–3 days.

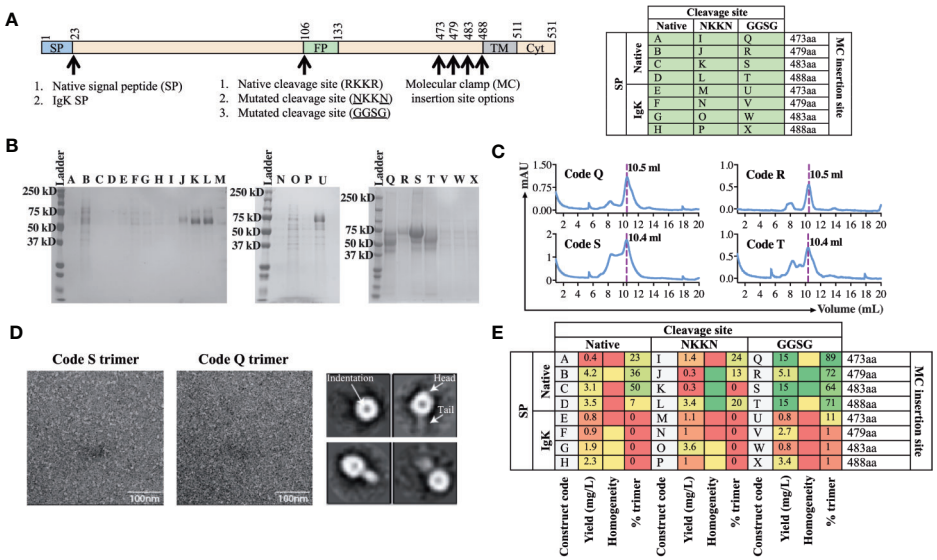

FIGURE 3 | Production and analysis of AchPV2-Fclamp antigens. **(A)** The cloning strategy delineating the 24 different constructs that were desginated to be cloned for the generation of the AchPV2-Fclamp. The table shows the alphabetized (A–X) construct codes based on the different permutations of introducing signal peptides, cleavage sites, and the Molecular Clamp following removal of the transmembrane and cytosolic regions of the F. The mutations introduced to the putative native cleavage site are underlined. **(B)** SDS-PAGE analysis of the expressed AchPV2 Fclamp proteins under reducing conditions. **(C)** Representative size exclusion chromatographs for construct codes Q, R, S, and T which were the four highest expressing constructs. The dotted line overlaps with the trimer peak and the elution volume of this peak is indicated. **(D)** Representative 30,000× images of the TEM negative stain analysis of the trimeric fraction from the size excluded AchPV2-Fclamp codes S (left) and Q (middle). Representative 2D averages for Q, characteristic indentation was visible in the head domains, some particles appear with head and tail features. **(E)** Summary of the purification and *in vitro* analysis of the AchPV2 Fclamp constructs highlighting the expression yield, percentage of the purified protein in the trimeric conformation and the homogeneity of the proteins on an SDS-PAGE. For the homogeneity analysis: red shaded = least homogenous or no proteins corresponding to AchPV2-Fclamp, yellow shaded = intermediate homogeneity, and green shaded = highly homogenous.

visible cleavage products detected for some constructs (**Figures 3C, E**). All constructs including the IgK SP were found to express to a much lower level than those including the native SP. However, the constructs Q, R, S, and T comprising of the native SP and the GGSG cleavage site mutation had the highest expression yield. Interestingly, mutation of the cleavage site to ^{103}NKKN^{106} did not appear to prevent proteolytic cleavage and mutation to ^{103}GGSG^{106} resulted in a cleavage profile that varied between constructs with distinct C-terminal lengths (**Figure 3B**). The SEC analysis showed that these protein constructs were mostly in the desired trimeric conformation: % trimer = 89% (Q), 72% (R), 64% (S), and 71% (T) (**Figures 3C, E**). We analyzed the size excluded trimer fraction (**Figure 3C**) from codes Q and S using negative TEM which suggested that the analyzed trimer fraction appeared to be in the prefusion stabilized trimeric conformation. The trimeric fraction from Q and S are highly homogeneous, both in size and shape (16 nm) (**Figure 3D**, left and middle panel). The 2D averaging analysis showed that some particles had a globular shaped head with an indentation in the center, while some had a globular head with a short tail (**Figure 3D**, right panel). This difference in appearance is likely caused by a different view of the same protein conformation, consistent with previous reports of the pre-fusion conformation for fusion proteins of viruses of the paramyxoviridae family (4).

The Use of the WENV GPclamp Vaccine

For the second test of the platform, we aimed to develop a subunit vaccine for WENV using the same workflow established for AchPV2 (**Figure 2**). Again we designed a panel of 24 variant antigens that included (*i*) two SP variants (native and insertion of the IgK SP), (*ii*) three variants at the cellular subtilisin kexin isozyme-1 (SKI-1)/site-1 protease (S1P) cleavage site (native, mutation to ^{253}GGLLG^{257} or ^{1253}GGSSG^{257} to prevent cleavage), and (*iii*) incorporation of the Molecular Clamp aminoacid sequence at four distinct sites at the C-terminus (aa413, 418, 423 and 428) (**Figure 4A**).

Following purification of the expressed proteins, the yield of the recovered GPclamp proteins ranged from 0.2 to 25 mg/L (**Figure 4E**). Insertion of the IgK SP in place of the native SP resulted in a higher yield and the longest C-terminus (aa428) also gave the highest yield. Subsequently, we prioritized the protein characterization analysis to encompass the 10 highest expressing constructs (D, E, H, L, N, P, T, V, W, and X) which expressed ≥10 mg/L of protein. While some background host cell proteins or low molecular weight degradation products were visible by SDS-PAGE, the purified antigen displayed good homogeneity and no obvious cleavage to GP1 and GP2 with either native or mutated cleavage site variants (**Figure 4B**). SEC analysis revealed a large degree of variation between constructs, with many showing the presence of significant aggregation while for

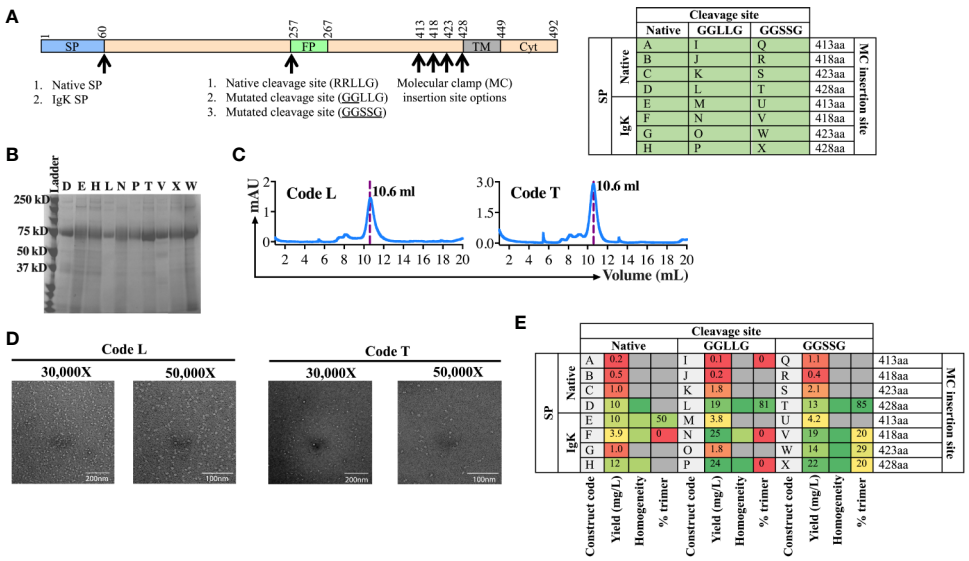

FIGURE 4 | Production and analysis of WENV GPclamp antigens. **(A)** The cloning strategy delineating the 24 different WENV GPclamp constructs and the table shows the alphabetized (A–X) construct codes based on the different permutations of signal peptides, cleavage sites, and the Molecular Clamp insertion. The mutations introduced to the putative native cleavage site are underlined. **(B)** SDS-PAGE analysis of the 10 highest expressing WENV GPclamp proteins under reducing conditions. **(C)** Representative size exclusion chromatographs for construct codes L and T which were the two highest expressing constructs also comprising of the highest proportion of trimer among the purified GPclamp protein solution. The dotted line overlaps with the trimer peak and the elution volume of this peak is indicated. **(D)** Representative 30,000× and 50,000× images of the TEM negative stain analysis of the trimeric fraction from the size excluded WENV GPclamp codes L and T. **(E)** Summary of the purification and *in vitro* analysis of the WENV Fclamp constructs highlighting the expression yield, percentage of the purified protein in the trimeric conformation, and the homogeneity of the proteins on an SDS-PAGE. For the homogeneity analysis: red shaded = least homogenous or no proteins corresponding to WENV-GPclamp, yellow shaded = intermediate homogeneity, and green shaded = highly homogenous. Gray shaded boxes indicate when the respective protein analysis were not performed.

constructs T and L, >80% of protein was present as soluble trimer (**Figures 4C, E**). Interestingly, while the IgK SP resulted in higher yield it appeared that the native SP was required for formation of the native soluble trimer. Following TEM analysis of the trimer fraction, constructs T and L appeared to be in the pre-fusion stabilized trimeric conformation of the protein revealing a monodisperse population with a diameter of 17 nm (**Figure 4D**). The class average showed undefined borders. This could be due to preferential binding of the samples on the EM grid or a high degree of glycosylation heterogeneity of the samples (data not shown).

DISCUSSION

In the midst of the COVID-19 pandemic, the importance of platform technologies for vaccine development that are applicable to rapid response has never been more evident. Notably, the majority of front-runners in the race to develop a vaccine are predominantly nucleic acid or viral vector approaches (16–18). These approaches are generally more broadly applicable, however they still need to encode and express *in situ* a target viral protein in the correct conformation that will induce an appropriate protective immune response. Subunit vaccines face their own set of challenges which are unique to each pathogen. Whereas viral vector and nucleic acid based platforms both express their target antigen within the body following vaccination, in subunit vaccines the target antigen itself must be reliably and consistently manufactured with a desired conformation that is stable throughout manufacturing, transport, and storage, right up until the moment of vaccination.

The viral surface proteins responsible for membrane fusion and viral entry are the primary target for a protective immune response, however such proteins are poorly conducive to subunit vaccine manufacture due to their inherent instability. Viral fusion proteins catalyse the merger between the viral envelope and the target cell's membrane during viral entry. To drive this energetically unfavorable event, viral fusion proteins undergo a major conformational change from a metastable pre-fusion form present on the surface of the live virus to a more highly stable post-fusion form. Manufacture of these proteins in their inherently unstable but crucially important pre-fusion conformation is complicated by this tendency to fall toward the lower free energy post-fusion conformation. Structure-based modifications have been shown to successfully facilitate stabilization of some viral fusion proteins (4–6), and stabilization approaches previously identified for MERS-CoV Spike and SARS-CoV Spike were able to be successfully incorporated into SARS-CoV-2 Spike (19). However, while

early structural data has assisted in one vaccine approach for COVID-19 (11), such structure-based antigen design is generally not conducive to a rapid response pipeline.

The proprietary Molecular Clamp platform technology we have developed has two major advantages making it uniquely suited to an emergency vaccine response. Firstly, the hyper-stable, six-helical bundle structure of the Molecular Clamp imparts sufficient stability to reliably constrain the prefusion conformation in the absence of structure-based physically incorporated constraints, and secondly, the availability of a mAb to the Molecular Clamp enaples the use of a consistent, first-pass, purification method irrespective of the viral antigen. In the current study we have outlined a process through which antigen design can be reduced down to three weeks and present two successful proof of concept studies with novel viral pathogens. The rapidness of the antigen design process also facilitates the screening of a larger panel of antigens based on the permutations described in **Figure 1** or for conducting subsequent screening if the initial study does not identify a suitable lead candidate. Furthermore, the process described for antigen design and development (**Figures 1** and **2**), was utilized to rapidly develop a SARS-CoV-2 Spike subunit vaccine, SARS-CoV-2 Sclamp (Watterson et al., under review).

Central to the process of accelerated vaccine design was the parallel cloning, expression, and characterization of a small panel of antigen designs. Our previous work, as well as the results of this study have shown that modifications at certain sites can have a large impact on the expression level, homogeneity, and the strucutural integrity of the prefusion conformation. Of equal importance are the assays used to screen candidates. These assays are well suited to a rapid assessment of key candidate biophysical and antigenic parameters and facilitates selection of a lead candidate that can proceed into further preclinical development including mouse immunogenicity analysis and simultaneous manufacturing process optimization. Of note, SEC facilitates a first-pass correlate of pre-fusion stabilization as transition to the post-fusion form results in the exposure of the hydrophobic fusion peptide and aggregate formation.

Based purely on sequence information of these relatively uncharacterised viruses and homology to more well studied relatives allowed us to select three regions to selectively target for modification (SP, cleavage site, and MPER). While we were unable to predict which of these constructs would be most successful, simple empirical analysis of the expressed products identified lead candidates. Ultimately, the native SP proved to be preferable for inclusion in both AchPV2 and WENV. Replacement of the native cleavage site with a flexible linker was also optimal for both viruses, however for AchPV2 the most truncated MPER was optimal, while for WENV the longer MPER was preferable.

Whilst the purpose of this study was to optimize the screening and rapid production of trimeric Class I fusion proteins using the Molecular Clamp in a Disease X scenario, it is important to demonstrate that the lead candidates screened are immunogenic and capable of eliciting virus neutralizing antibodies in pre-clinical models to progress into clinical trials. We are currently developing a pseudovirus neutralization assay for this purpose and to overcome this caveat of the current study. For the Molecular Clamp based subunit vaccine we developed against SARS-CoV-2, this was not an issue given the availability of live virus neutralization assays and animal challenge models shortly after the announcement of the virus sequence (Watterson et al., under review).

Stable trimer formation of expressed fusion protein ectodomains, as assessed by SEC analysis, is not definitive confirmation of the integrity of a pre-fusion protein structure. However we, and others, have empirically found that SEC confirmed trimer formation is highly indicative of a pre-fusion class I protein structure (20). Furthermore, members of the *Paramyxoviridae* have been shown in TEM to aggregate in rosettes and/or form "golf tee" shaped structures upon transition to the post-fusion conformation (21, 22), and members of the *Arenaviridae* have been shown to dissociate into monomers (23, 24). In addition, recent studies (20, 22), including our own experience with developing subunit vaccines using the Molecular Clamp for class I fusion proteins, suggest that TEM analysis confirms that the peak corresponding to the protein trimer in SEC analysis is indeed in its pre-fusion conformation.

We do not anticipate that the learnings from the optimal design of vaccine candidates targeting these viruses can be directly translated to others, unless there is a high degree of sequence homology. Therefore, the process we have defined necessitates the production of a small panel for the selection of a lead candidate. During an emergency response, any problem necessitating changes to the antigen sequence would constitute a substantial time delay, so we anticipate this emphasis on rapid screening and lead selection is likely to translante into valuable improvements during manufacture. Fortunately, we were able to undertake these two mock tests of our process during 2019. The learnings and personel training from this exercise proved invaluable to our SARS-CoV-2 response in 2020 and resulted in the generation of a Molecular Clamp stabilized subunit vaccine that is currently being tested in a Phase I clinical trial.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

KC, DW, and PY conceptualized the project. KC edited and finalized the manuscript draft. DKW designed the experiments and the manuscript figures and wrote the initial draft. SA and JL executed the experiments, performed data analysis, and wrote the initial draft. NM led the design of TEM experiments and contributed to the drafting process of the manuscript. AI performed the TEM analysis with NM. All authors contributed to the article and approved the submitted version.

FUNDING

The work was carried out with a project grant (CFP2) from the Coalition for Epidemic Preparedness Innovations (CEPI).

ACKNOWLEDGMENTS

The illustration in **Figure 2** was generated using BioRender.com.

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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.

frontiers in Immunology

MINI REVIEW
published: 06 November 2020
doi: 10.3389/fimmu.2020.589833

Adjuvants for Coronavirus Vaccines

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Vaccine development utilizing various platforms is one of the strategies that has been proposed to address the coronavirus disease 2019 (COVID-19) pandemic. Adjuvants are critical components of both subunit and certain inactivated vaccines because they induce specific immune responses that are more robust and long-lasting. A review of the history of coronavirus vaccine development demonstrates that only a few adjuvants, including aluminum salts, emulsions, and TLR agonists, have been formulated for the severe acute respiratory syndrome-associated coronavirus (SARS-CoV), Middle East respiratory syndrome-related coronavirus (MERS-CoV), and currently the SARS-CoV-2 vaccines in experimental and pre-clinical studies. However, there is still a lack of evidence regarding the effects of the adjuvants tested in coronavirus vaccines. This paper presents an overview of adjuvants that have been formulated in reported coronavirus vaccine studies, which should assist with the design and selection of adjuvants with optimal efficacy and safety profiles for COVID-19 vaccines.

Keywords: coronavirus disease 2019, SARS-CoV-2, adjuvant, coronavirus vaccine, aluminum salt

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: *31 July 2020*
Accepted: *14 October 2020*
Published: *06 November 2020*

Citation:
Liang Z, Zhu H, Wang X, Jing B, Li Z, Xia X, Sun H, Yang Y, Zhang W, Shi L, Zeng H and Sun B (2020) Adjuvants for Coronavirus Vaccines. Front. Immunol. 11:589833. doi: 10.3389/fimmu.2020.589833

INTRODUCTION

Coronaviruses (CoVs) are single-stranded RNA viruses characterized by club-like spikes that can potentially cause severe respiratory disease in humans (1, 2). The outbreak of severe acute respiratory syndrome (SARS) caused by the SARS-CoV resulted in more than 8000 confirmed infections, with an overall case fatality rate of 10% in 2002 (3). The Middle East respiratory syndrome (MERS)-CoV continues to cause deaths with increasing geographical distribution and a 34.4% case fatality rate, according to the World Health Organization (WHO) (4). Most recently, the coronavirus disease 2019 (COVID-19) caused by SARS-CoV-2 has spread globally, with over 33 million confirmed cases as of October 2020 (5). Considering the challenges to global health systems and the far-reaching consequences on the world economy, there is an urgent need to develop effective and safe vaccines that can be quickly deployed on a global scale (2, 6).

Vaccine candidates are currently under development using different platforms, such as inactivated vaccines, recombinant protein vaccines, live-attenuated vaccines, viral vector (adenovirus) vaccines, DNA vaccines, and mRNA vaccines (2, 6, 7). Adenovirus-vector could induce potent immunological responses due to the presence of viral proteins and stimulation of innate immunity sensors, *e.g.*, toll-like receptors (8). Nucleic-acid vaccines, *e.g.*, DNA and mRNA vaccines, encode the virus's spike protein, intrinsically could engage innate immunity that instructs

induction of immune protection (9). However, these platforms haven't been used in licensed human vaccines before. In other platforms, subunit or inactivated antigens were used, but these antigens lack the immunological profiles that mediate the enhanced adaptive immunity. Thus, in these CoV vaccines, they require the addition of adjuvants for directing the types and magnitude of immune responses (10). In previously reported exploratory and pre-clinical CoV vaccine studies, adjuvants such as aluminum salts, emulsions, and toll-like receptor (TLR) agonists, have been used in vaccine formulations for studies with various animal models (**Table 1**). The adjuvants AS03, MF59, and CpG 1018 have already been used in licensed vaccines (28) and have been committed by GlaxoSmithKline, Seqirus, and Dynavax to be available for COVID-19 vaccine development (29). When combined with subunit and specific inactivated antigens (30, 31), adjuvants with various characteristics elicit distinctive immunological profiles with regard to the direction, duration, and strength of immune responses. Thus far, there are at least 40 candidate vaccines in clinical trials and 149 vaccines in preclinical evaluation, of which 67 subunit and 15 inactivated COVID-19 vaccines have being developed (32). Among these adjuvants, alum have been formulated with S protein or RBD to induce neutralizing antibody production (17, 18), which has suggested to be associated with protection against SARS-CoV-2 (15, 16, 24). However, alum lacks the capability to promote the activation of $CD4^+$ and $CD8^+$ T cell responses, which has been demonstrated to coordinate with the antibody responses to provide protective immunity against the SARS-CoV-2 (33). Other adjuvants, *e.g.*, emulsion adjuvants and TLR agonists, which have been shown to induce both humoral and cellular immune responses could be more favorable. However, no phase III clinical trial results of COVID-19 vaccines are published so far, thus, there is no direct evidence to indicate which type of immune response induced by vaccine plays a more critical protective role in SARS-CoV-2 infection. Knowing these uncertainties, an overview of previous CoV vaccine studies using different adjuvants would be indispensable for the design and development of a COVID-19 vaccine.

The SARS-CoV-2 is a novel strain of the coronavirus, and very little is known about its epidemiology and pathogenesis. Therefore, extreme cautions should be taken when considering vaccine formulations that can achieve the desired efficacy and safety profiles. The selection of adjuvants should consider the magnitude, affinity, isotype, and durability of antibodies that are critical for coronavirus vaccine developments (34). It should be noted that low antibody production may lead to antibody-dependent enhancement (ADE) manifested by severe liver damage and enhanced infection (35), while high affinity neutralizing antibodies could help to avoid ADE. Additionally, the proper application of adjuvants also depends on the choice of antigens. The full-length S protein is more likely to trigger ADE due to mild antibody production (36). In comparison, the N protein is generally highly conserved, and it is associated with the ability to induce cytotoxic T lymphocytes (CTL). However, N protein could potentiate pro-inflammatory cytokine production and lead to severe lung pathology (37). In addition, previous study on respiratory syncytial virus (RSV) vaccine also indicated that immunization with whole inactivated virus could lead to vaccine-associated enhanced respiratory disease (VAERD), manifested by allergic inflammation and Th2 type immune responses (38). Altogether, these studies suggest that vaccines formulated with various antigen isotypes may require proper adjuvant selection to achieve the desired immune protection. In this paper, we reviewed adjuvants that have already been incorporated in the coronavirus vaccines under exploratory and pre-clinical investigations. By reviewing the vaccine formulations and the types of immune responses that were induced, we provide information that will enable proper adjuvant selection for COVID-19 vaccines to facilitate rapid vaccine delivery.

ALUMINUM SALT-BASED ADJUVANTS

Aluminum salt-based adjuvants (alum) were the first adjuvants used in licensed human vaccines. They are still the most widely used because of their wide-spectrum ability to strengthen immune responses and their excellent track record of safety (39–41). In limited coronavirus vaccine studies, it has been suggested that neutralizing antibody against the spike protein might be mechanistically correlated with immune protection (42). When alum was formulated with S protein or receptor-binding domain (RBD), it significantly enhanced humoral immune responses. This was demonstrated by higher titers of serum IgG_1, increased high affinity viral neutralizing antibodies, and the generation of long-lasting memory B cells in mice (13, 17–19). Additionally, Alum was formulated with the inactivated and VLP vaccines containing E, M, and N proteins (11, 12, 14) (**Table 1**) that showed enhanced IgG_1 and neutralizing antibody titers (14) and prolonged durability (12). Studies also demonstrated that alum adjuvant plays an essential role in the dose-sparing of CoV vaccines. In a SARS S protein subunit vaccine, the alum-adjuvanted S protein (1 μg) group showed neutralizing antibody titers similar to or higher than the non-adjuvanted S protein (50 μg) group. The alum-adjuvanted S protein (5 μg) group showed a geometric mean titer (GMT) twice as high as the non-adjuvanted S protein (50 μg) group (20). It should also be noted that different types of alum were selected in the studies, including Alhydrogel, which is chemically crystalline aluminum oxyhydroxide (43), aluminum hydroxide (11), aluminum phosphate (22), and Imject™ Alum (23), which is a mixture of aluminum hydroxide and magnesium hydroxide. Even though there is no specific description regarding the aluminum hydroxide in reported literature (11, 18, 21), it can also be referred to Aluminum oxyhydroxide (44). However, these studies lacked systematic comparisons with regards to their adjuvanticity and how various alum-based adjuvants differed in their ability to induce neutralizing antibodies.

It is worth noting that inactivated SARS-CoV or S protein-based vaccines are associated with Th2-type immunopathology, which is characterized by an increase in eosinophils and inflammatory infiltrates (14, 30, 37, 45). Moreover, the

TABLE 1 | Alum based adjuvants used in the coronavirus vaccine formulations under exploratory and pre-clinical investigations.

Adjuvant Type	Platform	Adjuvant	Antigen	Antigen Dose	Immunological response								Route	Immunization Schedule	Safety	Animal model	Ref.
					Nab	IgG_1	IgG_{2a}	IgA	Th1 cytokines	Th2 cytokines	Th17 Cytokines	$CD8^+$ T cell response					
Alum based adjuvants	Inactivated vaccines	Aluminum hydroxide	Doubly inactivated (formalin and UV) whole SARS virus	0.125/0.25/0.5/1 μg	√	N/A	N/A	N/A	N/A	N/A	N/A	N/A	i.m.	2	Pulmonary immunopathology	BALB/c or C57BL/6 mice	(11)
	Inactivated vaccines	Aluminum hydroxide	Purified beta propiolactone inactivated whole SARS virus	2 μg	√	N/A	N/A	N/A	N/A	N/A	N/A	N/A	i.m.	2	N/A	BALB/c or C57BL/6 mice	(11)
	Inactivated vaccines	Aluminum hydroxide gel	UV-inactivated purified SARS-CoV	10 μg	√	√	×	×	√	√	N/A	N/A	s.c.	2	N/A	BALB/c mice	(12)
	Inactivated vaccines	Aluminum hydroxide	SARS-CoV (formaldehyde and U.V. inactivation)	0.3~1 μg	√	N/A	N/A	N/A	√	√	N/A	N/A	s.c.	2	N/A	CD1 mice	(13)
	Inactivated vaccines	Alum	Double-inactivated SARS-CoV (DIV)	0.2 μg	√	√	×	N/A	×	√	N/A	N/A	Footpad injection	2	Lung immunopathology	BALB/c AnNHsd mice	(14)
	Inactivated vaccines	Aluminum hydroxide	SARS-CoV-2	Mice/Rats:1.5/3/6 μg; Monkeys:1.5/6 μg	√	N/A	N/A	N/A	×	×	N/A	N/A	i.m./i.p.	Mice/Rats:2; Monkeys:3	×	BALB/c mice; Wistar rats; Rhesus macaques	(15)
	Inactivated vaccines	Aluminum hydroxide	SARS-CoV-2	2/4/8 (μg/dose)	√	N/A	N/A	N/A	N/A	N/A	N/A	N/A	i.p./i.m	1/2/3	No acute toxicity/No Systemic anaphylax/ No long-term toxicity	Rats; Mice guinea pigs; Rabbits, Cynomol-gus monkeys; Rhesus macaques	(16)
	Subunit vaccines	Alhydrogel	SARS S protein	3 μg	√	N/A	N/A	N/A	N/A	N/A	N/A	N/A	i.m.	2	N/A	BALB/c mice	(17)
	Subunit vaccines	Alhydrogel	MERS S protein	1/3/10 μg	√	N/A	N/A	N/A	N/A	N/A	N/A	N/A	i.m.	2	N/A	BALB/c mice	(17)
	Subunit vaccines	Aluminum hydroxide	MERS-CoV S rRBD Protein	10 μg	√	√	√	N/A	×	√	N/A	N/A	i.m.	3	N/A	BALB/c mice	(18)
	Subunit vaccines	Alhydrogel	SARS-CoV S318–510 fragment	8 μg	√	√	×	N/A	×	N/A	N/A	N/A	s.c.	2	N/A	129S6/SvEv mice	(19)
	Subunit vaccines	Alhydrogel	Ectodomain of SARS-CoV S glycoprotein (ΔTMS)	1//5 μg	√	N/A	N/A	N/A	N/A	N/A	N/A	N/A	i.m.	1	N/A	CD1 mice	(20)
	Subunit vaccines	Aluminum hydroxide	S glycoprotein of SARS-CoV	0.25/0.5/1/2 μg	√	N/A	N/A	N/A	N/A	N/A	N/A	N/A	i.m.	2	N/A	BALB/c or C57BL/6 mice	(11)
	Subunit vaccines	Aluminum hydroxide	MERS-CoV rRBD protein S367-606	100/50 μg (primed) +50/25 μg (boosted)	√	N/A	N/A	N/A	N/A	N/A	N/A	N/A	i.m.	3	N/A	Monkeys	(21)
	Subunit vaccines	Aluminum phosphate	MERS-CoV S1 protein	10 μg	√	N/A	N/A	N/A	N/A	N/A	N/A	N/A	i.m.	2	N/A	BALB/c mice	(22)
	Subunit vaccines	Imject™ Alum	MERS-CoV RBD-dimer	10 μg	√	N/A	N/A	N/A	×	×	N/A	N/A	i.m.	3	N/A	BALB/c mice	(23)
	Subunit vaccines	Aluminum hydroxide gel	RBD of SARS-CoV-2 spike protein	0.1-20 μg	√	N/A	N/A	N/A	N/A	N/A	N/A	N/A	i.m	1/2/3	Safe	BALB/c and C57BL/ 6mice; Rabbits;Non-human primates (Macaca mulatta)	(24)
	VLP vaccines	Aluminum hydroxide	SARS-CoV S glycoprotein and E, M, and N proteins	2 μg	×	N/A	N/A	N/A	N/A	N/A	N/A	N/A	i.m.	2	N/A	BALB/c or C57BL/6 mice	(11)

Annotation:

S protein *is the spike glycoprotein of coronavirus and a main target for neutralizing antibodies (25);*

RBD *is the receptor-binding domain (RBD) of S protein, which could directly interact with the angiotensin-converting enzyme 2 (ACE2) receptor on host cells (25);*

N protein *is the highly conserved nucleocapsid protein of coronavirus and regulates RNA replication and transcription (26);*

M protein *(membrane protein) is the most abundant protein on the coronavirus surface and mediates virus assembly (25);*

E protein *(envelope protein) is an integral membrane protein and plays a pivotal role in virus envelope formation (27).*

Nab *represents the neutralizing antibodies, and N/A means not available.*

addition of alum adjuvant exacerbated the immunopathologic reactions (14, 45). In alum-adjuvanted SARS-CoV double-inactivated vaccine (DIV), there was a skew in the N or S protein-specific antibodies toward IgG_1, when compared with the more balanced antibody production in the nonadjuvanted DIV vaccine (14). These observations raise significant concerns regarding the safety of adjuvanted coronavirus vaccines. On the other hand, it has been shown that alum can reduce immunopathology in SARS-CoV vaccines containing either a double-inactivated virus or S protein (11). Furthermore, in a recent study, a purified inactivated SARS-CoV-2 vaccine (PiCoVacc) adjuvanted with aluminum hydroxide conferred complete protection in non-human primates (rhesus macaques) with potent humoral responses but without lung immunopathology (15). This finding raises the question of the mechanism of eosinophilic immunopathology. While commonly thought of as the product of Th2 responses, recent studies have indicated that tissue eosinophilia can also be controlled by Th17 responses (46). Thus, the proper selection of CoV antigens and adjuvants that can shift host responses away from a Th17-bias appears to be critical. In addition, other studies have demonstrated that the Th2 immunopathology may be associated with SARS N or S protein that results in enhanced eosinophilic immunopathology (11, 37, 47). However, more studies are required, as the preliminary data is limited. Additionally, the Th-2–biased immune responses may raise the concern on vaccine-enhanced respiratory disease (VAERD) (38, 48), however, there are no evidences that alum-adjuvanted CoV vaccines show the effect.

When alum was used as an adjuvant in CoV vaccines (**Table 1**), there was a lack of Th1 $CD4^+$ T cell and cytotoxic $CD8^+$ T cell immune responses, which is typical for alum-adjuvanted vaccines (49). However, recent study has demonstrated that the SARS-CoV-2–specific adaptive immune response correlated with milder disease, indicating that coordinated $CD4^+$ and $CD8^+$ T cell responses play a synergistic effect in the protective immunity of COVID-19 (33). Several other adjuvants, which are capable of inducing more balanced Th1/Th2 or Th1-biased immune responses, have been formulated in CoV vaccines and will be discussed in the following sections.

EMULSION ADJUVANTS

The emulsion adjuvants, MF59, and AS03 have already been used in licensed human vaccines to improve the immunogenicity of the antigens (50, 51). Compared with alum that lacks the capability to mediate cell-mediated immunity (49), MF59 and AS03 can elicit more balanced immunity, possibly by improving antigen uptake, recruiting immune cells, and promoting the migration of activated antigen-presenting cells (28, 50, 52). Emulsion adjuvants have already been used in preclinical studies of vaccines against coronavirus. MF59 used in inactivated SARS and MERS vaccines, as well as vaccines containing the RBD domain of the MERS-CoV spike (S) protein, has exhibited excellent adjuvanticity, with potent humoral immune responses, *i.e.*, high titers of neutralizing antibodies, and cell-mediated immunity in the coronavirus vaccines (53–55). In addition, depending on the types of antigen, cell-mediated immunity induced by MF59 differs. When formulated with the MERS-CoV S protein, MF59 enhanced both effective $CD4^+$ and $CD8^+$ T-cell immune responses. In comparison, when combined with inactivated SARS CoV, MF59 induced significant $CD4^+$ T cell, but not $CD8^+$ T cell responses (56, 57). However, in another study by Zhang et al., it was demonstrated that when MERS S protein was adjuvanted with MF59, it induced higher IgG_1 and IgG_{2a} antibodies with a slightly Th2-biased response (54). Subsequent studies also showed that ferritin-based MERS-CoV S protein, adjuvanted with MF59, promoted multiple antibody responses, including high levels of IgA antibody titers that resulted in potent mucosal immune responses (58). A study by Tang *et al.* has indicated that there are no significant differences in the neutralizing activity of the serum derived from mice immunized with MERS S377-588 at 1, 5, and 20 μg in the presence of MF59, suggesting the dose-sparing effect of MF59 when it was formulated with MERS S protein (57). However, an immunopathologic lung reaction, as well as an increase in IL-5 and IL-13 cytokines, was seen in animal studies using both MF59-adjuvanted and adjuvant-free inactivated MERS-CoV vaccines (53). It has shown that eosinophil infiltrations with higher Th2-type cytokine secretion aggravated the hypersensitivity-type pulmonary immunopathology when vaccinated with MF59-adjuvanted inactivated virus vaccines as compared with the inactivated virus vaccines alone (53).

Another emulsion adjuvant, AS03, elicits both potent humoral and cellular immune responses to an inactivated whole virion SARS-CoV (WI-SARS) vaccines (59) compared with the virion without adjuvants. Moreover, in the presence of the AS03 adjuvant, an identical trend toward specific $CD4^+$ T cell responses was observed when immunized with SARS-CoV containing the equivalent of 0.5 or 1.5 μg of S protein (59). Therefore, the addition of AS03 tends to potentiate the immune responses with a lower dosage of antigen. Considering its capability to induce both arms of the immune system, S protein, RBD domain, and N protein can also be formulated with AS03. Currently, GSK is sharing its AS03 adjuvant with COVID-19 vaccine developers globally (29).

Besides MF59 and AS03, other emulsion-based adjuvants such as Freund's adjuvant and Montanide ISA51 have also been formulated in CoV vaccines (54). By evaluating the titers of specific serum antibody responses, it has been demonstrated that Freund's adjuvant and ISA51 elicited significant Th1 antibody responses (IgG_{2a}) with no clear Th2 responses (IgG_1) (54, 59).

TLR AGONISTS AND OTHER ADJUVANTS

Toll-like receptors (TLRs), a category of pattern-recognition receptors, are critical to pathogen recognition. This allows for

rapid activation of innate immunity, and subsequently, effective adaptive immunity. TLR agonists have been extensively studied as vaccine adjuvants (60, 61). CpG, Poly I:C, glucopyranosyl lipid A (GLA), and resiquimod (R848) are agonists for TLR9, TLR3, TLR4, and TLR7/8, respectively. These adjuvants have been evaluated in candidate vaccines against SARS CoV (62, 63).

In addition to neutralizing antibodies and $CD4^+$ T cells, optimal protection against coronavirus probably involves the synergistic effect of $CD8^+$ T cells (64). Memory $CD8^+$ T cells solve the problem of neutralizing antibodies only existing for short periods and providing long-term protective cellular immunity (64). Among the TLR agonists, CpG significantly augments the $CD8^+$ T cell immune response higher than the others (63). Indeed, it has been demonstrated that CpG can also stimulate enhanced IgG production in animals immunized with an inactivated SARS-CoV vaccine (62). In addition to IgG, IgA production was also enhanced, only when CpG was administered *via* intranasal (*i.n.*) administration (62), indicating immune activation in the mucosal compartment. Although CpG is capable of inducing both cellular and humoral immune responses, it preferentially induces responses that are Th1-biased. Moreover, CpG can divert pre-existing Th2 responses to a Th1 phenotype, which has laid a foundation for the combination of CpG with other adjuvants, most commonly alum (65). In SARS-CoV or MERS-CoV subunit vaccines, studies have found that the combination of alum and CpG elicited higher neutralization antibody titers and a more robust cellular immune response compared with alum alone or alum with other TLR agonists (18, 19). In addition to alum, CpG is combined with Montanide ISA-51, a type of water-in-oil emulsion adjuvant. When the combined adjuvants were formulated with SARS S or N protein, they were capable of promoting robust neutralizing antibody production (66). However, vaccinated with only SARS N protein, animals showed immune responses biased dramatically toward Th1 (67). In addition, it is reported that R848 could enhance antigen-specific CTL response and induce a fast, robust and durable IFN-α production *in vivo* among humanized mice, which is distinct from the experimental findings based on common mouse models (68). However, further studies on R848 adjuvanticity should stress more on vaccine formulation. A recent study by Gadd *et al.* indicated that only when R848 was conjugated with DOPE (1,2−di−(9Z−octadecenoyl)−sn−glycero−3−phosphoethanolamine):DDA (dimethyldioctadecylammonium bromide salt) multilamella liposomes rather than linear mixed, a high potency of immunostimulatory activity was observed (69). Moreover, an R848-encapsulating PLGA nanoparticle can bring down the excessive level of inflammatory cytokines induced by free R848, which could be benefit to provide long-term safety and appropriate immune response (70).

Although CpG had been shown to exhibit considerable potential as a coronavirus-specific adjuvant, studies have found that it might be a poor inducer of long-term immune memory (46). A recent study indicated that single-stranded RNAs (ssRNAs) derived from the Cricket paralysis virus (CrPV) intergenic region (IGR) internal ribosome entry sites (IRES) could function as vaccine adjuvants endowing long-lasting immunity. This adjuvant significantly activates innate immune response through activating TLR7 and enhancing the chemotaxis of professional antigen-presenting cells (APC) (71). Moreover, some novel adjuvants such as STING agonist, Advax, and $AS01_B$, which is an adjuvant formulated in recombinant zoster vaccine Shingrix, exhibit advantages for long-lasting immune responses (46, 59, 72). Advax, a delta inulin microparticle adjuvant, augmented the induction of neutralizing antibodies along with the existence of memory B cells and a robust, long-lasting T-cell IFN-γ response when it was formulated in recombinant or inactivated SARS-CoV vaccines (46). Moreover, Matrix M1, a saponin-based adjuvant, has been demonstrated to be more effective than alum adjuvant in inducing neutralizing antibodies to SARS S protein or MERS S protein (17). This might address the concern that S protein may lead to antibody-dependent enhancement (ADE), which is more likely to be triggered by mild antibody production (36).

The SARS-CoV-2 infections occur at the mucosal surface of the upper respiratory tract (73). Thus, the elicitation of protective immune responses at the mucosa is critical. TLR agonists, such as flagellin (74) and CpG ODN (62), have been used as mucosal adjuvants. As discussed above, the CpG ODN can elicit neutralizing antibodies in mucosal compartments (62) when formulated with inactivated SARS-CoV. Additionally, the STING agonist, bis-(3′,5′)-cyclic dimeric guanosine monophosphate (c-di-GMP or cdGMP), has been reported as a potent mucosal vaccine adjuvant that induces Th1 and Th17 cytokines in a plant-derived H5 influenza vaccine after intranasal vaccination (75). In a very recent study, it was demonstrated that pulmonary surfactant–biomimetic liposomes encapsulating STING agonists could be used as mucosal adjuvants for universal influenza vaccines that trigger rapid humoral and cellular immune responses and exhibit sustained cross-protection against influenza (76). Though cdGMP in polymeric nanoparticle formulations has been used as adjuvants with MERS-CoV S-RBD protein, its ability to induce mucosal immunity was not specifically examined (72). Thus, further studies are warranted to examine both the efficacy and safety of mucosal adjuvants in coronavirus vaccines.

CONCLUSION AND PERSPECTIVES

In this article, we provided an overview of previously studied adjuvants in candidate inactivated and subunit coronavirus vaccines with a focus on the types of adjuvants in the vaccine formulations and the nature of immune responses to the formulated vaccines. These previous studies provided a convenient basis for the screening of adjuvants required to develop coronavirus vaccines. In-depth reviews of the various adjuvants, a comprehensive understanding of their impacts on the extent and types of immune responses, and an exploration of their combinations with various antigen types and vaccine platforms will facilitate the selection of adjuvants that provide the required immunological protection of coronavirus vaccines.

In the absence of a cure for COVD-19, effective and safe vaccines are urgently required. Adjuvants such as aluminum-based salts, TLR agonists, emulsions, and other novel adjuvants

TABLE 2 | Emulsion adjuvants used in the coronavirus vaccine formulations under exploratory and pre-clinical investigations.

Adjuvant Type	Platform	Adjuvant	Antigen	Antigen Dose	Immunological response								Route	Immunization Schedule	Safety	Animal model	Ref.
					Nab	IgG_1	IgG_{2a}	IgA	Th1 cytokines	Th2 cytokines	Th17 Cytokines	$CD8^+$ T cell response					
Emulsion adjuvants	Inactivated vaccines	MF59-like	Inactivated MERS virus	100 μl (1×10^7 $TCID_{50}$/ml)	√	N/A	N/A	N/A	√	√	N/A	N/A	i.m.	2	Lung immunopathology	hCD26/DPP4 Tg mice	(53)
	Inactivated vaccines	MF59	Inactivated SARS virus	5 μg	√	N/A	N/A	N/A	√	N/A	N/A	×	i.m.	2	N/A	BALB/c mice	(56)
	Inactivated vaccines	AS03	Inactivated SARS virus	100 μL of WI-SARS containing the equivalent of 0.5/1.0/1.5 μg of S protein	√	N/A	N/A	N/A	√	N/A	N/A	×	i.m.	2	N/A	BALB/c mice	(59)
	Subunit vaccines	MF59	MERS-CoV S-RBD-Fc protein	10 μg	√	√	√	N/A	×	N/A	N/A	×	s.c.	3	N/A	BALB/c mice; Ad5-hDPP4 mice	(54)
	Subunit vaccines	MF59	MERS-CoV S-RBD-Fc protein	1/5/20 μg (optimal: 1 μg)	√	√	√	N/A	√	N/A	N/A	√	s.c.	3	N/A	BALB/c mice	(57)
	Subunit vaccines	MF59-like	MERS-CoV S-RBD-Fc protein	10 μg	√	N/A	N/A	N/A	N/A	N/A	N/A	N/A	i.m.	2	Weight loss and death of mice	hCD26/DPP4 Tg mice	(78)
	Subunit vaccines	MF59	MERS-CoV S-RBD-Fc protein	10 μg	√	√	√	N/A	N/A	N/A	N/A	N/A	s.c.	2	N/A	BALB/c mice; hDPP4-Tg mice	(79)
	Subunit vaccines	MF59-like	MERS-CoV S-RBD-Fc protein	10 μg	√	N/A	N/A	N/A	√	N/A	N/A	N/A	i.m.	2	N/A	CD26/hDPP4 Tg mice	(55)
	Subunit vaccines	MF59	MERS-CoV S-RBD-Fd protein	10 μg	√	√	√	N/A	N/A	N/A	N/A	N/A	s.c.	4	N/A	BALB/c mice; hDPP4-Tg mice	(80)
	Subunit vaccines	MF59-like	MERS-CoV S-RBD protein NPs	20 μg	N/A	√	√	√	√	N/A	N/A	N/A	i.m.	3	N/A	BALB/c mice	(58)
	Subunit vaccines	MF59-like	MERS-CoV S-RBD protein	10 μg	N/A	√	√	N/A	×	N/A	N/A	×	s.c.	2	N/A	C57BL/6 mice	(72)
	Subunit vaccines	MF59-like	MERS-CoV/ SARS-CoV/ SARS-CoV-2 RBD-sc-dimer	10 μg	√	N/A	N/A	N/A	×	×	N/A	×	i.m.	3	N/A	BALB/c mice	(23)
	Subunit vaccines	Montanide ISA51	MERS-CoV S-RBD-Fc protein	10 μg	√	×	√	N/A	×	N/A	N/A	×	s.c.	3	N/A	BALB/c mice; Ad5-hDPP4 mice	(54)
	Subunit vaccines	Freund's adjuvant	MERS-CoV S-RBD-Fc protein	10 μg	×	×	√	N/A	×	N/A	N/A	×	s.c.	3	N/A	BALB/c mice; Ad5-hDPP4 mice	(54)
	Subunit vaccines	Ribi adjuvants	MERS-CoV S1 protein	10 μg	√	√	√	N/A	N/A	N/A	N/A	N/A	i.m.	2	N/A	BALB/c mice	(22)
	Subunit vaccines	Alum-stabilized Pickering emulsion (PAPE)	Recombinant RBD of SARS-CoV-2	5 μg	N/A	√	√	N/A	√	√	N/A	N/A	i.m.	2	Acceptable biosafety	BALB/c mice	(81)

Annotation:

MF59 adjuvant *an emulsion adjuvant composed of an oil phase (squalene:4.3%): and an aqueous phase (polysorbate 80:0.5%, sorbitan trioleate:0.5%);*

MF59-like *(AddaVax): a squalene-based oil-in-water nano-emulsion based on the formulation of MF59 (squalene:5%, polysorbate 80:0.5%, and sorbitan trioleate:0.5%);*

AS03 adjuvant*: an emulsion adjuvant composed of an oil phase (10.69 mg squalene, 11.86 mg DL-α-tocopherol) and an aqueous phase (4.86 mg polysorbate 80) each 0.5-mL adult dose;*

Ribi adjuvant*: an oil-in-water emulsion containing 2% squalene-Tween 80-water, 0.5 mg monophosphoryl lipid A, and 0.5 mg synthetic trehalose dicorynomycolate;*

Montanide ISA-51*: a water-in-oil (w/o) emulsion adjuvant composed of a mineral oil and a surfactant from the mannide monooleate family;*

Freund's adjuvant*: heat-killed mycobacterium tuberculosis in non-metabolizable oils (paraffin oil and mannide monooleate);*

Nab *represents the neutralizing antibodies, and N/A means not available.*

TABLE 3 | TLR agonists and other adjuvants used in the coronavirus vaccine formulations under exploratory and pre-clinical investigations.

Adjuvant Type	Platform	Adjuvant	Antigen	Antigen Dose	Immunological response								Route	Immunization Schedule	Safety	Animal model	Ref.
					Nab	IgG_1	IgG_{2a}	IgA	Th1 cytokines	Th2 cytokines	Th17 Cytokines	$CD8^+$ T cell response					
TLR agonists	Inactivated vaccines	CpG ODN 2006	inactivated SARS-CoV (SARS-CoV Z-1 strain virus)	10 μg	√	N/A	N/A	N/A	N/A	N/A	N/A	√	i.n./i.p.	3	N/A	BALB/c mice	(62)
	Subunit vaccines	Monophosphoryl lipid A	MERS-CoV S-RBD-Fc protein	10 μg	√	√	×	N/A	×	N/A	N/A	×	s.c.	3	N/A	BALB/c mice; Ad5-hDPP4 mice	(54)
	Subunit vaccines	CpG ODN 1826	SARS S peptide (HLA-A*0201 restricted)	20 μg	N/A	N/A	N/A	×	×	N/A	√	N/A	s.c.	2	N/A	HLA-A*0201 Tg mice	(63)
	Subunit vaccines	Poly(I:C)	SARS S peptide (HLA-A*0201 restricted)	20 μg	N/A	N/A	N/A	×	×	N/A	√	N/A	s.c.	2	N/A		(63)
	Subunit vaccines	R848	SARS S peptide (HLA-A*0201 restricted)	20 μg	N/A	N/A	N/A	×	×	N/A	√	N/A	s.c.	2	N/A		(63)
	Subunit vaccines	CpG ODN 1826 + Alhydrogel	SARS S protein	8 μg	√	√	√	√	√	N/A	N/A	√	s.c.	2	N/A	129S6/SvEv mice	(19)
	Subunit vaccines	CpG+aluminum oxyhydroxide	MERS S protein	10 μg	√	√	√	√	√	N/A	N/A	√	i.m.	3	N/A	BALB/c mice	(18)
	Subunit vaccines	CpG+ Montanide ISA-51	SARS S protein	30 μg	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	s.c.	3	N/A	BALB/c mice	(66)
	Subunit vaccines	CpG+ Montanide ISA-51	SARS N protein	50 μg	N/A	√	√	√	N/A	N/A	√	N/A	s.c.	3	N/A	BALB/c mice; Macaques	(67)
	Subunit vaccines	GLA+ Alhydrogel or aluminum phosphate	RBD (S318-510) of the SARS-CoV S protein	N/A	√	√	√	N/A	N/A	N/A	N/A	√	i.m.	2	N/A	BALB/c mice	(82)
	Subunit vaccines	ssRNA+ aluminum hydroxide	MERS-CoV S protein	1 μg	√	√	N/A	√	N/A	N/A	√	√	i.m.	2	N/A	hDPP4 Tg mice	(71)
Others	Inactivated vaccines	$AS01_B$	SARS inactivated whole virus	0.5/1 μg	√	N/A	N/A	N/A	N/A	N/A	×	√	i.m.	2	N/A	BALB/c mice; Hamsters	(59)
	Inactivated vaccines /Subunit vaccines	Advax	SARS S protein/inactivated whole virus	1 μg	√	√	√	√	√	√	√	√	i.m.	2	N/A	BALB/c mice	(46)
	Subunit vaccines	Matrix M1	SARS S protein/MERS S protein	1/3/10 μg	√	N/A	N/A	N/A	N/A	N/A	N/A	√	i.m.	2	N/A	BALB/c mice	(17)
	Subunit vaccines	NP-cdGMP	MERS-CoV S-RBD protein	10 μg	N/A	√	√	N/A	√	√	√	√	s.c.	2	N/A	C57BL/6 mice	(72)

Annotation:

MPLA *(monophosphoryl lipid A): a low-toxicity derivative of lipopolysaccharide (LPS), that retains the immunologically active lipid A portion of the parent molecule;*

CpG ODN*: synthetic oligodeoxynucleotides containing unmethylated CpG motifs;*

PolyI:C *(Polyinosinic-polycytidylic acid): a synthetic analog of double-stranded RNA (dsRNA), a molecular pattern associated with viral infection;*

R848 *(resiquimod): a small molecular weight imidazoquinoline compound, an immune response modifier with potent antiviral and antitumor activities;*

GLA*: glucopyranosyl lipid A, a synthetic Toll-like receptor 4 (TLR4) agonist;*

Advax*: a novel microcrystalline polysaccharide particle engineered from delta inulin;*

$AS01_B$*: a liposome-based emulsion adjuvant system containing two immunostimulants, 3-O-desacyl-4′-monophosporyl lipid A (MPL) and the saponin QS-21;*

Matrix M1*: consists of two individually formed 40-nm-sized particles, each with a different and well-characterized saponin fraction (Fraction-A and Fraction-C);*

NP-cdGMP*: cyclic diguanylate monophosphate (cdGMP), a canonical STING (stimulator of interferon genes) agonist, encapsulated into PLGA-based hollow nanoparticles.*

Nab *represents the neutralizing antibodies, and N/A means not available.*

have distinctive physicochemical properties, which can be significant in regulating the strength, duration, and types of immune responses (19, 63, 77). Studies have suggested that neutralizing antibodies are critical for immune protection (34, 42).While mechanistic studies are still being conducted, emerging evidence has suggested that SRAS-CoV-2-specific $CD4^+$ and $CD8^+$ T cells in coordination with neutralizing antibodies are required for generating protective immunity against SARS-CoV-2 (33). Thus, the appropriate adjuvants should be selected to formulate specific antigens that will achieve optimal immunogenicity profiles. Current available studies have demonstrated the feasibility of formulating S protein, RBD domain, M protein and N protein with specific adjuvants.

It should be noted that the development of a COVID-19 vaccine has been on a fast track. Thus far, four non-replicating viral vector vaccines, three inactivated vaccines and two mRNA vaccines being under clinical phase III stage, with more are on the way (32). Though different types of adjuvants have been used in exploratory and pre-clinical studies (**Tables 1**–**3**), considering the need for rapid deployment of COVID-19 vaccines for the pandemic, alum, which had been formulated in many other licensed vaccines, have been prioritized (15, 16). In addition to the adjuvants described above, engineered nanomaterials also shed light adjuvant development. It has been shown that physicochemical characteristics of aluminum oxyhydroxide could affect the optimal immunogenicity profiles of vaccine formulations (41, 83, 84). Moreover, a recent study has shown that an alum-stabilized Pickering emulsion (PAPE) showed robust RBD-specific IgG_1 and IgG_{2a} titers and a high level of inducing IFN-γ-secreting T cells in a COVID-19 vaccine. Additionally, it has been shown that a natural and potent STING agonist encapsulated by pulmonary biomimetic liposomes triggered rapid humoral and cellular immune responses and exhibited a sustained cross-protection against influenza (76). However, more comprehensive mechanistic studies, including the nature of protective immune responses and screening of the various combinations of antigens and adjuvants, are needed for the successful development of a safe and effective COVID-19 vaccine.

AUTHOR CONTRIBUTIONS

ZLiang, HZhu, XW, BJ, and XX wrote the manuscript. BS, LS and HZeng conceived and revised the manuscript. HS, YY, and WZ provided critical suggestions. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Natural Science Foundation of China (31870919), Natural Science Foundation of Liaoning Province (No. 20180550597), LiaoNing Revitalization Talents Program (XLYC1807113), Dalian Science and Technology Innovation Fund (No. 2020JJ25CY015), and Fundamental Research Funds for the Central Universities (DUT19TD12).

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Conflict of Interest: WZ was employed by NCPC Genetech Biotechnology Co., Ltd. LS was employed by Shanghai Zerun Biotechnology Co., Ltd.

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.

frontiers in Immunology

MINI REVIEW
published: 08 January 2021
doi: 10.3389/fimmu.2020.601170

Sex Differences in Immunity: Implications for the Development of Novel Vaccines Against Emerging Pathogens

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: 31 August 2020
Accepted: 30 November 2020
Published: 08 January 2021

Citation:
Fathi A, Addo MM and Dahlke C (2021) Sex Differences in Immunity: Implications for the Development of Novel Vaccines Against Emerging Pathogens. Front. Immunol. 11:601170. doi: 10.3389/fimmu.2020.601170

Vaccines are one of the greatest public health achievements and have saved millions of lives. They represent a key countermeasure to limit epidemics caused by emerging infectious diseases. The Ebola virus disease crisis in West Africa dramatically revealed the need for a rapid and strategic development of vaccines to effectively control outbreaks. Seven years later, in light of the SARS-CoV-2 pandemic, this need has never been as urgent as it is today. Vaccine development and implementation of clinical trials have been greatly accelerated, but still lack strategic design and evaluation. Responses to vaccination can vary widely across individuals based on factors like age, microbiome, co-morbidities and sex. The latter aspect has received more and more attention in recent years and a growing body of data provide evidence that sex-specific effects may lead to different outcomes of vaccine safety and efficacy. As these differences might have a significant impact on the resulting optimal vaccine regimen, sex-based differences should already be considered and investigated in pre-clinical and clinical trials. In this Review, we will highlight the clinical observations of sex-specific differences in response to vaccination, delineate sex differences in immune mechanisms, and will discuss the possible resulting implications for development of vaccine candidates against emerging infections. As multiple vaccine candidates against COVID-19 that target the same antigen are tested, vaccine development may undergo a decisive change, since we now have the opportunity to better understand mechanisms that influence vaccine-induced reactogenicity and effectiveness of different vaccines.

Keywords: vaccine, hormones, genetic, X-linked gene products, X-chromosome inactivation, miRNAs, emerging infections, sex differences

INTRODUCTION

Vaccination has been one of the most successful public health interventions to date. Every year, vaccines prevent millions of deaths worldwide (1). Despite this remarkable achievement, recent outbreaks of emerging viruses such as Ebola, Zika, and the coronaviruses (CoV) SARS-CoV, MERS-CoV, and SARS-CoV-2 underline the importance of a more rapid and systematic vaccine

development. In this context, a deeper understanding of host-specific mechanisms that influence vaccine-induced immunity is urgently needed to rapidly develop optimal vaccine strategies for heterogeneous populations.

Classic vaccine development has been rather empirical and employs three vaccine types: i) live attenuated, ii) inactivated, and iii) subunit vaccines. They represent "one bug, one drug" approaches as their respective safety and immunogenicity profiles are pathogen-specific. Their development is both time- and cost-intensive. To accelerate the response to emerging pathogens, so-called "plug-and-play" vaccine platforms have been generated, in which e.g. a carrier system can be adapted to express antigens of interest. These platforms can serve as blueprints to swiftly create vaccine candidates against emerging infectious diseases (EID).

Vaccine development traditionally follows a "one-size-fits-all" approach, although it is well understood that host factors like age, co-morbidities, co-infections, the microbiome and sex influence individual responses to vaccination. With regard to sex, a growing body of evidence indicates its significant role: Women generally develop stronger innate and adaptive immune responses than men (2–4), which can lead to a more rapid clearance and control of infection (5). They often express higher antibody levels and greater T-cell activation, and are thus likely to be more resistant to infections (6). While the trend toward an elevated vaccine-induced humoral and/or cellular immunogenicity in women (7, 8) may be beneficial with regard to efficacy, this may on the other hand lead to increased reactogenicity and negatively impact vaccine safety (6).

Here, we will provide insights gained from clinical trials with regard to sex-specific responses to vaccination and delineate underlying hormonal and genetic mechanisms that are described to affect immunity.

INFLUENCE OF SEX ON OUTCOMES OF VACCINATION

Findings from clinical trials have underlined that sex might have a crucial influence on the vaccine response. A higher magnitude of immune responses in adult women has been observed for a variety of vaccine candidates, including vaccines against influenza, yellow fever, rubella, measles, mumps, hepatitis A and B, herpes simplex 2, rabies, dengue and smallpox (6). Generally, adverse events are more common in adult females than males, which has been shown for influenza, hepatitis B, and yellow fever vaccines (9). The inactivated trivalent influenza vaccine (TIV) has, for example, been described as more reactogenic in women, who reported more adverse events such as injection site pain, myalgias and headaches than men after vaccination (10). Simultaneously, TIV was more immunogenic in women, and half a dose of TIV already induced humoral responses comparable to those in men who received the full dose (10). The importance to take sex into account as a biological variable when assessing vaccine efficacy was further depicted in a phase 3 trial of a glycoprotein D herpes simplex vaccine candidate (8, 11). This vaccine had shown no efficacy in the pre-specified overall analysis of both sexes. However, in a post-hoc sex-stratified analysis, the vaccine was highly protective against genital herpes in females while showing no protective effect in male vaccinees (11). Notably, humoral and cell-mediated immune responses did not differ between sexes, which illustrates the complexity of this issue.

NON-SPECIFIC EFFECTS OF VACCINATION

Vaccination may also modulate immune responses that are not antigen-specific, and experts have raised awareness for non-specific effects (NSE) of vaccination on immunity. These NSE may impact subsequent morbidity and even mortality from non-vaccine-related infectious diseases. It is assumed that these can be sex-specific (2). In areas where childhood mortality was high, clinical trials of high-titer measles vaccine and subsequent administration of diphteria-tetanus-pertussis or inactivated poliovirus vaccine found that mortality in girls was elevated as compared to boys who received the same vaccination regimen (12). A post-hoc analysis of a phase 3 malaria vaccine trial testing the recombinant protein vaccine RTS,S has also described a sex-differential mortality after receipt of the vaccine (13). Here, an increase in all-cause mortality was observed in girls who had received RTS,S compared to a control group of girls. Those differences were not observed in boys. The results were interpreted with caution and the possibility that they were incidental was raised. The WHO shared this interpretation and recommended the vaccine for use (14), however, others indicate that the findings were highly significant and may be due to NSE and emphasize that the observed safety signals need to be further monitored in phase 4 post-licensure studies, and should also be investigated in preclinical studies (15).

VIRAL VACCINES

Findings from clinical trials assessing viral vaccines are of particular interest in the field of EID vaccine research, since an array of recombinant viral vectors are now in development for novel vaccine candidates. These candidates include replication-deficient vectors that deliver the antigenic insert into the cell and thereby induce antigen-specific immune responses. In comparison, replication-competent vectors do replicate and, therefore, may induce a stronger immune response to the vector and tend to be more reactogenic and immunogenic.

Modified Vaccinia virus Ankara (MVA) is a replication-deficient viral vector, has been licensed as a smallpox vaccine (Imvamune®/ Imvanex®), and has been extensively tested. Males showed on average 27% higher anti-MVA titers (16), and stronger T-cell responses to Dryvax®, another poxvirus vaccine than females, in addition to statistically significant sex-related differences in interleukin (IL)-2, IL-1β, and IL-10 secretion (17). There was no reported sex difference in adverse events to Dryvax® vaccination (18). During the last decade, MVA has been further developed as a promising recombinant viral

vector for a multitude of pathogens (19–21). A MVA-based vaccine candidate against MERS-CoV has successfully completed phase 1 testing (22) and the MVA-based filovirus vaccine MVA-BN Filo® has been approved in 2020 as part of a heterologous prime-boost regimen against Ebola virus (EBOV) (23). MVA-vectored vaccine candidates are currently also evaluated against Coronavirus Disease 2019 (COVID-19), and the candidate MVA-SARS-2-S has recently entered clinical trials (NCT04569383). So far, these studies have not reported on sex differences.

Adenoviruses (Ad) are widely used as recombinant vaccine vectors and both chimpanzee and human adenovirus vectors, including different serotypes like Ad5 and Ad26, have entered clinical trials (24–27). In a clinical trial of the anti-EBOV vaccine Ad5-EBOV, fever was significantly more prevalent in men than in women (28). For another Ad5-vectored vaccine expressing HIV-antigens, a study that assessed predictors of cellular immune responses against this vaccine revealed that female sex was correlated with a higher number of vaccine responders (29). The most developed viral vector vaccine candidates against COVID-19 are Ad-based (30); these include the chimpanzee adenovirus (ChAd)-vectored vaccine ChAdOx1 nCoV-19, a heterologous Ad26/Ad5-vectored vaccination regimen, as well as an Ad5-vectored candidate; and phase 1/2 (ChadOx1, Ad26/Ad5) and phase 2 results (Ad5) have been published (27, 31, 32). The clinical studies of these candidates all included both sexes and the candidates were found to have an acceptable safety profile and to be immunogenic. Sex-differences could not be inferred from the phase 1/2 trials, which are small by design. However, the phase 2 trial, assessing a single dose of Ad5 vectored vaccine against placebo, included 508 participants with a balanced sex ratio. Humoral and cellular immunogenicity outcomes did not differ between men and women, but fever was more common in women post-vaccination (27).

The yellow fever virus strain 17D (YF-17D) is a live-attenuated virus vaccine that is highly effective against YF, but has a rather reactogenic profile. In rare cases, YF-17D can cause serious adverse events (SAE). Local and early reactions after YF-17D vaccination have been reported more often in women than in men (33). It has been postulated that this observation may be due to an enhanced innate immune response, as a higher number of TLR-associated genes that activate interferon pathways have been found to be upregulated in women post-YF-17D immunization (6). Notably, YF-17D vaccine-associated neurotropic disease (YF-AND) and YF vaccine-associated viscerotropic disease (YF-AVD)—both rare but highly lethal SAE—have occurred more frequently in men than in women (33). The underlying reasons remain to be elucidated. Currently, YF-17D is also under investigation as a carrier for EID vaccines, like the dengue virus vaccine candidate Dengvaxia® (34) and as a COVID-19 vaccine candidate (30).

SEX-SPECIFIC MECHANISMS IN IMMUNITY

Hormonal Factors

Clinically observed sex-differential responses to vaccination may partially be explained by hormonal factors. Women exhibit higher levels of estrogen and progesterone, while testosterone is more highly expressed in men, and circulating hormones are assumed to play a relevant role in immunity (35). Sex steroid hormone levels do not only vary greatly between the sexes, but also throughout the life span. The quality and magnitude of immune response may, therefore, vary between men and women, pre- and post-menopausal women, or adults compared to children. Pre-menopausal adult women generally have stronger immune responses than children, men or women during the post-menopause (36). Hormonal levels also fluctuate during the menstrual cycle: estrogen levels increase during the follicular phase, and progesterone remains low, while the luteal phase is characterized by high estrogen 17ß-oestradiol (E2) and progesterone plasma concentrations (37). While data are scarce, there is evidence that the menstrual cycle might affect immune cell numbers and modulate their activity throughout the 4-week period (38). Regulatory T-cells have been observed to be expanded during the follicular phase (38), while B-cell numbers and activity might increase in the periovulatory period. Monocyte numbers and their Tumor Necrosis Factor (TNF)α production increase during the luteal phase, while their IL-1 levels develop in opposing fashion. Data on Natural Killer (NK) cell number and activity have been conflicting, but a study by Souza and colleagues observed no correlation between progesterone and NK cell activity, while cytotoxicity was higher in the follicular than in the luteal phase of the menstrual cycle (38). However, the question whether and to what extent hormonal fluctuations are relevant for the vaccine response has not been comprehensively investigated so far.

The mechanisms of sex steroids that influence immune responses are generally driven by their binding to receptors, which in turn directly influence pro- and anti-inflammatory signaling pathways (39). Numerous immune cells express estrogen (ERs), androgen (ARs) and progesterone receptors (PRs) to varying degrees. The binding of sex steroids can directly affect the immune cell. Estrogens have been implicated in plasmacytoid dendritic cell (pDC) homeostasis, which is a key cell in antiviral immunity. pDCs produce large amounts of interferon (IFN)-α in response to a wide range of viruses, but also other microbial stimuli (40), and probably vaccines (41, 42). The induction of type I IFN drives the activation and anti-viral effector functions of immune cell populations and plays a pivotal role in inducing adaptive immunity. Human female peripheral blood mononuclear cells (PBMCs) and pDCs produce significantly more IFN-α in response to viral nucleic acids or synthetic Toll-like receptor (TLR)7 ligands than PBMCs and pDCs in men (43, 44). The precise functional mechanisms by which sex hormones regulate the IFN-α response of pDCs are unknown, but are thought to involve ERα signaling (45), and an increased level of estrogen may lead to an increased amount of TLR7-mediated IFN-α secretion by pDCs (46).

The E2 has a strong influence on the functional activity of innate immune cells, and it can greatly influence the quality and extent of adaptive immune responses (47). Elevated E2 levels strengthen type 2 helper T-cell (Th2) reactions, augment humoral immunity and regulate pro-inflammatory responses (5, 6, 48). In comparison,

low E2 concentrations may result in Th1-type and cell-mediated responses (5, 49). Notably, E2 levels correlated with the number of antibody-secreting cells and antibody levels, as they are highest before ovulation in females (50). An *in vitro* study could further show that estrogen enhanced antibody production and increased the survival rate of B-cells (51).

In comparison to estrogen, several studies reported that testosterone induces rather immunosuppressive effects. The Y-chromosome includes the sex-determining region (SRY), which is responsible for gonad development and the main driver for higher levels of androgens such as testosterone in men. This immunosuppressive effect has been observed in the context of the seasonal TIV. Higher serum testosterone concentrations correlated with reduced neutralizing antibody responses following influenza vaccination (52). In particular, males with elevated levels of serum testosterone and high expression of genes participating in lipid metabolism were significantly less likely to respond to TIV.

Genetic and Epigenetic Factors

Sex differences in vaccination can already be observed in children, which suggests that not only hormonal but also genetic and epigenetic factors may influence outcomes of vaccination. For example, vaccines against hepatitis B, diphtheria, pertussis, pneumococcus, rabies, malaria and human papillomavirus induced a greater immune response in female than in male children (3).

An important effect is mediated through the gene dosage between male and female cells. Dosage compensation for X-linked gene products may occur via random epigenetic silencing of one of the two X-chromosomes in females. However, 15-23% of X-linked human genes escape X-chromosome inactivation (XCI) resulting in simultaneous expression of both alleles (53). Since many genes encoded on the X-chromosome regulate immune functions, including TLR7, TLR8, IL-2, IL-3, Forkhead-box-P3 (FOXP3), and C-X-C chemokine receptor 3 (CXCR3), distinct gene expression levels may influence immune- and hence vaccine-specific responses (54).

Interestingly, the key immune receptor TLR7 is linked to the pathophysiology of the autoimmune disorder systemic lupus erythematosus (SLE) (54). In addition to pDCs, TLR7 is also present on monocytes/macrophages and B-cells. The gene encoding for TLR7 can escape from XCI and be biallelically expressed on B-cells, which then display higher TLR7-driven functional responses. This may increase susceptibility to TLR7-dependent autoimmune syndromes in females.

In a mouse model for leishmania infection, biallelic expression was likewise observed for CXCR3. XCI escape led to increased levels of CXCR3 in T-cells that consequently produced more IFN-γ, IL-2, and expressed more CD69 compared with T-cells that expressed CXCR3 only from one allele (55). XCI escape by CXCR3 potentially contributes to enhanced Th1 responses in females, which may affect the sex-associated bias observed during leishmania infection (55). Notably, the importance of the CXCR3 ligand CXCL10 (IP10) in response to a vaccine has been shown by our group in a first-in-human trial testing the recombinant viral vector vaccine against EBOV rVSV-ZEBOV (now renamed VSV-EBOV; tradename: Ervebo®) (56), as increased CXCL10 levels in the blood correlated with higher antibody titers. Whether a sex-bias toward CXCL10 in VSV-EBOV vaccinees exists is unknown, as studies with large cohorts including a balanced male:female ratio to evaluate innate immune responses and CXCL10 expression have not yet been performed.

Sex-differential expression of microRNAs (miRNAs) represents another gene-related factor that affects immunity. The X-chromosome contains 10% of the 800 miRNAs, whereas the Y chromosome encodes for only two miRNAs (57). MiRNAs are important regulators of messenger RNA (mRNA) stability and translation, and it is assumed that expression of about 60% of protein-coding genes are regulated by miRNAs (58). In the last decade, the role of miRNAs on the innate and adaptive immune response has been discussed extensively [reviewed in (59)]. Due to the high density of miRNAs encoded by the X-chromosome, it can be concluded that females may express more miRNAs due to incomplete XCI. The expression level of miR-223, for example, which is encoded on the X-chromosome, differs between men and women, either due to a skewed inactivation or an escape of gene silencing. High levels of miR-223 can limit recruitment of innate immune cells due to downregulation of CXCL2 and CCL3 and thereby modulate the magnitude of downstream adaptive immune responses (60). Of note, in a study that evaluated measles vaccination, the expression of miR-223 in B-cells was correlated to the induction of neutralizing antibodies, highlighting the potential impact of miRNAs on vaccine efficacy (61).

DISCUSSION

Hormonal and genetic differences between men and women might be the main drivers of sex-specific responses to vaccines affecting safety, immunogenicity and efficacy. Results from clinical trials, however, generally may not explain the factors that account for observed sex-specific differences due to their non-mechanistic nature and bear significant limitations, as they often originate from post-hoc analyses, and include a heterogeneous group of vaccine products and regimen.

The importance to gain insight into optimal vaccination strategies for men and women has been increasingly recognized and was recently addressed by the European Union's Horizon 2020 expert group that emphasized the need to analyze sex-specific side effects in the context of SARS-CoV-2 vaccine research (62). In EID vaccine development, the investigation of sex-differential outcomes of vaccination can be challenging, as early clinical trials evaluate vaccines in small and often homogeneous groups and little data exist on sex-specific immunity induced by the novel platform technologies. However, with more than 200 vaccine candidates against SARS-CoV-2 currently underway, of which over 45 have advanced to clinical trials—up to phase 3 (30) – we now have the unique opportunity to comprehensively investigate sex-specific immune responses induced by various vaccine candidates in pre-clinical and clinical trials (**Figure 1**). In this context, the impact of hormonal cycling on vaccine response should be further investigated.

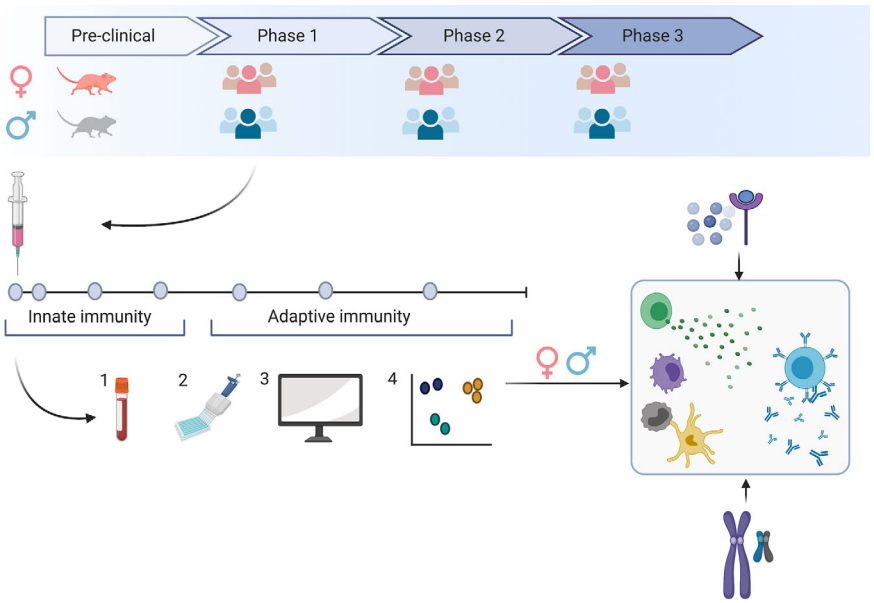

FIGURE 1 | Sex-specific immune responses can lead to vaccine responses that differ in safety, immunogenicity or efficacy. To evaluate the impact of sex on the specific vaccine candidate, we need to include both sexes as early as during the preclinical development stage and aim for a balanced sex ratio in clinical phase 1-3 trials. A detailed snapshot of immune responses to the vaccine can be achieved by frequent blood sampling following vaccination (1) and the application of various technologies (2). Here, we can evaluate responses to the vaccine on the transcriptome, epigenome and proteome level. Using bioinformatic tools (3), we may gain a comprehensive insight into multiple levels of immune responses that may be different in men and women (4). By comprehensively studying innate and adaptive immune responses in men and women, we may better understand how genetic or hormonal differences affect the number and functionality of immune cells.

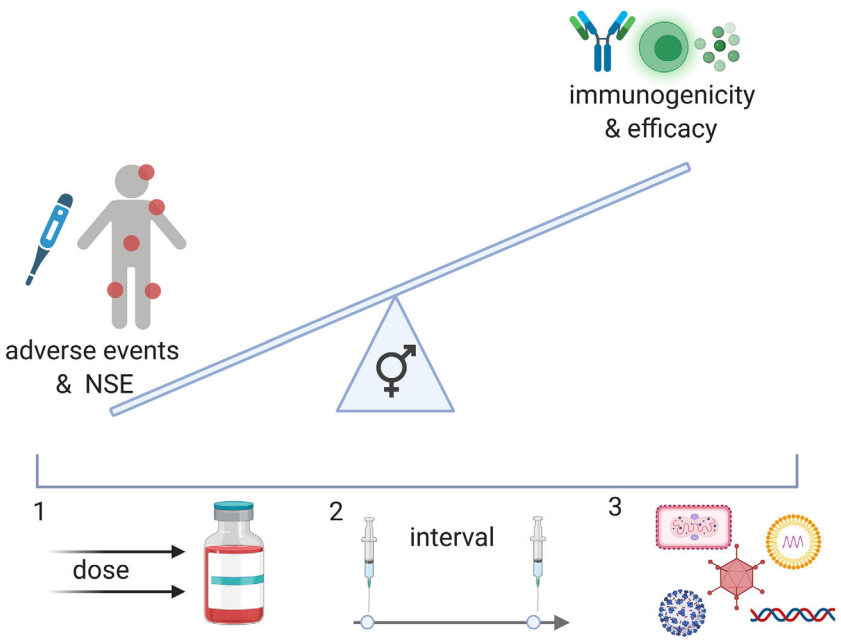

FIGURE 2 | A better understanding of the effect of vaccines in men and women can result in a balanced vaccine response. While NSE of vaccination may improve immune responses to pathogens, negative effects on immunity have also been described in females in specific contexts. In addition, reactogenicity is generally increased in females and may affect the safety profile of vaccines for women and girls. Immunogenicity, however, seems to also generally be increased in females and may therefore affect vaccine efficacy in this population. To achieve an equally beneficial vaccine for men and women, we may administer different doses (1), different intervals (2) or different vaccine candidates (3) (viral vector, nucleosid vaccines, inactivated viruses, proteins).

Frequent blood sampling during clinical trials and the implementation of an array of technologies such as multiple bead assays, flow cytometry, single cell RNA and transposase-accessible chromatin sequencing, multiplex mass, or chip cytometry will allow us to assess sex-differences at multiple levels like the transcriptome, epigenome, and proteome. We can achieve a detailed snapshot of immune responses upon vaccination (**Figure 1**) and correlate these data with measures of outcome. The findings may then guide future vaccine strategies resulting in potentially different dosage levels, different prime-boost intervals or specific vaccine platforms for men and women (**Figure 2**).

We are now at a critical point in time, with an array of new vaccine candidates on the way and state-of-the-art technologies at hand to evaluate host factors influencing vaccine response. If we use this moment right, we may be able to revolutionize vaccine development and strategically design vaccination regimens for distinct populations. Ultimately, this will enable us to optimize vaccination outcomes for the individual.

AUTHOR CONTRIBUTIONS

AF and CD conducted a review of articles and wrote the manuscript. MMA reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the German Center for Infection Research (DZIF).

ACKNOWLEDGMENTS

We thank Drs. Anneke Novak-Funk and Julie Sellau for proof-reading of the manuscript. Figures were generated using the software Biorender.

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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.

frontiers in Immunology

ORIGINAL RESEARCH
published: 26 January 2021
doi: 10.3389/fimmu.2020.602254

Induction of SARS-CoV-2 Protein S-Specific CD8+ T Cells in the Lungs of gp96-Ig-S Vaccinated Mice

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Given the aggressive spread of COVID-19-related deaths, there is an urgent public health need to support the development of vaccine candidates to rapidly improve the available control measures against SARS-CoV-2. To meet this need, we are leveraging our existing vaccine platform to target SARS-CoV-2. Here, we generated cellular heat shock chaperone protein, glycoprotein 96 (gp96), to deliver SARS-CoV-2 protein S (spike) to the immune system and to induce cell-mediated immune responses. We showed that our vaccine platform effectively stimulates a robust cellular immune response against protein S. Moreover, we confirmed that gp96-Ig, secreted from allogeneic cells expressing full-length protein S, generates powerful, protein S polyepitope-specific CD4+ and CD8+ T cell responses in both lung interstitium and airways. These findings were further strengthened by the observation that protein-S -specific CD8+ T cells were induced in human leukocyte antigen HLA-A2.1 transgenic mice thus providing encouraging translational data that the vaccine is likely to work in humans, in the context of SARS-CoV-2 antigen presentation.

Keywords: heat shock protein, glycoprotein 96, vaccine, lungs, COVID-19, SARS-CoV-2 protein S, CD8+ T cells

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Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: *02 September 2020*
Accepted: *17 December 2020*
Published: *26 January 2021*

Citation:
Fisher E, Padula L, Podack K, O'Neill K, Seavey MM, Jayaraman P, Jasuja R and Strbo N (2021) Induction of SARS-CoV-2 Protein S-Specific CD8+ T Cells in the Lungs of gp96-Ig-S Vaccinated Mice. Front. Immunol. 11:602254. doi: 10.3389/fimmu.2020.602254

INTRODUCTION

The rapid spread of the global COVID-19 pandemic has put pressure on the development of a SARS-CoV-2 vaccine to address global health concerns. We generated a gp96-Ig-secreting vaccine expressing full-length spike or "S" glycoprotein of SARS-CoV-2 *via* a cell-delivered platform. Targeting SARS-CoV-2 spike (S) protein remains the favorable vaccine choice as it is one of the most abundant and immunogenic proteins translated from the SARS-CoV-2 genome (1). Antibodies targeting S protein aim to neutralize mammalian host-cell interaction, thereby minimizing viral multiplicity of infection, however, recent studies have shown that "antibodies are not enough" to protect against COVID-19 for a variety of reasons, including S-protein glycosylation, which shields the antibody from eliciting an optimal neutralization response (2). Antibody decay has also been detected in individuals after recovery from COVID-19, and this decline was more rapid than reported for the first SARS infection in 2003 (3, 4).

T-cell immunity plays a pivotal role in generating a durable immune memory response to protect against viral infection. Prior studies have shown that memory B-cell responses tend to be short lived

after infection with SARS-CoV-1 (5, 6). In contrast, memory T-cell responses can persist for many years (7). Recent data confirm that SARS-CoV-2-specific memory CD8+ T cells are present in the vast majority of patients following recovery from COVID-19 (7–10), and their protective role has been inferred from studies in patients who have had both SARS and MERS (11–13). Recent reports show that patients who have recovered from a severe SARS-CoV-2 infection have T-cell responses against viral spike protein and other structural and nonstructural proteins; in some patients, T-cell responses were present regardless of symptoms or antibody seropositivity (14–16). Here, we generated a COVID-19 vaccine based on the proprietary secreted heat shock protein, gp96-Ig vaccine strategy, that induces antigen-specific CD8+ T lymphocytes in epithelial tissues, including lungs.

Tissue-resident memory (TRM) T cells have been recognized as a distinct population of memory cells that are capable of rapidly responding to infection in the tissue, without requiring priming in the lymph nodes (17–20). Several key molecules important for CD8+ T cell entry and retention in the lung have been identified (21–26) and recently CD69 and CXCR6 (20, 27–29) have been confirmed as core markers that define TRM cells in the lungs. Furthermore, it was confirmed that CXCR6-CXCL16 interactions control the localization and maintenance of virus-specific CD8+ TRM cells in the lungs (20). It has also been shown that, in heterosubtypic influenza challenge studies (30–32), TRM were required for effective clearance of the virus. Therefore, vaccination strategies targeting generation of TRM and their persistence may provide enhanced immunity, compared with vaccines that rely on circulating responses (32).

Our platform technology consists of a genetically engineered construct of gp96, fusion protein gp96-Ig, wherein the C-terminal KDEL-retention sequence was replaced with the fragment crystallizable (Fc) portion of immunoglobulin G1 (IgG1), and then encoded within a plasmid vector that is transfected into a cell line of interest. The cell serves as the antigen supply to secreted gp96-Ig. Complexes of gp96-Ig and antigenic peptides lead to specific cross-presentation of cell-derived antigens by gp96-Ig *in vivo* (33, 34). A crucial advantage offered by this gp96-based technology platform is that it allows for any antigen (such as SARS-CoV-2 S peptides) in the complex with gp96 to drive a potent and long-standing immune response. Over the last 2 decades, we have established that gp96-Ig, secreted from allogeneic or xenogeneic cells containing selected infectious antigens, generates potent, disease antigen specific, polyepitope, multifunctional CD8+ T cells in epithelial tissues (33–39). Here we generated a COVID-19 vaccine based on the secreted heat shock protein gp96-Ig vaccine strategy, and demonstrated vaccine-induced SARS-CoV-2 protein S-specific CD8+ and CD4+ T lymphocytes in epithelial tissues, including lungs and airways. The secreted gp96-Ig-COVID-19 vaccine has the potential to elicit robust long-term memory T-cell responses against multiple SARS-CoV-2 antigens and is designed to work cohesively with other treatments/vaccines (as boosters or as second-line defense) with large-scale manufacturing potential.

METHODS

Generation of Vaccine Cell Lines

Human embryonic kidney (HEK)-293 cells, obtained from the American Tissue Culture Collection (ATCC, #CRL-1573) and human lung adenocarcinoma cell lines (AD100) (40, 41) (source: University of Miami, FL, USA) were transfected with 2 plasmids: B45 encoding gp96-Ig (source: University of Miami) and pcDNA^{TM} 3.1(–) (Invitrogen), encoding full-length SARS-CoV-2 protein S gene (Genomic Sequence: NC_045512.2; NCBI Reference Sequence: YP_009724390.1 GenBank Reference Sequence: QHD43416). A B45 plasmid expressing secreted gp96-Ig has been approved by the Food and Drug Administration and Office of Biotechnology Activities for human use and is currently employed in a clinical study for the treatment of nonsmall cell lung cancer (NSCLC) (NCT02117024, NCT02439450) (42). The histidinol-selected, B45 plasmid, replicates as multicopy episomes and provides high levels of expression. Full-length SARS-CoV-2 protein S is based upon published SARS-CoV-2 protein S sequence from the original Wuhan strain (GenBank Reference Sequence: QHD43416) and cloned into the neomycin-selectable eukaryotic expression vector, pcDNA 3.1(-). HEK-293 and AD100 cells were simultaneously transfected with B45 and pcDNA 3.1 plasmid by Lipofectamine 3000 (Invitrogen) following the manufacturers' protocols. Transfected cells were selected with 1 mg/ml of G418 (Life Technologies, Inc.) and with 7.5 mM of L-Histidinol (Sigma Chemical Co., St. Louis, MO, USA). After stable transfection, a cell line was established. Single cell cloning by limiting dilution assay was performed, and all the cell clones were first screened for gp96-Ig production and then for protein S expression. Vaccine cells sterility testing and IMPACT^{TM} II polymerase chain reaction evaluation was performed for: Ectromelia, mouse rotavirus (EDIM), lymphocytic choriomeningitis virus (LCMV), lactate dehydrogenase-elevating virus (LDEV), mouse adenovirus (MAV1, MAV2), mouse cytomegalovirus (MCMV), mouse hepatitis virus (MHV), murine norovirus (MNV), mouse parvovirus (MPV), minute virus of mice (MVM), mycoplasma pulmonis, Mycoplasma sp., Polyoma, pneumonia virus of mice (PVM), Reovirus 3 (REO3), Sendai, Theiler's murine encephalomyelitis virus (TMEV). All test results were negative.

Western Blotting and Enzyme-Linked Immunosorbent Assay

Protein expression was verified by SDS-page and Western blotting using rabbit anti-SARS-CoV-2 spike glycoprotein antibody (MBS 150780) at 1/1000 dilution and secondary antibody: Peroxidase AffiniPure F(ab')$_2$ Fragment Donkey Anti-Rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories) horseradish peroxidase conjugated anti-rabbit IgG (Jackson ImmunoResearch) at 1/10,000 dilution. S protein was visualized by an enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ, USA) (**Figure 1C**). Recombinant human coronavirus SARS-CoV-2 spike glycoprotein S1 (Fc Chimera) (ab272105, Abcam) was used as

positive control (loaded 2.4 ug/lane). One million cells were plated in 1 ml for 24 hours (h) and secreted gp96-Ig production was determined by ELISA using anti-human IgG antibody for detection and human IgG1 as a standard (**Figure 1B**).

Immunofluorescence

AD100-gp96-Ig cytospins were fixed in pure cold acetone (VWR chemicals, BDH®, Catalog #BDH1101) for 10 min followed by three washes of 5 min each with phosphate-buffered saline (PBS). The slides were left in blocking media [5% bovine serum albumin (BSA) in PBS] at room temperature for 2 h. Rabbit anti-SARS-CoV-2 spike glycoprotein antibody (Abcam ab272504) and Donkey anti-rabbit IgG FITC, (BioLegend Cat# 406403) fluorescent antibody—were added in 1/50 and 1/100 dilutions of the antibodies combined in 5% BSA in PBS and/or rabbit isotype control (Abcam Ab172730 diluted 1/50), and incubated overnight at 4° C in a dark moisture chamber. The next day, slides were washed 3 times for 5 min with PBS and mounted with

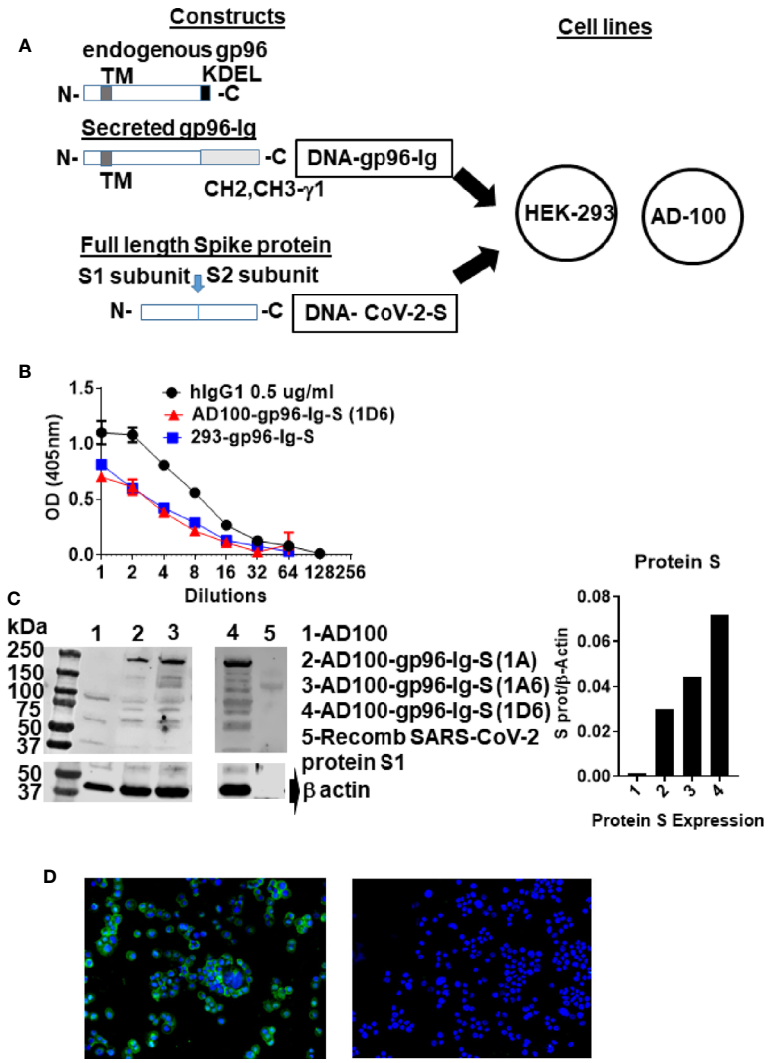

FIGURE 1 | Schematic of gp96-Ig and SARS-CoV-2 protein S constructs used to generate vaccine cells HEK-293-gp96-Ig-S and AD-100-gp96-Ig-S. **(A)** Each panel presents the protein expressed by the DNA (black outline) for the gp96-Ig and SARS-CoV-2 protein S vaccine antigen. Gp96-Ig and SARS-CoV-2-S DNA were cloned into the mammalian expression vectors B45 and pcDNA 3.1, which are transfected into HEK-293 and AD100. Stably transfected vaccine cell clones (1A, 1A6, 1D6) were generated after selection with L-Histidinol and Neomycin; **(B)** One million 293-gp96-Ig-S and AD-100-gp96-Ig-S (1D6) cells were plated in 1 ml for 24 h and gp96-Ig production in the supernatant was determined by ELISA using anti-human IgG antibody for detection with human IgG1 (0.5 ug/ml) as a standard; **(C)** Cell lysates were analyzed under reduced conditions by SDS-PAGE and Western blotting using anti protein S antibody and recombinant protein S1 as a positive control; **(D)** IF for protein S (in green) expressed in AD100-gp96-Ig-S cells using rabbit anti-SARS-CoV-2 S antibody and anti-rabbit Ig-AF488 as secondary antibody. AD100 was used as a negative control and β-actin for protein quantification. Original magnification 40× with DAPI nuclear staining shown in blue. DNA, deoxyribonucleic acid; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; N, amino terminus; C, carboxy terminus; IF, immunofluorescence; TM, transmembrane domain; KDEL, retention signal; CH2 CH3 gamma 1, heavy chain of IgG1. See text for explanation.

Prolong Gold antifade reagent with DAPI from Invitrogen (Catalog #36935), covered with a coverslip, and allowed to cure. The slides were then sealed with nail polish and taken to the KEYENCE microscope for examination. The following filter cubes were used: DAPI (for nuclear stain), FITC (for protein S), and images were acquired on KEYENCE microscope (BZ-X Viewer).

Animals and Vaccination

Mice used in this study were colony-bred mice (C57Bl/6) and human leukocyte antigen (HLA)-A02-01 transgenic mice [C57BL/6-Mcph1Tg (HLA-A2.1)1Enge/J, Stock No: 003475] purchased from JAX Mice (the Jackson Laboratory for Genomic Medicine, Farmington, CT, USA). Homozygous mice carrying the Tg (HLA-A2.1)1Enge transgene express human class I major histocompatibility complex (MHC) Ag HLA-A2.1. The animals were housed and handled in accordance with the standards of the Association for the Assessment and Accreditation of Laboratory Animal Care International under University of Miami Institutional Animal Care & Use Committee-approved protocol. Both female and male mice were used at 6–10 weeks of age.

Equivalent number of 293-gp96-Ig-protein S and AD100-gp96-Ig-protein S cells that produce 200 ng gp96-Ig or PBS were injected *via* the subcutaneous (s.c.) route in C57Bl/6 and HLA-A2.1 transgenic mice. Mice received vaccination on days 0 and 30 and were sacrificed 5 and 30 days after vaccination. Spleen, lungs, and bronchoalveolar lavage (BAL) were collected and processed into single-cell suspension.

BAL and Lung Harvest and Cell Isolation

For mouse samples, spleens were collected, and tissues processed into single-cell suspension. Leukocytes were isolated from spleen by mechanical dissociation and red blood cells were lysed by lysing solution. BAL was harvested directly from euthanized mice *via* insertion of a 22-gauge catheter into an incision into the trachea. Hanks' Balanced Salt Solution (HBSS) was injected into the trachea and aspirated 4 times. Recovered lavage fluid was collected and BAL cells were gathered after centrifugation.

To isolate intraparenchymal lung lymphoid cells, the lungs were flushed by 5 ml of prechilled HBSS into the right ventricle. When the color of the lungs changed to white, the lungs were excised avoiding the peritracheal lymph nodes. Lungs were then removed, washed in HBSS and cut into 3-mm pieces, and incubated in Iscove's Modified Dulbecco's Medium containing 1 mg/ml collagenase IV (Sigma) for 30 min at 37°C on a rotary agitator (approximately 60 rpm). Any remaining intact tissue was disrupted by passage through a 21-gauge needle. Tissue fragments and majority of the dead cells were removed by a 250-micrometer mesh screen, and cells were collected after centrifugation.

Ex Vivo Stimulation and Intracellular Cytokine Staining

Spleen and intraparenchymal lung lymphocytes from immunized and control animals were analyzed for protein S-specific CD8+ T cell responses. 1–1.5 × 10^6 cells were incubated for 20 h with 2 protein S peptide pools (S1 and S2, homologous to vaccine insert) (JPT Peptide Technologies, Berlin, Germany; PM-WCPV-S1). Peptide pools contain pools of 15-meric peptides overlapping by 11 amino acids covering the entire S protein (UniProt: P0DTC2) of SARS-CoV-2. Pool of 315 peptides (delivered in two subpools of 158 and 157 peptides) derived from a peptide scan through Spike glycoprotein. Peptide pools were combined (S1+S2) and used at a final concentration of 1.25 ug/ml of each peptide, followed by addition of Brefeldin A (BD GolgiPlug™; BD Biosciences, San Diego, CA, USA) (10 ug/ml) for the last 5 h of the incubation. Stimulation without peptides served as background control. The results were calculated as the total number of cytokine-positive cells with background subtracted. Peptide stimulated and non-stimulated cells were first labeled with live/dead detection kit (Thermo Fisher Scientific, Waltham, MA, USA) and then resuspended in BD Fc Block (clone 2.4G2) for 5 min at room temperature prior to staining with a surface-stain cocktail containing the following antibodies purchased from BioLegend® (San Diego, CA, USA): antigen presenting cell (APC)Cy7 CD45: Clone: 2D1; AF700 CD3: Clone: 17A2; APC CD4: Clone : RM4-5; PerCP CD8: Clone:53-6.7; PE Dazzle CD69: Clone:H1.2F3; BV 605 CD44: Clone : IM7; BV510 CD62L: Clone: MEL-14; PerCP/Cy5.5 CXCR6 (CD186): Clone: SA051D1. After 30 min, cells were washed with a flow cytometry staining buffer and then fixed and permeabilized using BD Cytofix/Perm fixation/permeabilization solution kit (according to manufacturer instructions), followed by intracellular staining using a cocktail of the following antibodies purchased from BioLegend: Alexa Fluor 488 interferon (IFN) gamma: Clone: XMG1.2; PE interleukin 2 [IL-2]: Clone: JES6-5H4 PE Cy7 tumor necrosis factor alpha (TNFα): Clone: MPG-XT22.

Data were collected on Spectral analyzer SONY SP6800 instrument (Sony Biotechnologies, Inc, San Jose, CA, USA). Analysis was performed using FlowJo™ software version 10.8 (Tree Star Inc, Ashland, OR, USA). Cells were first gated on live cells and then lymphocytes were gated for CD3+ and progressive gating on CD8+ T cell subsets. Antigen-responding CD8 (cytotoxic) T cells (IFNγ, or IL-2, or TNFα-producing/expressing cells) were determined either on the total CD8+ T cell population or on CD8+ CD69+ cells.

HLA-A2 Pentamer Staining

A total of 1–2 x 10^6 spleen, BAL, or lung cells were labelled with peptide-MHC class I (HLA 02-01) pentamer-APC (ProImmune, Oxford, UK) and incubated for 15 min at 37°C. Cells were labelled with LIVE/DEAD™ Fixable Violet—Dead Cell Stain Kit (Invitrogen, Carlsbad, CA, USA) and then stained with the following antibody cocktail: APCCy7 CD45: Clone:2D1; AF700 CD3: Clone: 17A2; PECy7 CD4: Clone : RM4-5; FITC CD8: Clone: KT15 (ProImmune, Oxford, UK) or PerCP CD8: Clone:53-6.7; PE Dazzle CD69: Clone:H1.2F3; BV 605 CD44: Clone : IM7; BV510 CD62L: Clone: MEL-14; PerCP/Cy5.5 CXCR6: Clone: SA051D1. Spleen and lung cells that were stimulated overnight with peptide pools (as described under

ex vivo stimulation and intracellular staining) were fixed and permeabilized with Cytofix/Perm solution (BD) and then stained for intracellular cytokines: IFNγ, and IL-2. Cells were acquired on SP6800 Sony instrument and data analyzed using FlowJo software version 10.8. Data were analyzed using forward side-scatter single-cell gate followed by CD45, CD3, and CD8 gating, then pentamer gating within CD8+ T cells. These cells were then analyzed for expression of markers using unstained and overall CD8+ population to determine the placement of the gate. Single-color samples were run for compensation and fluorescence minus 1 control sample were also applied to determine positive and negative populations, as well as channel spillover.

STATISTICS

All experiments were conducted independently at least 3 times on different days. Comparisons of flow cytometry cell frequencies were measured by the 2-way analysis of variance (ANOVA) test with Holm-Sidak multiple-comparison test, *p<0.05, **p<0.01, and ***p<0.001, or unpaired T-tests (2-tailed) were carried out to compare the control group with each of the experimental groups (alpha level of 0.05) using the Prism software (GraphPad Software, San Diego, CA, USA). Welch's correction was applied with the unpaired T test, when the p-value of the F test to compare variances were ≤0.05. Data approximately conformed to Shapiro-Wilk test and Kolmogorov-Smirnov tests for normality at 0.05 alpha level. Data were presented as mean ± standard deviation in the text and in the figures. All statistical analysis was conducted using GraphPad Prism 8 software.

RESULTS

AD100 and HEK-293 Express gp96-Ig and Protein S

Cell-based secreted heat shock protein technology has been previously validated in numerous animal models and in humans (37–39, 42). The secreted form of gp96 protein (gp96-Ig) was generated by replacing the c-terminal, KDEL-retention sequence of human gp96 gene, with hinge region and constant heavy chains (CH2 and CH3) of human IgG1 (43) (**Figure 1A**). The pcDNA 3.1(–) vector was used to express SARS-CoV-2 spike (S) protein (in this manuscript referred as protein S) (**Figure 1A**), due to its propensity to constitutively express large amounts of the protein in mammalian cells. Complementary (c) DNA encoding the full-length SARS-CoV-S glycoprotein included Kozak sequence (GCCACC) to optimize expression in eukaryotic cells and the open-reading frame contained endogenous leader sequence, transmembrane, and cytosolic domains.

Vaccine cells, 293-gp96-Ig-S and AD100-gp96-Ig-S, were generated by cotransfection of AD100 and HEK293 cells with plasmids encoding gp96-Ig (B45) and protein S (pcDNA 3.1) and selection with G418 and L-histidinol as described in *Methods*. We confirmed by ELISA that both stable transfected cell lines secreted gp96-Ig into culture supernatants at a rate of 125 ng/ml/24 h/10^6 vaccine cells (**Figure 1B**). Our previous data indicate that gp96-Ig accumulation in cell culture supernatant is linear and time dependent (35, 43).

Protein S expression by the vaccine cells was confirmed by analyzing vaccine cell lysates on SDS page, blotting with anti-SARS-CoV-2 S antibody (**Figure 1C**) and by immunofluorescence (**Figure 1D**). We observed expression of full-length protein S (250 kDa) only in AD100 transfected cell lines (lanes 2–4) but not in nontransfected AD100 cell line (lane 1). In addition, we observed molecular weight bands of 120 and 130 kDa that could represent cleavage products of full length protein S (protein S1 and S2) and/or gp96-Ig fusion protein chaperoning the protein S peptides. The expected molecular weight of gp96-Ig fusion protein is 116 kDa. Additional bands, of ~70 kDa, were found to be expressed only in the transfected cell line and were not observed in the nontransfected AD100 cells. However, nonspecific bands of 100, 60, and 40 kDa were observed in the AD100 parental cell line. Recombinant protein S1 130 kDa was used as a positive control. We calculated the ratio of protein S to β-actin expression (**Figure 1C**) and confirmed the expression of protein S by immunofluorescence (**Figure 1D**). We observed cytoplasmic and transmembrane distribution of protein S in AD100-gp96-Ig-S cell line. We therefore confirmed the expression of gp96-Ig and S protein in our AD100 cell line and used it for immunogenicity studies as described below.

Secreted gp96-Ig-S Vaccine Induces CD8+ T Cell Effector Memory Responses in the Lungs

Our vaccination strategy is based on the quantity of gp96-Ig-S secreted by the vaccine cells to stimulate CD8+ CTL responses *via* APC cross-presentation. The vaccination dose, is therefore, standardized to a set amount of gp96-Ig secreted by 10^6 vaccine cells within 24 h. It has been well established from our previous vaccine immunogenicity studies that the optimal dose for induction of CD8+ T cell specific responses in mice is 200–500 ng/ml (33, 35, 38, 39, 43). Here, we used 200 ng/ml to immunize mice with AD100-gp96-Ig-S vaccine. Mice were vaccinated *via* the s.c. route and, after 5 days, the frequency of T cells within spleen, lungs (lung parenchyma), and BAL cells (lung airways) was determined. We observed significant increase in the frequencies of CD8+ T cells in the spleen and lungs, but not within the BAL of vaccinated mice (**Figure 2A**). Frequency of CD4+ T cells was unchanged between vaccinated and control mice in all analyzed tissues. It is well established that vaccination with gp96-Ig induces CD8+ TEM differentiation (33, 37, 39). Here, we confirmed that gp96-Ig-S vaccine primes strong effector memory CD8+ T-cell responses as determined by analysis of CD44 and CD62L expression (**Figure 2B**). Whereas the frequency of naïve (N), CD44-CD62L+CD8 T cells and central memory (CM), CD44+CD62L+ CD8+ T cells was unchanged, we found statistically significant increase of TEM CD44+ CD62L- CD8+ T cells within the spleen and lungs (**Figure 2B**). In addition, we observed a trend of more TEM CD8+

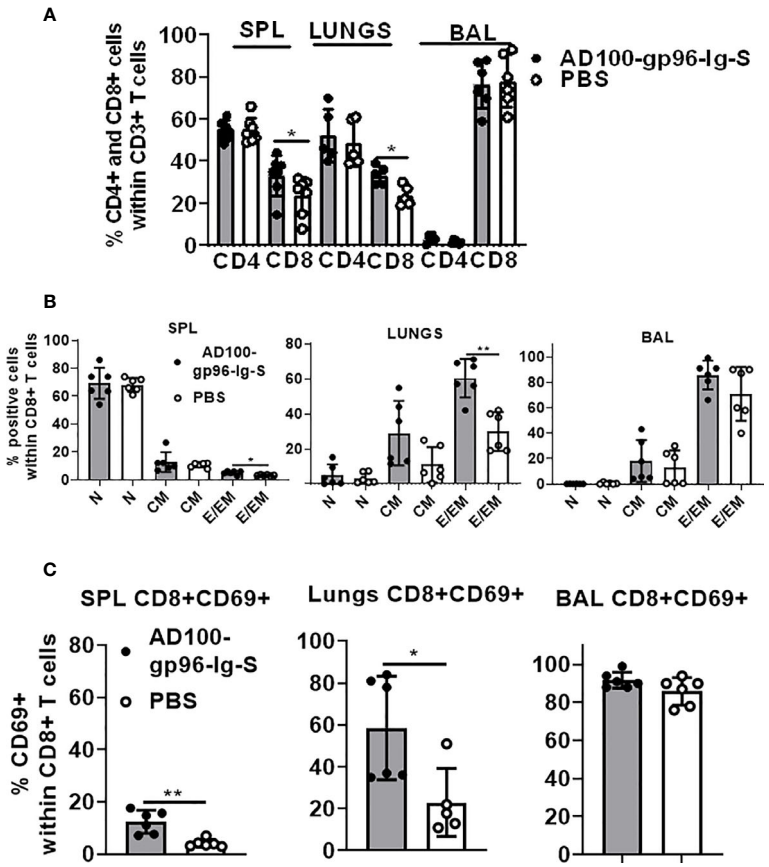

FIGURE 2 | Secreted gp96-Ig-S vaccine induces CD8+ TEM and TRM responses in the lungs. Equivalent number of AD100-gp96-Ig-S vaccine cells that produce 200 ng/ml gp96-Ig or PBS were injected by s.c. route in C56Bl/6 mice. 5 days later, mice were sacrificed and spleen, lungs, and BAL were isolated and **(A)** frequency of CD4+ and CD8+ T cells; **(B)** naive (N) CD44-CD62L+, CM CD44+CD62L+ and EM CD44+CD62L- CD8+ T cells; and **(C)** TRM CD69+ cells were determined by flow cytometry after staining the cells with antibodies against the following surface markers: CD45, CD3, CD4, CD8, CD44, CD62L, and CD69 antibodies. Bar graph shows percentage of CD4+ and CD8+ cells within CD3+ cells or CD8+ T cell memory subset within CD8+ T cells. Data represent at least two technical replicates with three to six independent biological replicates per group. *p<0.05, **p<0.01. **(A, B)** Mann-Whitney tests were used to compare two experimental groups. To compare >2 experimental groups, Kruskal-Wallis ANOVA with Dunn's multiple comparison tests were applied). BAL, bronchoalveolar lavage; CM, central memory; EM, effector memory; TEM, T cell effector memory.

T cells within the CD8+ T cells in the BAL (**Figure 2B**). TRM cells are a distinct memory T cell subset compared to CM and EM cells (44) that are uniquely situated in different tissues, including lungs (30, 31). One of the canonical markers of TRM T cells is CD69 (20, 44, 45). We found that there was a significant increase in the frequency of CD8+CD69+ T cells in vaccinated mice compared to control, non-vaccinated mice in both spleen and lungs (**Figure 2C**). Even though the frequency of CD8 +CD69+ T cells was the highest in the BAL compared to spleen and lungs, we did not observe a difference in their frequencies between vaccinated and control mice. Overall, vaccination with AD100-gp96-Ig-S induced robust TEM and CD69+ CD8+ T cell responses in both spleen and lungs. Our vaccine can therefore successfully elicit both systemic and tissue-specific immune response, which is pivotal in conferring robust immunity against infection such as against SARS-CoV-2.

Both Protein S-Specific CD8+ and CD4+ T Helper 1 T Cell Responses Are Induced by gp96-Ig-S Vaccine

To evaluate polyepitope, protein S-specific CD8+ and CD4+ T-cell responses induced by gp96-Ig-S vaccination, we used pooled S peptides (S1+S2) and a multiparameter intracellular cytokine-staining assay to assess Th1 (IFNγ+, IL-2+, and TNFα+), CD8+ and CD4+ T cells (**Figure 3**). Spleen and lung cells were tested for responses to the pool of overlapping protein S peptides (S1 + S2) and all of the vaccinated animals showed significantly higher magnitude of the protein S-specific T cell responses against S1 and S2 epitopes compared with non-vaccinated controls (**Figures 3A–E**). Increase in the vaccine-induced Th1 CD8+ T cell responses (IFNγ+, IL-2+, and TNFα+) was noted in both spleen and lungs (**Figures 1A, B**), whereas Th1 CD4+ T cell responses (IFNγ+, IL-2+, and TNFα+) were induced only in lungs (**Figures 3C, D**). The proportion of the protein S-specific CD8+ T cells that produce

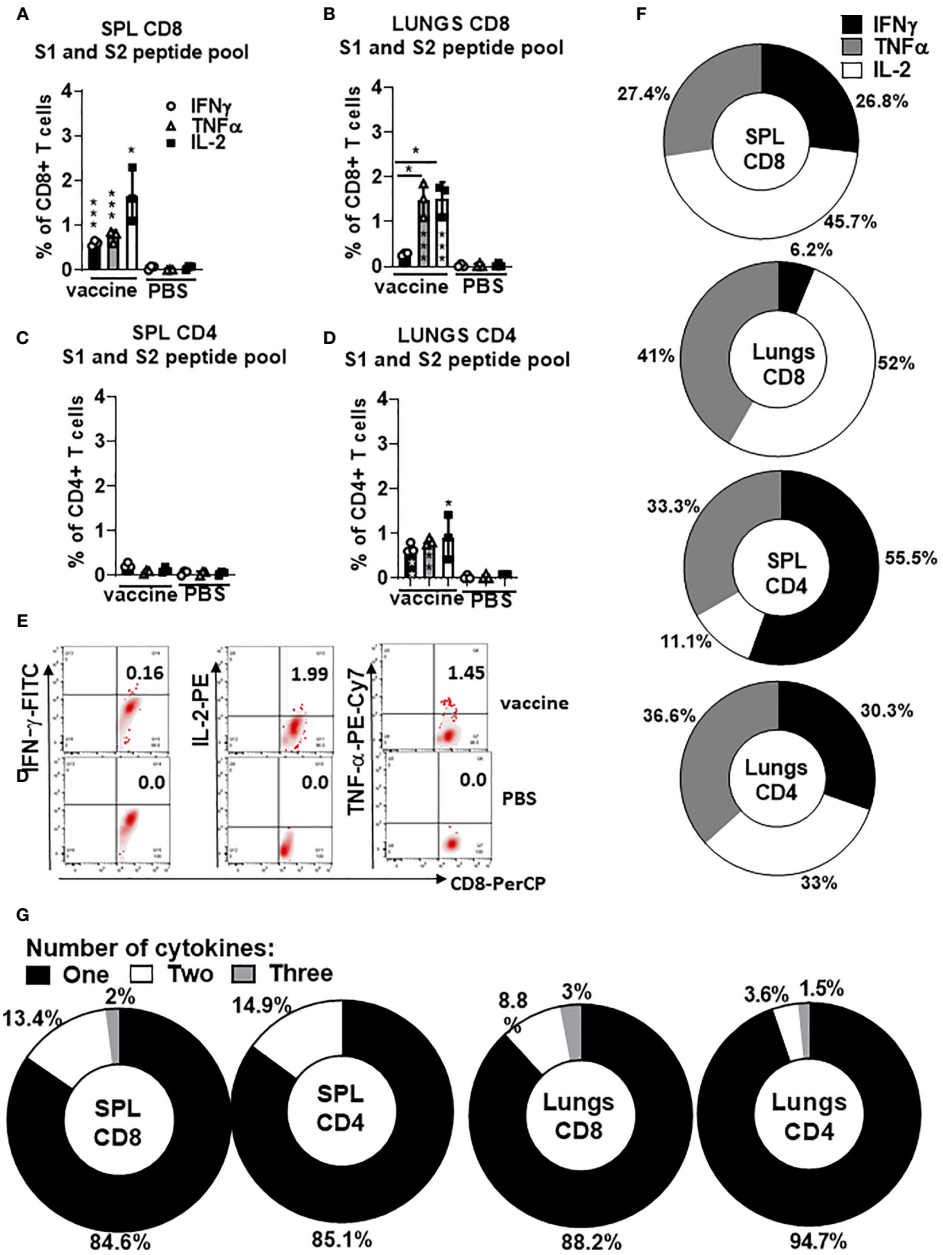

FIGURE 3 | Secreted gp96-Ig-S vaccine induces protein S specific CD8+ and CD4+ T cells in the spleen and lung tissue. Five days after the vaccination of C57Bl6 mice, splenocytes and lung cells were isolated from vaccinated and control mice (PBS) and in vitro restimulated with S1 and S2 overlapping peptides from SARS-CoV-2 protein in the presence of protein transport inhibitor, brefeldin A for the last 5 h of culture. After 20 h of culture, ICS was performed to quantify protein S-specific CD8+ and CD4+ T-cell responses. Cytokine expression in the presence of no peptides was considered background and it was subtracted from the responses measured from peptide pool stimulated samples for each individual mouse. **(A, B)** CD8+ T cells from spleen and lungs expressing IFNγ, TNFα, and IL-2 in response to S1 and S2 peptide pool; **(C, D)** CD4+ T cells from spleen and lungs expressing IFNγ, TNFα, and IL-2 in response to S1 and S2 peptide pool; **(E)** Representative dot plot of gated CD8+ T cells in lungs expressing indicated cytokines (IFNγ, IL-2, and TNFα) in vaccinated and non-vaccinated (PBS, control) HLA-A2 mice at day 5 **(F)** Proportion of antigen (protein S)-experienced CD8+ and CD4+ T cells isolated from spleen and lung tissue expressing IFNγ, TNFα, or IL-2 after o/n stimulation with S1 + S2 peptides. Pie charts corresponding to cytokine profiles of CD8+ and CD4+ T cells isolated from spleen and lung tissue; **(G)** Pie charts corresponding to cytokine profiles of CD8+ CD4+ T cells isolated from spleen and lung tissue after o/n stimulation with S1 + S2 peptides. Assessment of the mean proportion of cells making any combination of one to three cytokines (IFN-γ, TNFα, IL-2). Data represent at least two technical replicates with three to six independent biologic replicates per group. *p<0.05, ***p<0.001. Kruskal-Wallis ANOVA with Dunn's multiple comparisons tests were applied. Asterisks (*) above or inside the column denote significant differences between indicated T cells producing cytokines in vaccine versus control (PBS) at 0.05 alpha level. ANOVA, analysis of variance; ICS, intracellular cytokine staining; IFN, interferon; IL, interleukin; PBS, phosphate-buffered saline; TNF, tumor necrosis factor.

IFNγ (26.8%) was significantly reduced in the lungs (6.2%), while both TNFα and IL-2 productions were increased in the lungs (41% and 52%, respectively) compared to spleen (27.4% and 45.7%, respectively) (**Figure 3F**). We found that the proportion of the protein S-specific CD4+ T cells that produce IFNγ was higher in the spleen than in the lungs [55.5% (spleen) versus 30.3% (lungs)], whereas IL-2 production was higher in the lungs than in the spleen [33% (lungs) versus 11.1% (spleen)] (**Figure 3F**). Further assessment of protein S-specific CD8+ and CD4+ T cells in the spleen and lungs revealed that the vast majority of protein S-specific CD8+ and CD4+ T cells, irrespective of their location, synthesized only 1 cytokine (**Figure 3G**).

It was therefore confirmed that a polyepitope, S-specific, CD4+, and CD8+ T cell response was generated in the spleen and lungs to different extents, providing a strong vaccine-induced Th1 cellular immune responses.

Induction of SARS-CoV-2 Protein S Immunodominant Epitope-Specific CD8+ T Cells in the Lungs and Airways of Vaccinated HLA-A2.1-Transgenic Mice

Recently, it was reported that SARS-CoV-2-specific memory CD8+ T cell responses generated against cognate antigens positively correlate with a number of symptom-free days after infection (14, 16). Therefore, it is important to develop vaccines that can elicit SARS-CoV-2-specific CD8+ T cells. Having identified overall T-cell responses to SARS-CoV-2 protein S (**Figure 3**), we wanted to determine whether gp96-Ig-S vaccine induced HLA class I-specific cross-presentation of immunodominant SARS-CoV-2 protein S epitopes. In order to do this, we used transgenic HLA-A2.1 mice and HLA class I pentamers as probes to detect CD8+ T cells specific for two immunodominant SARS-CoV-2 protein S epitopes: YLQPRTFLL (YLQ) (aa 269-277) and FIAGLIAIV (FIA) (aa 1220-1228) in vaccinated mice (**Figures 4A–C**). We found that the vaccine effectively induces both YLQ+CD8+ T cells, as well as FIA+CD8+ T cells in the spleen, lungs, and BAL (**Figures 4A–C**). Interestingly, we found the highest frequency of YLQ+CD8+ T cells in the BAL of vaccinated mice and the lowest frequency of YLQ+ and FIA+ CD8+ T cells was observed in the lungs 5 days after primary vaccination. Importantly, we observed that after contraction of vaccine induced YLQ+CD8+ and FIA+CD8+ T cell responses in spleen, lungs and BAL, frequency of S-specific CD8+ T cell responses is preserved and it is significantly higher in BAL compared to non-vaccinated controls (**Figures 4A–C**).

Upon further phenotype analysis of YLQ+CD8+ T cells, it was confirmed that they express both CD69 and CXCR6 (**Figure 4D**). Particularly, we found that during primary response (day 5) all YLQ+CD8+ T cells in the BAL were also CD69+ and CXCR6+, and the frequency of YLQ+CD8+CXCR6+ cells was significantly higher in the BAL compared to the lungs. However, during contraction phase (day 30) YLQ+CD8+ cells (**Figures 4A, B** and **Supplementary Figure 1**) in the SPL, lungs and BAL express both, CD69 and CXCR6 (**Figure 4D**).

We confirmed that S-specific CD8+ and CD4+ T cells are present in significantly higher frequencies compared to unvaccinated controls 30 days after single dose vaccination (**Figures 5A–E**). The proportion of the protein S-specific CD8+ T cells that produce IFNγ was comparable in the spleen and lungs (32.7% and 28%, respectively) while we observed increased proportion of the cells that produce TNFα in the lungs (47.3%) compared to spleen (24.5%) (**Figure 5C**). In addition, we found that the proportion of the protein S-specific CD8+ T cells that produce IL-2 was higher in the spleen than in the lungs (42.6% and 24.5%, respectively) while the proportion of S-specific CD4+ T cells that produce IL-2 was higher in lungs than in spleen (44.4% and 33.3%, respectively) (**Figure 5C**).

Furthermore, we observed increase in the frequency of YLQ+ CD8+ T cells in the spleen, lungs and BAL in all animals after receiving second dose of gp96-S vaccine at day 30 (**Figure 5F**). Despite the similar magnitudes of primary and secondary CD8+ T cell expansion, contraction of vaccine induced Ag-specific CD8+ T cell responses was different during primary and secondary response. We observed more rapid contraction of CD8+ T cells after primary vaccination, with 68% loss of Ag-specific CD8+ T cells from day 5 to 30 (**Figure 5G**), compared to 25% decrease in Ag-specific CD8+ T cells during secondary response (**Figure 5G**). Thus, the program of contraction in CD8+ T cells that are responding to second dose of vaccine was prolonged, resulting in increased frequencies of memory S-specific CD8+ T cells.

DISCUSSION

Our vaccine approach is based on the gp96-Ig platform technology that elicits potent, antigen-specific CD8+ T-cells. This proprietary secreted heat shock protein platform has been successfully used to induce immunogenicity against tumors, HIV/SIV, Zika, and malaria in different animal models (37–39, 46–48). Importantly, this vaccine strategy has shown success in delaying virus acquisition, as well as in improving the survival of NSCLC patients in clinical trials (38, 42).

The principle of a cell-based vaccine relies on the ability of gp96-Ig to chaperone antigenic proteins to be efficiently endocytosed and cross-presented by activated dendritic cells (DC) to CD8+ T cells, thereby stimulating an avid, pathogen-specific T-cell response (33, 34, 37–39). We adapted this cell-based technology to create a vaccine that delivers SARS-CoV-2 spike (S) protein directly to DCs, so that primed and activated SARS-CoV-2 protein S-specific CD8+ T cells can identify and kill SARS-CoV-2 infected lung epithelial cells. We generated vaccine cells by co-expressing secreted gp96-Ig and full-length protein S. Gp96-Ig is an endoplasmic reticulum chaperone that, together with TAP (transporter associated with antigen processing) and calreticulin in the endoplasmic reticulum, is thought to constitute a relay line for antigenic peptide transfer from the cytosol to MHC class I molecules in a concerted and regulated manner (49, 50). The gp96-antigenic peptide complexes are predominantly internalized by subsets of APCs through cell surface receptor CD91. Internalized gp96 can effectively present the associated peptides to MHC class I and II molecules and thus activate specific CD8+ and CD4+ T-cell responses (34, 39, 51, 52). We expressed full-length protein

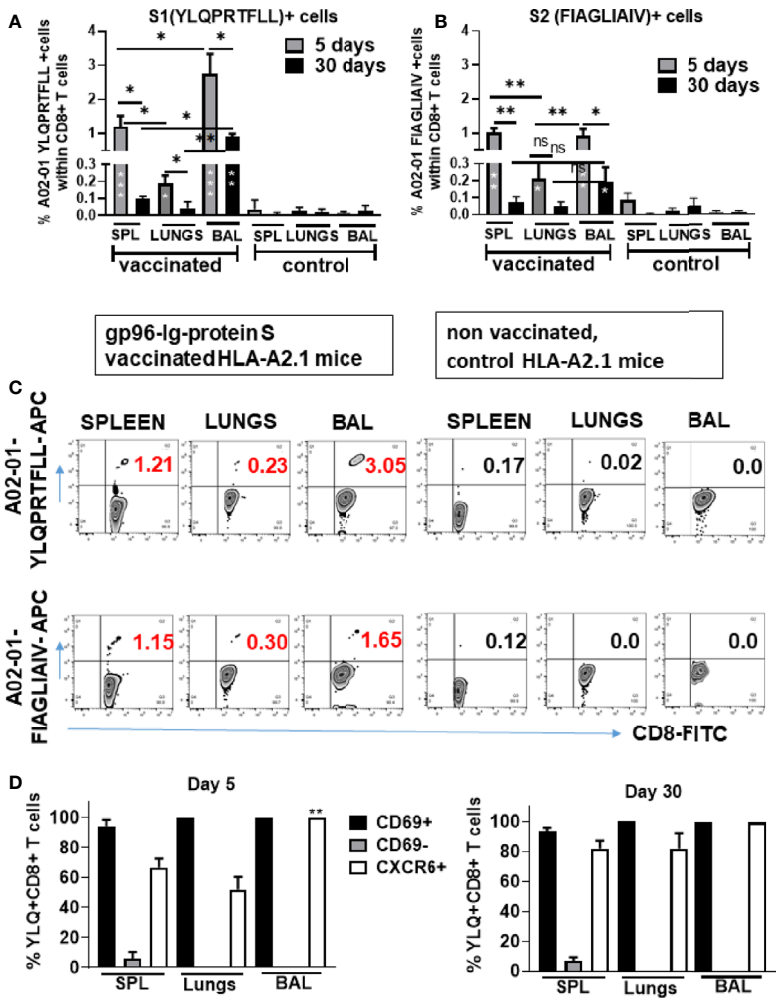

FIGURE 4 | Secreted Gp96-Ig-S vaccine induces S1- and S2-specific CD8 + CD69 + CXCR6 + cells in the spleen, lung tissue, and BAL. Five and 30 days after the vaccination of HLA-A2.1 transgenic mice, splenocytes, lung cells and BAL were isolated form vaccinated and control mice (PBS). Cells were stained with HLA-A2 pentamer containing FIAGLIAIV and YLQPRTFLL peptides, followed by surface staining for CD45, CD3, CD4, CD8, CD69, CXCR6. **(A, B)** Bar graphs represent percentage of the pentamer positive cells within CD8+ T cells; **(C)** Representative zebra plots of gated CD8+ T cells expressing indicated pentamer-specific TCR+ CD8+ T cells in vaccinated and non-vaccinated HLA-A2.1 mice at day 5 **(D)** Bar graphs represent percentage of CD69+, CD69-, and CXCR6+ cells within YQL-pentamer positive cells at days 5 and 30; (Data represent at least two technical replicates with three to six independent biologic replicates per group. *p<0.05, **p<0.01, ***p<0.001. Kruskal-Wallis ANOVA with Dunn's multiple comparisons tests were applied. Asterisks (*) inside the column denote significant differences between indicated pentamer+CD8+ T cells in the vaccinated group and control (PBS) **(A, B)** at 0.05 alpha level. ANOVA, analysis of variance; BAL, bronchoalveolar lavage; PBS, phosphate-buffered saline.

S in the vaccine cells to ensure broad representation of all immunodominant protein S peptides (S1- and S2-derived peptides) by secreted gp96-Ig. Since coronaviruses assemble in the compartment between the endoplasmic reticulum and Golgi apparatus (53, 54) and the S leader directs it to the endoplasmic reticulum, the native leader sequence of protein S was retained, as well as transmembrane and cytosolic domain. We confirmed in previous studies that secreted gp96-Ig provides immunologic specificity for the antigenic repertoire expressed inside the cells, including surrogate antigen ovalbumin, as well as numerous tumor or infectious antigens, but does not cross-immunize to different cell-derived antigens (35, 37–39). Our data are consistent with the explanation that S1 and S2 peptides associated with secreted gp96-Ig are transferred to and presented by class I and II MHC and stimulate a S1- and S2-specific CD8+ and CD4+ T cell response. We confirmed that vaccination with AD100-gp96-Ig-S induces CD8+ T cells specific for S1- and S2-immunodominant epitopes in both lungs and airways. Most importantly, this is a proof-of-concept study that will be applied to other structural proteins such as nucleocapsid protein, membrane protein, and nonstructural proteins such as NSP-7, NSP-13 of ORF-1 that all have been reported to be important in induction of SARS-CoV-2-specific CD4+ and CD8+ T cell responses in convalescents (7, 10, 11, 55).

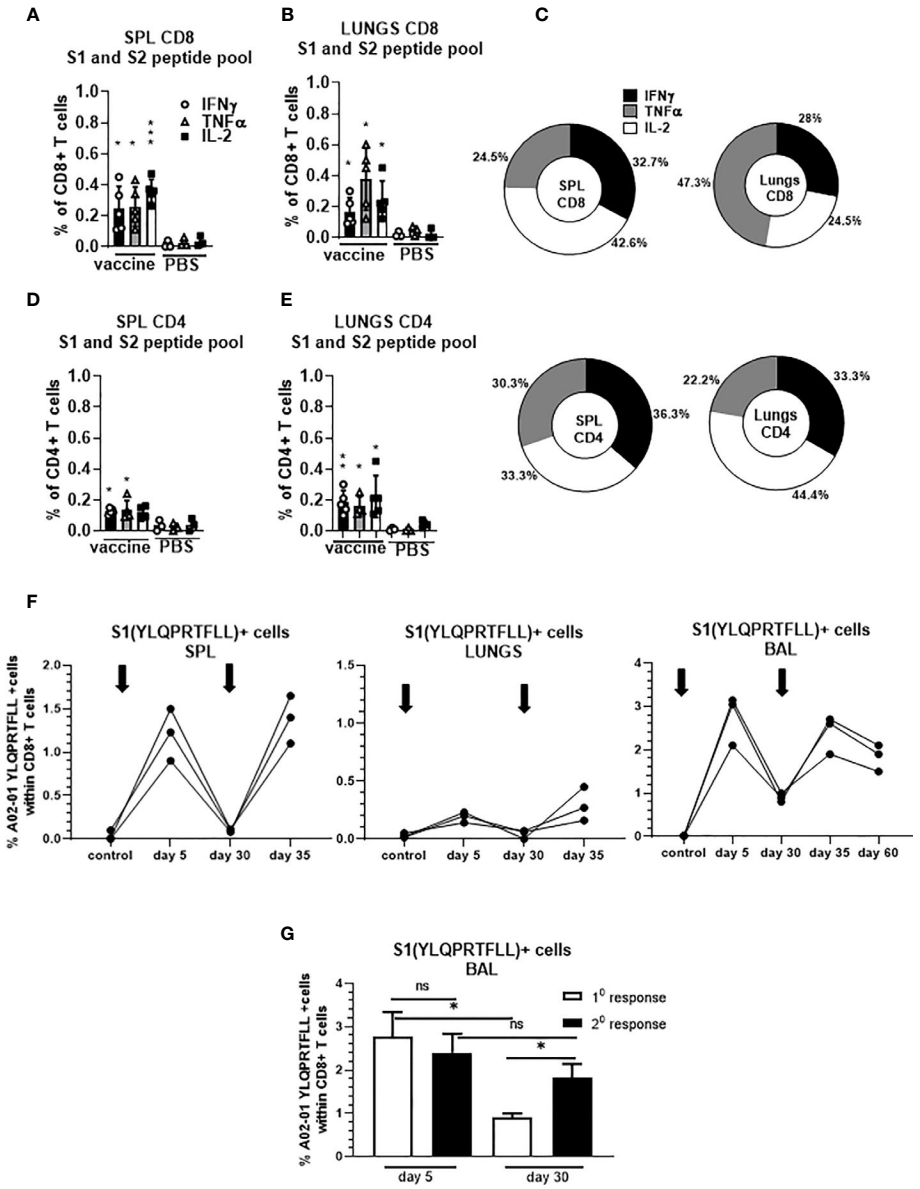

FIGURE 5 | Secreted gp96-Ig-S vaccine induces protein S specific memory CD8+ and CD4+ T cells in the spleen and lungs. Thirty days after the vaccination of HLA-A2.1 transgenic mice, splenocytes and lung cells were isolated from vaccinated and control mice (PBS) and in vitro restimulated with S1 and S2 overlapping peptides from SARS-CoV-2 protein in the presence of protein transport inhibitor, brefeldin A for the last 5 h of culture. After 20 h of culture, ICS was performed to quantify protein S-specific CD8+ and CD4+ T-cell responses. Cytokine expression in the presence of no peptides was considered background and it was subtracted from the responses measured from peptide pool stimulated samples for each individual mouse. **(A, B)** CD8+ T cells from spleen and lungs expressing IFNγ, TNFα, and IL-2 in response to S1 and S2 peptide pool; **(D, E)** CD4+ T cells from spleen and lungs expressing IFNγ, TNFα, and IL-2 in response to S1 and S2 peptide pool; **(C)** Proportion of antigen (protein S)-experienced CD8+ and CD4+ T cells isolated from spleen and lung tissue expressing IFNγ, TNFα, or IL-2 after o/n stimulation with S1 + S2 peptides. Pie charts corresponding to cytokine profiles of CD8+ and CD4+ T cells isolated from spleen and lung tissue; **(F)** 5 days after primary and secondary vaccination of HLA-A2.1 transgenic mice, splenocytes, lung cells and BAL were isolated form vaccinated and control mice (PBS). Time of primary and secondary vaccination is indicated with black arrows. Cells were stained with HLA-A2 pentamer containing YLQPRTFLL peptide, followed by surface staining for CD45, CD3, CD4, CD8. **(G)** BAL was analyzed 5 and 30 days after primary and secondary vaccination and frequency of HLA-A2 pentamer positive cells was determined. Graphs represent percentage of the pentamer positive cells within CD8+ T cells in individual mice; Data represent at least two technical replicates with three to six independent biologic replicates per group. *p<0.05, **p<0.01, ***p<0.001, ns, not significant. Kruskal-Wallis ANOVA with Dunn's multiple comparisons tests were applied. Asterisks (*) above or inside the column denote significant differences between indicated T cells producing cytokines in vaccine versus control (PBS) at 0.05 alpha level. ANOVA, analysis of variance; ICS, intracellular cytokine staining; IFN, interferon; IL, interleukin; PBS, phosphate-buffered saline; TNF, tumor necrosis factor.

In agreement with our previous findings (33, 38, 39), the gp96-Ig vaccine resulted in the preferential induction of CD8+ T cell responses systemically and in epithelial compartments. However, this is the first report about the increase in the frequencies of vaccine induced CD8+ T cells in the lungs (**Figure 1A**). TEM CD8+ T cells are considered to constitute the frontline defense within the different epithelial compartments including lungs and airways, which promptly recognize and kill infected cells. Our data suggest that after a single dose of AD100-gp96-Ig-S immunization there is preferential compartmentalization of TEM and TCM immune responses in the lungs and BAL compared to the spleen where a majority of cells are naïve CD8+ T cells (**Figure 1B**). However, additional memory cells without migratory potential such as TRM CD8+ T cells, exist within the tissues including lungs and airways (20, 44, 45, 56). Since TRM are uniquely situated in the lungs to immediately respond to reinfection, by inducing the protein-S-specific CD8+ T cells that home to the lungs, gp96-Ig-S vaccine provides an ideally balanced generation of both arms, TRM and TEM, of the memory response in the lungs. To further gauge the effect of gp96-Ig vaccination on the induction of epitope specific immunogenicity, we used pentamers to detect S1 and S2 epitope-specific CD8+ T cell responses. We found that gp96-Ig induced the highest frequencies of S1- and S2- epitope specific CD8+ T cells in the airways. In light of the new findings about exclusive highly clonally expanded SARS-CoV-2-specific CD8+ T cells with preferentially expressed tissue-resident genes (XCL1, CXCR6, and ITGAE) in the BAL of moderate COVID-19 cases (57) and not in the critical/severe COVID-19 patients, induction of SARS-CoV-2 specific CD8+ T cells that home to airway epithelium emphasizes the importance of developing vaccination strategies that induce TRM antigen-specific CD8+ T cells that will improve efficacy of vaccination against respiratory pathogens including SARS-CoV-2.

It is well appreciated that the antigen presenting cells at the site of immunization direct the imprinting of the ensuing T-cell response and control the expression of trafficking molecules (58). Priming of CD8+ T cells by CD103+ DC was found to promote TRM CD8+ T cell differentiation and migration into peripheral epithelial tissues, including lungs (56, 59). Our previous studies indicated that gp96-Ig immunization increases frequency of $CD11c^{high}$ MHC class II^{high} CD103+ cells at the vaccination site (33). In light of our previous findings and findings of Bedoui et al. (60) that CD103+ DCs are the main migratory subtype with dominant cross-presenting ability, induction of CD103+ DCs by gp96 represents an ideal vaccination strategy for priming effective and durable immunity in the epithelial tissues. We also hypothesize that secretory gp96-Ig can be captured by lung DC upon s.c. inoculation and in that way directly induce tissue memory responses and even more importantly support their longevity in the tissue. It was previously shown that, based on differences in the localization and functions, there are two different subsets of lung TRM cells: airway TRM and interstitial TRM (61–63). CXCR6-CXCL16 interactions are crucial in controlling the localization of virus-specific TRM CD8+ T cells in the lungs and maintaining the airway TRM cell pool (20). Moreover, blocking of CXCR6-CXCL16 interactions significantly decreases the steady-state migration of TRM cells into airways, so vaccine induced SARS-CoV-2 S specific CD8+ T cells that express CXCR6 fulfill one of the major requirements for continued CXCR6 signaling in maintaining the airway TRM pool (20). *In vivo* anti-CD3 labelling of the lymphocytes is the best method for distinguishing intravascular vs extravascular antigen specific cells. Future studies should include *in vivo* labelling to distinguish if vaccine induced S-specific immune responses are circulating/effector or local/tissue resident cells.

We have confirmed in different infectious vaccine models that gp96-Ig carries all peptides of a cell that are selected in the recipient/vaccinee for MHC I, having the broadest, theoretically possible antigenic epitope-spectrum for cross-priming of CD8+ T cells by any MHC I type. Here, we showed that AD100-gp96-Ig-S resulted in the polyepitope and polyfunctional protein S-specific CD8+ and CD4+ T cell responses (**Figure 3**). When stimulated *in vitro* with S1+S2 peptides, spleen and lung CD8+ T cells produce IFNγ, TNFα, and IL-2 cytokines (**Figure 3**) with CD8+ T cells in the lungs producing significantly less IFNγ than the CD8+ T cells in the spleen. It is known that enhanced activation, resulting from high levels of inflammation, induces CD8+ T cells entering the lungs to produce regulatory cytokines (64) that initiate "dampening" of the immune response in order to prevent any excessive damage of the lung tissue. In addition, we report that CD4+ T cells in the lungs produce all 3 Th1 cytokines in equal ratio (**Figure 3F**). This finding is in line with our previous report discussing gp96-Ig induced SIV-specific CD4+ T cells in the lamina propria which were almost the exclusive producers of IL-2 (33). Further studies will therefore be required to evaluate the role of protein-S specific CD4+ T cells in the induction of B cell and antibody responses. Previously, we have shown that gp96 is a powerful Th1 adjuvant for CTL priming and for stimulation of Th1 type antibodies in mice and nonhuman primates (37, 38). In addition, we will evaluate memory responses after a single and booster dose to establish the best vaccination protocol for future challenge studies.

In summary, we provide a paradigm for a novel vaccine development approach capable of induction of cellular immune responses in epithelial tissues such as the lungs. Structure-guided SARS-CoV-2 S protein combined with a safe and efficacious gp96-Ig vaccine platform can pave the way for a protective and durable immune response against COVID-19. This is a first demonstration of the utility and versatility of our proprietary secreted gp96-Ig SARS-CoV-2 vaccine platform that can be rapidly engineered and customized based on other and future pathogen sequences. Furthermore, the platform is proof of concept for the prototype vaccine approach for similar pathogens that require induction of effective TRM responses in epithelial tissues.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Miami Institutional Animal Care & Use Committee.

AUTHOR CONTRIBUTIONS

NS conceived and coordinated the experiments and obtained funding. EF, LP, KP, KO'N, and NS performed the experiments and analyzed the data. MS provided reagents. NS, EF, PJ, RJ, and MS wrote the paper. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by Heat Biologics, Inc (AWD-005840) (NS) and by Department of Microbiology and Immunology (NS) and University of Miami (NS).

ACKNOWLEDGMENTS

This manuscript has been released as a pre-print at *bioRxiv* (65). We dedicate this work to the late Dr. Eckhard Podack. We are grateful to all members of Strbo laboratory, Heat Biologics, Inc. CEO Jeff Wolf, Chief Scientific and Operating Officer, Jeff Hutchins, and Director Discovery Sciences, Eric Dixon, for their overall support, advice, and editorial contributions. We are grateful to Mr. William Strong for his generous gift for vaccine development. We thank Sylvester Comprehensive Cancer Center Flow Cytometry Shared Resource (SCCC FCSR) staff for their help.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.602254/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | Secreted Gp96-Ig-S vaccine induces S1-specific CD8 + cells in the spleen, lung tissue, and BAL. Thirty days after the vaccination of HLA-A2 transgenic mice, splenocytes, and lung cells and BAL were isolated from vaccinated and control mice (PBS). Cells were stained with HLA-A2 02-01 pentamer containing SARS-CoV2 YLQPRTFLL peptide, followed by surface staining for CD45, CD3, CD4, CD8, CD69, CXCR6. Representative zebra plots of gated CD8+ T cells expressing indicated pentamer-specific TCR+ CD8+ T cells in vaccinated and non-vaccinated HLA-A2 mice at day 30. HLA-A2 pentamer containing HIV/SIV (HIV-1 gag) SLYNTVATL pentamer was used as a negative control pentamer.

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Conflict of Interest: NS is inventor on the patent application No 62/983,783 entitled "Immune-mediated coronavirus treatments"; NS is a member of Heat Biologics COVID-19 Advisory Board. MS is the Executive Director of Special Projects. PJ is the Associate Director of Business Development, both are employed by Heat Biologics, Inc. RJ is the CEO of Pelican Therapeutics, a subsidiary of Heat Biologics, Inc. MS, PJ, RJ, and KP hold stock options in Heat Biologics, Inc.

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.

frontiers in Immunology

REVIEW
published: 29 January 2021
doi: 10.3389/fimmu.2020.607333

Adenoviral Vectors as Vaccines for Emerging Avian Influenza Viruses

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It is evident that the emergence of infectious diseases, which have the potential for spillover from animal reservoirs, pose an ongoing threat to global health. Zoonotic transmission events have increased in frequency in recent decades due to changes in human behavior, including increased international travel, the wildlife trade, deforestation, and the intensification of farming practices to meet demand for meat consumption. Influenza A viruses (IAV) possess a number of features which make them a pandemic threat and a major concern for human health. Their segmented genome and error-prone process of replication can lead to the emergence of novel reassortant viruses, for which the human population are immunologically naïve. In addition, the ability for IAVs to infect aquatic birds and domestic animals, as well as humans, increases the likelihood for reassortment and the subsequent emergence of novel viruses. Sporadic spillover events in the past few decades have resulted in human infections with highly pathogenic avian influenza (HPAI) viruses, with high mortality. The application of conventional vaccine platforms used for the prevention of seasonal influenza viruses, such as inactivated influenza vaccines (IIVs) or live-attenuated influenza vaccines (LAIVs), in the development of vaccines for HPAI viruses is fraught with challenges. These issues are associated with manufacturing under enhanced biosafety containment, and difficulties in propagating HPAI viruses in embryonated eggs, due to their propensity for lethality in eggs. Overcoming manufacturing hurdles through the use of safer backbones, such as low pathogenicity avian influenza viruses (LPAI), can also be a challenge if incompatible with master strain viruses. Non-replicating adenoviral (Ad) vectors offer a number of advantages for the development of vaccines against HPAI viruses. Their genome is stable and permits the insertion of HPAI virus antigens (Ag), which are expressed *in vivo* following vaccination. Therefore, their manufacture does not require enhanced biosafety facilities or procedures and is egg-independent. Importantly, Ad vaccines have an exemplary safety and immunogenicity profile in numerous human clinical trials, and can be thermostabilized for stockpiling and pandemic preparedness. This review will discuss the status of Ad-based vaccines designed to protect against avian influenza viruses with pandemic potential.

Keywords: adenovirus, adenoviral vector, vaccine, immunogenicity, influenza, avian influenza, highly pathogenic, highly pathogenic avian influenza

OPEN ACCESS

Edited by:
Neeltje van Doremalen, Rocky Mountain Laboratories (NIAID), United States

Reviewed by:
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Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: *16 September 2020*
Accepted: *07 December 2020*
Published: *29 January 2021*

Citation:
Kerstetter LJ, Buckley S, Bliss CM and Coughlan L (2021) Adenoviral Vectors as Vaccines for Emerging Avian Influenza Viruses. Front. Immunol. 11:607333. doi: 10.3389/fimmu.2020.607333

INTRODUCTION

Influenza viruses belong to the family *Orthomyxoviridae* and have a genome composed of eight single-stranded negative sense RNA (-ssRNA) segments. The natural reservoirs for influenza A viruses (IAV) are aquatic and migratory birds. However, these zoonotic viruses can also infect domesticated animals such as poultry and swine, as well as humans (**Figure 1**). The zoonotic nature of IAVs, coupled with humans encroaching on animal habitats (1, 2), has increased the likelihood for emerging avian influenza viruses to jump the species barrier and infect humans. As such, these viruses represent a major pandemic threat and vaccine development and pandemic preparedness are a global priority (2).

IAVs are phylogenetically sub-divided according to their surface glycoproteins, the viral hemagglutinin (HA) and neuraminidase (NA) (**Figure 2**). To date, 18 HA and 11 NA subtypes have been identified, although this includes two bat IAV-like HAs (H17, H18) and NAs (N10, N11) (3). Distinct HA subtypes are classified into two groups, group 1 (G1): comprised of H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, and the bat HAs, and group 2 (G2), which includes H3, H4, H7, H10, H14, and H15 HAs (4). The HA protein is immunodominant and is therefore a major target for neutralizing antibodies (NAbs). As a result, it is also the main focus for seasonal influenza virus vaccines. However, IAV viruses evolve and mutate using processes known as *antigenic drift* and *antigenic shift*. Antigenic drift is the accumulation of mutations in the HA (and other proteins) incurred by the error-prone viral RNA-dependent RNA polymerase, often in response to selective pressure from the host. This can result in the evasion of pre-existing NAbs elicited by natural infection or prior vaccination, leading to reduced vaccine effectiveness (5, 6). Alternatively, the segmented nature of the viral genome can result in genome reassortment if more than one IAV simultaneously infects the same cell, creating progeny viruses with a hybrid combination of segments (7). This internal shuffling of genome segments can result in the exchange or incorporation of a novel HA or NA glycoprotein on the virion surface, in a process known as *antigenic shift* (**Figure 2**). This has the potential to result in a novel subtype, for which the human population would be immunologically naïve. Unlike influenza B and C viruses which mainly infect humans and therefore limit this scenario, IAVs can infect many different species including poultry, swine, and other mammals (8). The majority of reassortments result in defective progeny viruses:

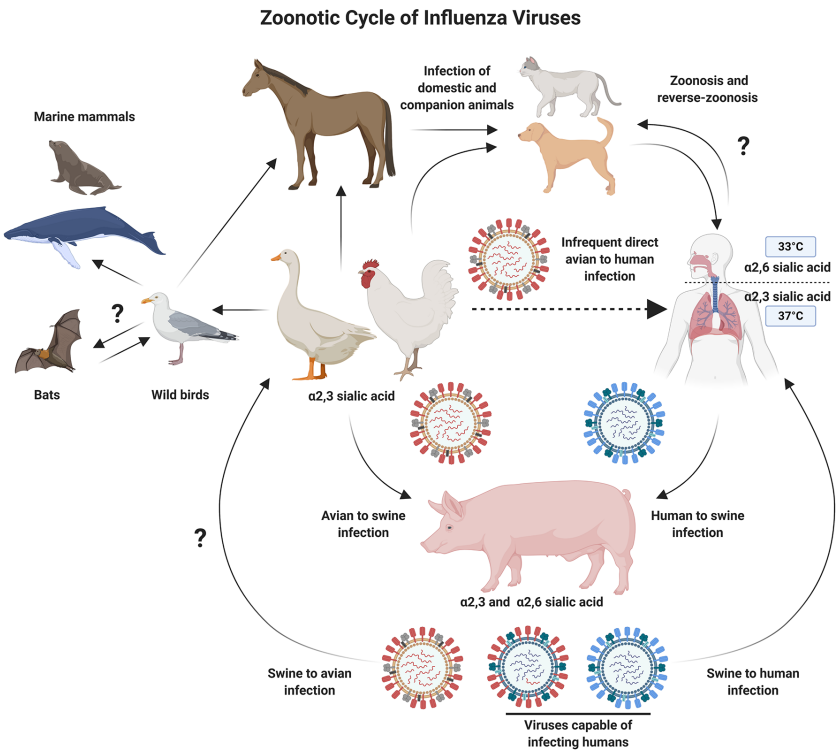

FIGURE 1 | Schematic Diagram Showing Zoonotic Cycle of Influenza Viruses. Influenza A viruses can infect multiple animal species, which increases the probability of cross-species transmission events. Migratory and aquatic birds represent natural reservoirs for avian influenza viruses, and pigs act as a mixing vessel, allowing the reassortment of diverse influenza viruses. The process of reassortment could lead to the emergence of novel influenza subtypes which are better adapted for infection and transmission in humans. Several barriers to this process also exist, including, but not limited to receptor usage preferences. Direct infection of humans with avian influenza viruses is an infrequent event. However, the potential for adaptation while maintaining high pathogenicity is a major concern and drives efforts to develop improved vaccines against emerging avian influenza viruses. Figure created with ©BioRender - Biorender.com.

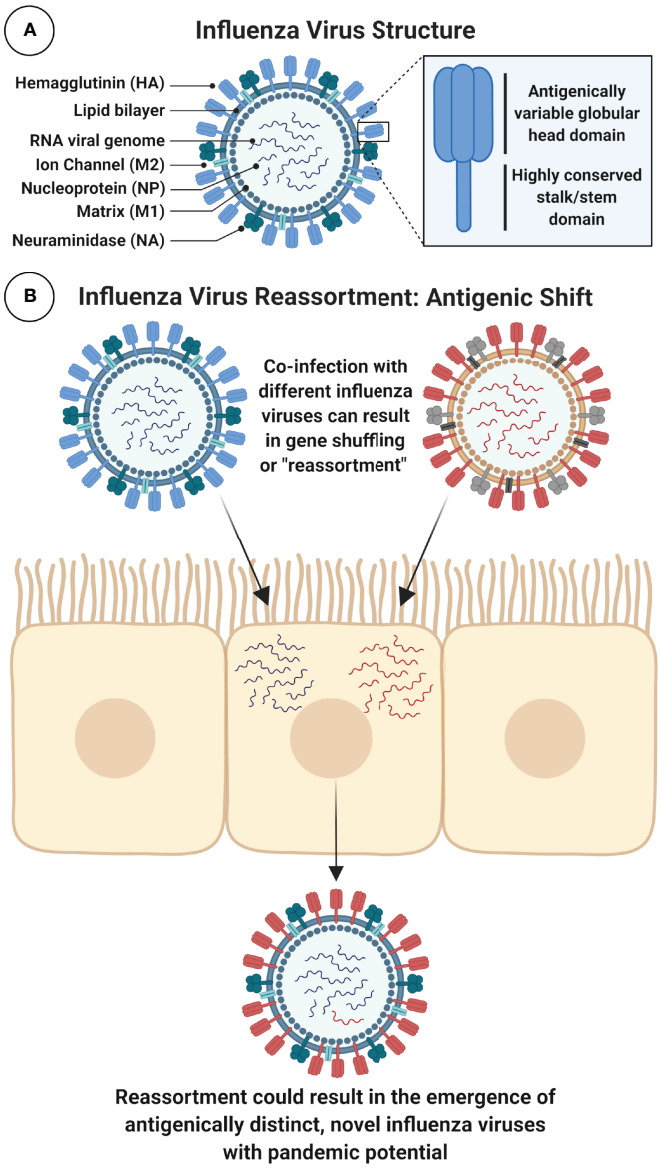

FIGURE 2 | Schematic Diagram of IAV Structure and Reassortment. **(A)** Figure shows a schematic cross-section of the influenza virus virion with main components labeled. Surface glycoproteins, trimeric hemagglutinin (HA) and tetrameric neuraminidase (NA), play important role in viral entry and egress and are major targets for immune responses following infection or immunization. In particular, the highly conserved stalk domain of HA is a target for universal influenza virus vaccines. *Note:* HA stalk and NA stalk are not shown as trimeric or tetrameric structures. Internal, highly conserved antigens matrix protein-1 (M1) and nucleoprotein (NP) are targets for cytotoxic T lymphocytes (CTLs). *Note:* icons for NP, which coats the viral RNA, and the viral ribonucleoproteins (vRNPs) which contain viral RNA, NP and polymerase are not shown. **(B)** Influenza A viruses (IAVs) can evolve to generate viruses with pandemic potential by antigenic shift, using a process of genome reassortment. Co-infection of susceptible cells with more than one distinct IAV can result in the selection of progeny with shuffled gene segments and potentially a new HA or NA, against which humans have no prior immunity. Figure created with ©BioRender - Biorender.com.

due to incompatibility between reassorted segments and virus-associated packaging constraints (9), or as a result of species-specific host restrictions which can negatively impact on multiple stages in the virus life cycle (10). For example, a crucial human host protein ANP32A, can confer species-specific restrictions on the avian influenza virus polymerase, limiting the ability of avian viruses to replicate efficiently in human cells (10, 11).

Host receptor tropism determinants can also restrict the occurrence of reassortment. IAV HA proteins bind to host cell sialic acid (SA) receptors, predominantly using SAs attached to galactose with α2,3 linkage (SA α2,3-Gal) or α2,6 linkage (SA α2,6-Gal) (12). Human and classical swine IAVs preferentially bind to α2,6 linked SAs, while avian IAVs preferentially bind to α2,3 linked SAs. SA receptors are mostly found on epithelial cells, with α2,3 linked SAs found in the intestines and respiratory

tract of birds (and the lower respiratory tract of humans) (13), while α2,6 linked SAs are mostly found in the respiratory tract of humans and pigs (2, 12, 14–16). Intermediary hosts, such as pigs, play a role in the adaptation of IAVs by acting as a "mixing vessel" and facilitating reassortment, as expression of both α2,3 and α2,6 linked SAs enables them to be simultaneously infected by both human and avian influenza viruses (17) (**Figure 2**). In addition, swine ANP32A has been shown to support replication with the avian virus polymerase (18), further supporting the role for pigs as "mixing vessels" for the emergence of reassortant viruses with pandemic potential (18). The process of antigenic drift can also contribute to the adaptation of avian influenza virus HAs, by facilitating a switch in preference for α2,3 linked SAs to α2,6 linked SAs, or in the viral polymerase (mutation PB2-E627K) (19, 20). If these modifications retained stability and compatibility with other IAV proteins, there is concern that this could facilitate sustained human-to-human spread of avian influenza viruses (21, 22).

AVIAN INFLUENZA VIRUSES

Avian influenza viruses are divided into two main categories on the basis of their pathogenicity in chickens. Highly pathogenic avian influenza (HPAI) viruses cause high mortality in poultry due to their capacity for disseminated, systemic infection (2). This pathogenicity is attributed to the presence of a multi-basic cleavage site within the IAV HA protein. The precursor HA protein, HA0, is cleaved into the HA1 and HA2 subunit, the latter of which is required for membrane fusion and viral entry. HA0 cleavage is normally mediated by trypsin-like proteases for HA0 from human IAVs and low-pathogenicity avian influenza (LPAI) viruses. Trypsin-like proteases are anatomically restricted to the respiratory tract in humans, and the gastrointestinal tract in birds. As such, viral replication following infection with human IAV and LPAI viruses is largely localized to these organs (2). In contrast, the polybasic cleavage site in HPAI viruses, restricted to H5 and H7 subtypes, can be cleaved by proteases which are ubiquitously expressed, facilitating disseminated, extra-pulmonary replication and consequently, severe disease. Although infrequent, sporadic instances of direct bird-to-human transmission of HPAI have occurred. The first report of such a spillover event was recorded in Hong Kong in 1997 following an outbreak of HPAI H5N1 (23, 24). Since 2003, H5N1 viruses have caused a total of 861 laboratory-confirmed cases and 454 deaths. The first report of human infection with HPAI H7N7 was in 2003 in the Netherlands, resulting in 89 confirmed infections and one death (25, 26). In 2013, H7N9 emerged in China and to date has resulted in 1568 laboratory-confirmed cases and 615 deaths (27).

Ducks are mostly migratory birds with α2,3 linked SAs on their intestinal epithelium (12). Studies have shown that several duck species can be infected with, and spread IAVs while showing no clinical signs (28). However, strains of IAV that ducks carry can be highly pathogenic to land fowl, including chickens. The migratory nature of certain water fowl, coupled with the absence of symptoms while carrying IAVs, has been implicated as a major, and unavoidable facilitator for the global spread of IAVs (29). While human contact with wild birds is uncommon, poultry are routinely farmed and present at live animal markets in many countries, which provide an opportunity for human and avian IAVs to co-infect and reassort (**Figure 2**). When farmed poultry are infected by HPAI, containment measures include mass culling, which can have a substantial financial impact. For example, the 2014/2015 H5N2 outbreak in the USA resulted in the death or culling of over 50 million poultry, and was estimated to have a negative economic impact of over $3 billion (30). In low income countries, financial implications can drive smallholder poultry farmers to respond to poultry deaths by rapidly selling stocks, often at markets, which does not help with containment of emerging viruses (31). Despite the obvious benefits of vaccinating poultry in terms of biosecurity, routine vaccination of poultry has cost implications, which means that flock depopulation is a more cost-effective control strategy in many countries (32, 33). It is clear that pandemic preparedness strategies including one-health vaccine development, global surveillance, and data sharing will be crucial in limiting the spread of emerging avian influenza viruses.

OVERVIEW OF ADAPTIVE HUMORAL AND CELLULAR IMMUNE RESPONSES AGAINST INFLUENZA VIRUS

Ideally, vaccines designed to protect humans against avian influenza viruses should be rapidly customizable and scalable, and should elicit broad, protective immune responses following a single shot. An overview of the types of immune responses which are important in protection from influenza virus infection and disease, and how those responses are measured, are provided below.

HUMORAL IMMUNITY

Several methods exist for measuring humoral immunity to influenza virus Ags. ELISA assays are a straightforward and quantitative assay to determine the breadth of, or concentration of antibody (Ab) binding to a range of viral proteins. In addition, they can be used for epitope mapping, which may aid in the identification of new vaccine targets. Adapted ELISAs which measure Ag-specific Ab isotypes or IgG subclasses can also be informative in evaluating the phenotype of response following immunization with different vaccine platforms: including mucosal Abs, or Ab subclasses which have a preference for engaging Fc-mediated effector functions. However, ELISA assays only measure binding-specificity but do not confirm whether Abs are functional and capable of preventing infection.

Protective and/or NAbs which bind to HA can block viral entry through a range of mechanisms (**Figure 3**). The HA protein is composed of the globular head, and the stalk/stem domain (**Figure 2A**). HA is responsible for entry, facilitated by binding to SA on the cell surface followed by membrane fusion: a process which is mediated following a drop in pH in the endosome, triggering conformational changes in the HA to expose a fusion peptide which fuses the viral envelope with the endosomal membrane. The receptor binding site (RBS) is located in the HA head. Abs recognizing the HA head can confer sterilizing protection by blocking viral entry and thereby preventing infection. NAbs can also recognize the HA stalk domain and can prevent viral entry or egress. In recent years, an important role for stalk-specific, *non-neutralizing* Abs which are broadly cross-reactive has been identified. Many of the latter Abs are non-neutralizing *in vitro* using classical microneutralization (MN) assays. However, it is important to emphasize that this class of Abs are protective

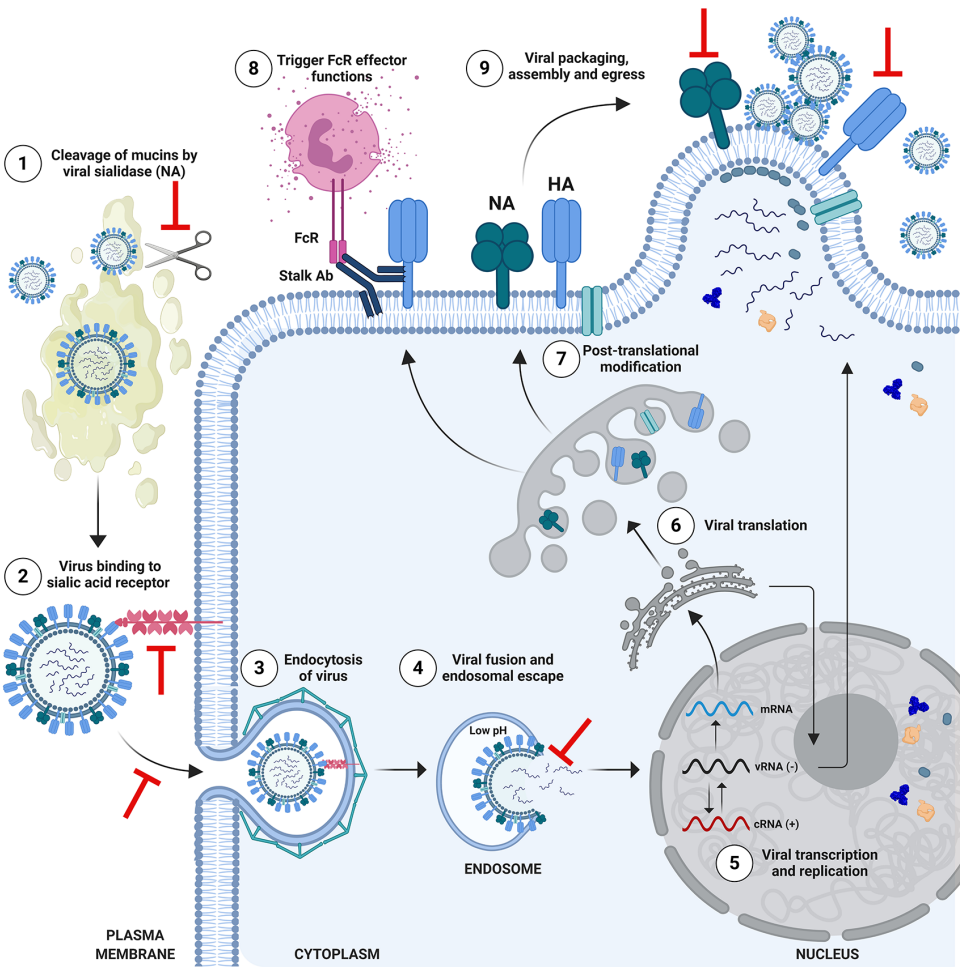

FIGURE 3 | Schematic of Influenza Virus Life Cycle and Targets for Protective Antibodies. The life cycle of influenza viruses has several major steps in which inhibition by neutralizing or protective antibodies can occur. (1) Viral entry in the respiratory tract is facilitated by the enzymatic activity of the viral neuraminidase (NA), which cleaves mucins to allow access to respiratory cells. Anti-NA antibodies, or anti-hemagglutinin (HA) antibodies which block the enzymatic function of NA could potentially inhibit this process. (2) Viral entry is mediated by binding of the head of HA to sialic acid receptors on the surface of cells, followed by endosomal escape by fusion of the viral and endosomal membrane. Antibodies which bind to the HA head domain can block this interaction and can confer sterilizing protection from infection. (3) Alternatively, neutralizing antibodies against HA can block the post-binding internalization of influenza virus, or (4) its' ability to fuse and escape from the endosome. (5) Viral ribonucleoproteins (vRNPs) are imported into the nucleus for viral transcription and replication. (6) mRNAs exported to the cytoplasm for translation. (7) HA and NA are trafficked to the Golgi for post-translational modification and subsequent presentation on the cell surface. Selected proteins return to the nucleus to participate in viral replication. Progeny vRNPs are exported out of the nucleus towards the plasma membrane for subsequent assembly and virion formation. (8) Anti-HA stalk antibodies can recognize HA on the surface of infected cells and engage Fc-mediated effector functions such as antibody-dependent cellular cytotoxicity, targeting the infected cell for degradation. (9) Viral packaging, assembly and egress takes place at the plasma membrane. This process can also be a target for anti-HA or anti-NA antibodies, which block egress. Anti-NA antibodies can do this by preventing new virions from being released from the surface of infected cells, or by the absence of NA activity causing new virions to aggregate. Figure is adapted from Krammer, 2019 (4). *Note:* icons are not to scale. HA stalk is trimeric (*not shown*) and NA stalk is tetrameric (*not shown*). Figure created with ©BioRender - Biorender.com.

in vivo and function by engaging Fc-mediated effector functions *in vivo*, such as Ab-dependent cellular cytotoxicity (ADCC) (34, 35) or Ab-dependent cellular phagocytosis (ADCP) (36).

Hemagglutination Inhibition Assay

The interaction between the HA head and SA receptors is the basis for the hemagglutinin assay (HA), or the hemagglutinin inhibition assay (HAI), which measures Abs that block this interaction. Influenza virus binding to red blood cells (RBCs) causes them to agglutinate and form a lattice. In the HAI assay, sera are first treated with receptor destroying enzyme (RDE) to remove non-specific inhibitors and serial dilutions of sera are pre-incubated with a known quantity of influenza virus, and standard amounts of RBCs subsequently added to the wells. Following an incubation period, the wells are read and the HAI titer identified as the last serum dilution where agglutination was inhibited, as observed by a dense pellet of RBCs in the well. A serum HAI^+ titer of 1:40 is considered to be a correlate of protection in humans on the basis of a 50% reduction in risk for influenza virus infection (37).

Microneutralization Assay

MN assays can be carried out as a multi-cycle replication assay (i.e., to measure inhibition of entry and/or egress following addition of Ab prior to, or after viral infection), or as a single-cycle replication assay (to measure inhibition of entry only). The multi-cycle MN assay is performed using a serial dilution of RDE treated sera with TPCK-trypsin, adding a set quantity of virus and pre-incubating before addition of the suspension to cells. Cell supernatants are collected and a HA assay is performed. Alternatively, the single-cycle MN assay is performed in the absence of trypsin and can identify Abs which prevent entry, using an immunostaining-based method as the readout, detecting viral nucleoprotein or HA expression.

Abs With Fc-Mediated Effector Functions

Several *in vitro* assays exist to measure Fc-mediated effector functions. Mononuclear leukocytes and polymorphonuclear leukocytes can both participate in ADCC, and can be evaluated *via* chromium-release, lactate dehydrogenase-release (38) and esterase-release assays (39), flow cytometry based viability (40) and perforin deposition assays (41). More recently, reporter assays have been developed to measure specific Fcγ-receptor (FcγR) activation. To evaluate the Fc-effector potential of broadly cross-reactive stalk Abs, cell lines expressing different HAs are used. Activation requires a two-contact interaction involving engagement of the Fc portion of the stalk-binding Ab with FcγR on the effector cell, in addition to binding of the HA head to SA on the cell surface (42–45). The ADCC reporter assay has been validated for both human serum and monoclonal Abs (mAbs) against measurement of CD107a cytotoxic degranulation marker on primary NK cells using a FACS-based readout (46). ADCP is typically measured using a cell-based assay, where Abs/serum are pre-mixed with virus to form immune complexes, followed by addition of phagocytes and quantification of internalized virus, which can be achieved *via* RT-PCR, or ELISA-based and fluorescence-based methodologies (36, 47).

CELLULAR IMMUNITY

Although T cells cannot confer sterilizing immunity, there is evidence in animal models (48, 49) and humans that they can contribute to limiting disease severity (50), reducing symptomatic infection and viral shedding (51–53). T cells have also been shown to correlate with the NAb response to influenza, with $CD4^+$ T cells potentially augmenting the NAb response (54). Considering their capacity for heterosubtypic reactivity, T cells may be the first line of defense and could have an impact at a population level (55) in mitigating the severity of early waves in an emerging avian influenza virus pandemic.

However, widespread implementation of T cell assays is limited by the fact that assays are complicated, require more extensive training, and expensive reagents and equipment. Antigen-presenting cells (APCs) sample exogenous viral Ags or debris from dead/dying cells, and can present epitopes to $CD4^+$ T helper cells *via* major histocompatibility complex (MHC) class II. Such helper cells differentiate into different T helper subsets depending on secondary signals (49). Ag-specific $CD8^+$ CTLs elicited by prior infection or immunization can recognize influenza-infected cells following presentation of viral peptides on the surface of cells *via* MHC class I (56). Following recognition, these infected cells are subsequently targeted for destruction, limiting viral replication and spread. APCs can also cross-present exogenous influenza Ag to $CD8^+$ T cells (57, 58). Popular methods for quantifying Ag-specific T cells are the Enzyme Linked ImmunoSpot (ELISpot) assay and a range of flow cytometry techniques to enumerate and phenotype the T cell response through fluorescently-tagged Ab staining.

ELISpot Assay

PBMCs can be pulsed with Ag (overlapping influenza peptides, protein or whole virus) to stimulate an existing T cell response to that Ag. The ELISpot is a sandwich ELISA using a capture Ab which binds molecules of interest (eg. cytokines) secreted from T cells undergoing stimulation, followed by use of biotinylated secondary Ab, enzyme-conjugated streptavidin and a development substrate. The readout is based on the formation of visible spots at the location of each responding Ag-specific T cell. Unlike flow cytometry-based T cell assays, ELISpot does not determine whether the responding T cell type is $CD4^+$ or $CD8^+$, however assay sensitivity is substantially higher (59). ELISpot assays can be modified to measure antigen-specific or total immunoglobulin from B cells, or adapted to use a fluorescent readout, termed a Fluorospot assay, which can detect multiple secreted molecules (60).

Intracellular Cytokine Staining (ICS) Assay

PBMCs can be stimulated with Ag followed by surface and intracellular staining, to enable the identification of $CD4^+$ and $CD8^+$ T cells and the cytokines or effector molecules they express (61, 62). Unlike the ELISpot assay that captures secreted effector molecules (eg. IFN-γ), this assay chemically inhibits protein secretion from the Golgi complex, resulting in the intracellular

accumulation of upregulated molecules during stimulation. Unlike ELISpot assays, flow cytometry uses a multi-parameter staining readout on a cell-by-cell basis, permitting the analysis of individual cell responses and polyfunctionality.

MHC Class I/II Multimer Staining

Ex vivo MHC multimer staining is a technique by which known epitopes to $CD4^+$ or $CD8^+$ T cells are targeted through use of specific peptides complexed with MHC (pMHC), and bound in multimeric (ie. tetrameric, pentameric) formation to a fluorescent tag. These multimeric complexes bind the corresponding T cell receptor of cells that recognize the influenza peptide in the pMHC, permitting identification of Ag-specific $CD8^+$ T cells using pMHC class I (63), or $CD4^+$ T cells using pMHC class II (64). This can be combined with surface staining (e.g., memory markers, CD45RA and CCR7) (65, 66) to provide more comprehensive phenotyping of the Ag-specific cells, without the need to detect a response *via* direct Ag stimulation.

BEYOND TRADITIONAL CORRELATES OF PROTECTION

As innovative universal influenza vaccine platforms and approaches are developed, the field needs to move beyond traditional assays to measure correlates of protection. For example, the HAI assay cannot quantify broadly-reactive Abs recognizing the HA stalk. Unlike IIV-based vaccines, many alternative vaccine platforms elicit robust cellular immune responses (67–70). Substantial differences in how assays to measure cellular immune responses are performed makes direct comparisons between pre-clinical and clinical studies challenging (71). This extends to differences in the specific Ags being evaluated, as well as differences in the cell number, stimulating peptide concentration used, all factors which can affect the results. Furthermore, the identification of a particular phenotype of cellular immune response does not confirm a role in protection. Therefore, much information remains to be learned from well-designed longitudinal cohort studies of natural infection, and human challenge studies (72). The licensure of new vaccine candidates will be dependent on the implementation and standardization of a broader range of assays to identify and measure correlates of protection (72).

APPROACHES FOR AVIAN INFLUENZA VIRUS VACCINES

HPAI viruses represent an ongoing pandemic threat, and unfortunately, it is difficult to predict which subtype will spillover and cause the next epidemic or pandemic. As a result, there is significant interest in developing vaccines which provide broad protection from a range of emerging IAVs. Conventional vaccine platforms used to protect against influenza virus, such as IIV or LAIV, predominantly rely on production in embryonated chicken eggs. However, many novel vaccine candidates are under development which do not rely on egg-based production (**Figure 4**).

IIV

Inactivated vaccines can comprise of several formulations including whole inactivated virus vaccines (WIV), split-virion or sub-unit vaccines, each with their advantages and disadvantages. WIV vaccines are generally chemically inactivated and are robustly immunogenic, thought to be due to their crude preparation and subsequent stimulation of innate immune signaling pathways by residual viral RNA (73). Depending on the method of inactivation used, WIV vaccines can retain the structural integrity or functional activity of the HA and NA, the two major targets for NAbs (4). Furthermore, as WIV vaccines retain internal Ags, these may also facilitate boosting of cross-reactive T cell responses to conserved viral proteins such as nucleoprotein (NP). However, WIV vaccines have fallen out of use in recent years due to their increased reactogenicity relative to more highly-purified formulations. Split-virion or sub-unit vaccines represent WIV vaccines which have undergone additional treatment with detergents to further purify virions into membrane components bearing both HA and NA (split-virion), or almost purely HA-based immunogens (sub-unit) (4). As a result of manufacturing processes which enrich for HA content, immune responses to the latter vaccines are almost exclusively skewed towards HA (74). Unfortunately, the use of IIV-based vaccines for avian influenza viruses with pandemic potential has been hampered by poor or variable immunogenicity requiring high Ag doses (75), multiple immunizations (75, 76) or the inclusion of adjuvants to achieve levels of Abs which would be considered protective (76–79).

LAIV

LAIV platforms are cold-adapted and are designed to be administered to the upper respiratory tract (URT) *via* intranasal (*i.n.*) immunization. Cold-adaptation allows the LAIV to undergo limited replication in the cooler environment of the URT, but does not facilitate dissemination to the lung. The aim of this vaccine is to stimulate a multi-faceted response, with mucosal immunity in addition to priming/boosting of cellular immunity (80–82). IIV and LAIV vaccine formulations are similar in that they aim to stimulate protective Abs directed towards HA, and to a lesser extent, NA. Although there is added potential to elicit cross-reactive immunity with LAIV as compared with IIV (82), there are safety concerns regarding the use of LAIV vaccines with avian HAs, as it could be argued that immunization might facilitate reassortment if the recipient became simultaneously infected with a circulating seasonal IAV (**Figure 2**). In addition, LAIV vaccines are not suitable for use in all populations (i.e., pregnant women, immunocompromised individuals).

However, sub-optimal immunogenicity and safety concerns are not the only challenges: employing conventional IIV/LAIV platforms in the development of avian influenza vaccines also

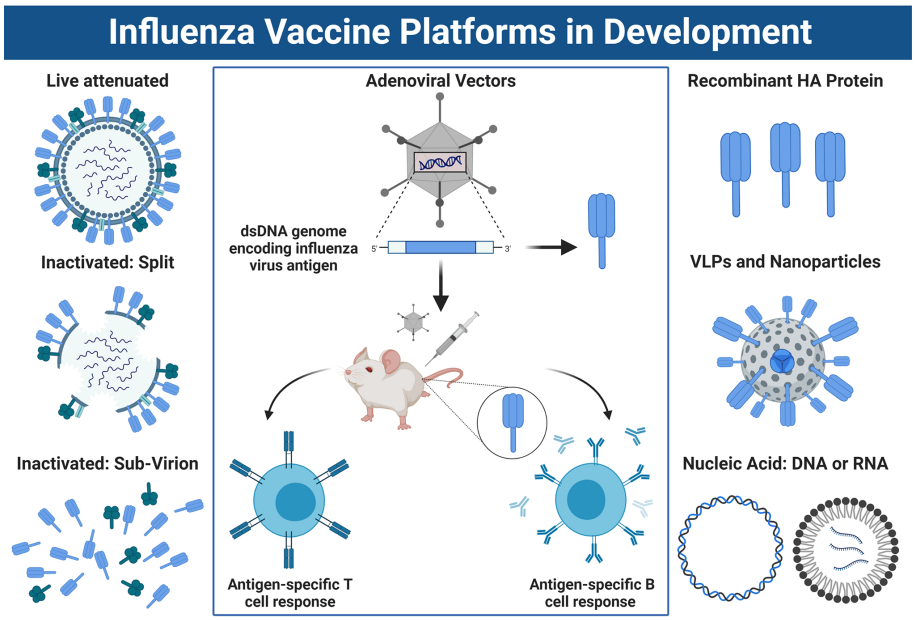

FIGURE 4 | Approaches for Influenza Vaccine Development. *Left panel*: A schematic overview of conventional influenza virus vaccine platforms, including the live attenuated vaccine (LAIV), the split virion inactivated influenza vaccine (IIV) or IIV sub-virion vaccine, which has HA>NA content. *Right panel:* Newer vaccines being developed include recombinant HA protein, virus-like-particles or nucleic acid-based vaccines such as DNA or mRNA platforms. *Center panel:* Schematic overview of how non-replicating adenoviral (Ad) vectored vaccines work. DNA sequence encoding an influenza virus antigen is inserted into the dsDNA genome of the Ad vector under the control of a powerful promoter to drive expression. Once immunized, the DNA sequence coding for the influenza antigen is transcribed into mRNA and translated into protein which is expressed inside the host cells at the site of injection and/or within draining lymph nodes. This results in a robust $CD8^+$ T cell response, as well as humoral immune responses directed towards the encoded transgene antigen. *Note:* the trimeric stalk of HA, or tetrameric stalk of NA are not shown in the diagram and icons are not to scale. Figure created with ©BioRender - Biorender.com.

presents several unique manufacturing hurdles. A major issue is the long manufacturing time, which is not conducive to rapid responsiveness in an emerging pandemic scenario. Strain selection usually takes place 7–8 months prior to influenza season (83), and production can be achieved within 5–6 months in the best-case scenario: when suitable seed stocks are identified and recommended by WHO on time, and when these viruses grow to sufficient titers. However, under unexpected circumstances the process can become protracted, with production ranging from 6–8 months (2, 84–86). Another issue is the over-reliance on production in embryonated eggs and the potential for significant reductions in supply should a HPAI epidemic result in decimation of poultry, and subsequently eggs needed for manufacturing. In parallel with this is the fact that HPAI viruses and derived vaccine seed stocks can be embryo-lethal, leading to challenges in propagating viruses in eggs to make vaccine stocks. Additionally, handling HPAI viruses intended for vaccine development in enhanced BSL-3 biocontainment facilities requires specialized staff and procedures which increases costs. To overcome this, non-pathogenic surrogate avian viruses can be used, or HPAI viruses can be genetically modified using reverse genetics (2). However, production would benefit from alternative platforms which are safe, easily adaptable, elicit robust and broad protective immunity and possess manufacturing characteristics which are compatible with stockpiling and pandemic preparedness.

Newer vaccines to the market, such as recombinant HA (rHA) produced in insect cells (i.e., Flublok®) or IIV vaccines grown in mammalian cells (i.e., Flucelvax®) could overcome the dependency on egg-based manufacturing and the protracted manufacturing process. Recombinant protein-based vaccines could certainly be scaled up more rapidly in response to an emerging pandemic. However, in the context of avian influenza vaccines, both rHA and IIV-based platforms may still be affected by inherently poor immunogenicity, in addition to the fact that these particular vaccines are limited in their ability to stimulate robust cellular immunity. Therefore, when designing vaccines to protect against HPAI viruses, we should consider platforms which can elicit immune responses with increased breadth, or which simulate both arms of the adaptive immune response to several antigen targets simultaneously, rather than over rely on HA as the sole target.

NOVEL VACCINATION STRATEGIES TO INCREASE INFLUENZA VACCINE BREADTH

The development of a universal influenza virus vaccine has become a significant research priority in recent years. Several position papers have outlined major gaps in our knowledge and have highlighted the need to invest in innovative approaches to

achieve this (87–89). One strategy is to expand the repertoire of vaccine platforms under investigation, which might help to overcome the reliance on egg-based manufacturing, as well as increase vaccine breadth and durability. Alternatively, as different vaccine delivery vehicles elicit a differential phenotype of immunity, distinct platforms could be used as tools to better understand which components of the immune response are desirable for broad efficacy, and could help to identify new correlates of protection. Although beyond the scope of this review, diverse vaccine platforms including conserved peptides (90), bivalent peptide conjugate vaccines (i.e., NCT00851266), DNA (91, 92), mRNA (70, 93), nanoparticle (94, 95), or virus-like-particle (VLP) (96, 97) based vaccines are undergoing evaluation as universal influenza virus vaccines, which could also protect against emerging pandemic HPAIs. Many of these alternative platforms are attractive because they can facilitate the delivery of multiple conserved Ags/epitopes simultaneously, some have inherent immunostimulatory or innate "adjuvanting" qualities which could increase breadth, some platforms present Ag in novel conformations such as repetitive particulate formulations (i.e., nanoparticle or VLP), and others are amenable to rapid customization and pandemic responsive scale-up. In particular, viral vectored vaccines fulfil many of these criteria. Their ability to enter cells and deliver their nucleic acid genome allows them to trigger immunostimulatory pathways, which can create an environment which enables increases in immunological potency once the transgene is expressed (98).

NON-REPLICATING ADENOVIRAL VECTORED VACCINES

Non-replicating Ad vectored vaccines are an attractive platform for vaccine development (**Figure 4**). They have a stable dsDNA genome, they can be rendered replication-incompetent (non-replicating) by deletion of the E1 region which is essential for viral replication, they can tolerate the insertion of large heterologous transgene Ags and promoters driving their expression (up to 7.5 kbp), and a number of vectors are available for vectorization (98). More importantly, they have a strong track record of use in human clinical trials (99, 100) and are well-established to be safe and immunogenic when used as vaccines for major infectious diseases in young infants (101–103), healthy adults (67, 68, 104–107), older adults (67, 68) and even immunocompromised individuals (108). In recent months, their suitability for rapid, pandemic responsiveness has been exemplified by the fact that several Ad vaccine platforms (i.e., Ad5 (109, 110), Ad26 (110, 111), and ChAdOx1 (112, 113)) have advanced through pre-clinical studies in mice (114), hamsters (115), pigs (114), and non-human primates (NHP) (111, 112), and are now leading the way in clinical trials for the newly emerged coronavirus, SARS-CoV-2 (i.e., NCT04324606, NCT04313127, NCT04436276, NCT04436471, and NCT04437875) (109, 110, 113).

Depending on the particular Ad serotype selected as a vaccine, Ad vectors elicit potent cellular immunity (largely $CD8^+$) (116), in addition to humoral immunity directed towards the encoded transgene Ag. This is due in part to their ability to stimulate multiple innate immune signaling pathways upon viral entry (117–121), as well as their capacity for persistent transgene expression *in vivo* (98, 122). Adenoviruses are classified into species groups A–G, with Ad vectors derived from species groups C, D, and E exhibiting the highest immunological potency when used as vaccines (98, 116). The most commonly used "prototype" Ad vector is human adenovirus type-5 (HAdV-C5, *referred to as Ad5 throughout this review*), a potently immunogenic vaccine which unfortunately has high seroprevalence in humans, possibly limiting its potential for broad clinical applications (98). Issues associated with pre-existing immunity in humans has driven scientists to vectorize a range of alternative, rare serotype human Ad vectors, or Ad vectors derived from NHPs, great apes and other animal species. Novel Ad platforms which have been evaluated in pre-clinical models as vaccines against avian influenza virus include species C vectors HAdV-C5, HAdV-C6 (123), species D vectors HAdV-D26 (124), HAdV-D28 (124) and HAdV-D48 (124), species E human virus HAdV-E4 (125, 126), along with species E viruses isolated from chimpanzees ChAdV-7 (ChAd7) (127), ChAdV-68 (AdC68) (128), and ChAdOx1 (129–131). Other novel vaccines include the use of porcine vector PAdV-3 (132), or bovine Ad vector BAdV-3 (133). Aside from the contribution of the specific Ag selected for incorporation into an Ad vaccine to overall immunogenicity or efficacy (*discussed in more detail below*), the relative immunological potency of the chosen Ad vector platform can vary significantly. It is considered that a combination of factors contribute to the hierarchy of immunogenicity when comparatively evaluating distinct Ad vaccines (98). These include the persistence of transgene expression *in vivo*, and subsequently, the magnitude of the ensuing immune response (122, 134, 135), as well as the preferential induction of key innate immune signaling pathways —combined with the avoidance of Type I IFN stimulation at early time-points post-immunization (134, 135). Additional factors such as the cellular tropism or receptor usage of the selected Ad vector, the route of vaccine administration and dose, can also play a role in modulating the inherent immunogenicity of different Ad vaccines (98). These concepts are the subject of a comprehensive review article recently published by our group (98).

ANTIGEN TARGETS FOR AVIAN INFLUENZA VACCINE DEVELOPMENT

The high mutability of the HA, a result of antigenic drift, ensures that conventional vaccine platforms (i.e., IIV) elicit largely strain-specific humoral immunity. A vaccine based on this premise would provide little or no protection against antigenically diverse avian influenza viruses, particularly if we consider the unpredictable nature of cross-species transmission events by these zoonotic viruses. Therefore, it is difficult to rely on current licensed vaccine platforms for pandemic preparedness against HPAI. Ideally, we need novel vaccines which are capable of stimulating broad, heterosubtypic immunity against a wide range of avian influenza viruses, in addition to developing platforms which are amenable to rapid production and scale-up, or suitable for

stockpiling. One way to achieve increased breadth of protection from a vaccine is to select viral Ags which are highly conserved as targets. Such Ags usually play crucial functional or structural roles in viral replication or assembly, making them unable to tolerate significant mutations without compromising viral fitness. Several key targets which are currently under investigation for universal influenza virus vaccine design are discussed below.

HA

HA is the most abundant glycoprotein on the surface of the influenza virion. Although HA is subject to antigenic variation which can negatively impact on vaccine effectiveness, it does possess a highly conserved domain which is an ideal target for universal influenza virus vaccines. HA is composed of two main structural domains, the *antigenically variable* and *immunodominant* HA head domain, and the *highly conserved*, but *immunosubdominant* HA stalk/stem domain (**Figure 2A**). As previously stated, conventional vaccines elicit largely strain-specific humoral immune responses predominantly focused on the antigenically variable HA head domain. The *immunosubdominance*, or poor immunogenicity of the HA stalk in this context is well documented (i.e., head > stalk) (136). However, advances in innovative HA immunogen design in recent years has enabled re-focusing of humoral immune responses towards this *immunosubdominant* HA stalk domain. A major step forward in facilitating the induction of robust stalk-specific immune responses was the development of chimeric HA (cHA) immunogens (137–141), in which the head domain of an exotic IAV HA is grafted onto the stalk domain of a common human HA, the use of mosaic HAs (mHAs), in which the major antigenic sites in the HA head domain have been silenced (142, 143), or the design of structurally stabilized headless HA immunogens (93, 94, 144–148) (**Figure 5**). Alternatively, hyper-glycosylation of the HA head domain through the introduction of N-linked glycosylation sites, can also re-focus humoral immunity away from the head and towards the stalk

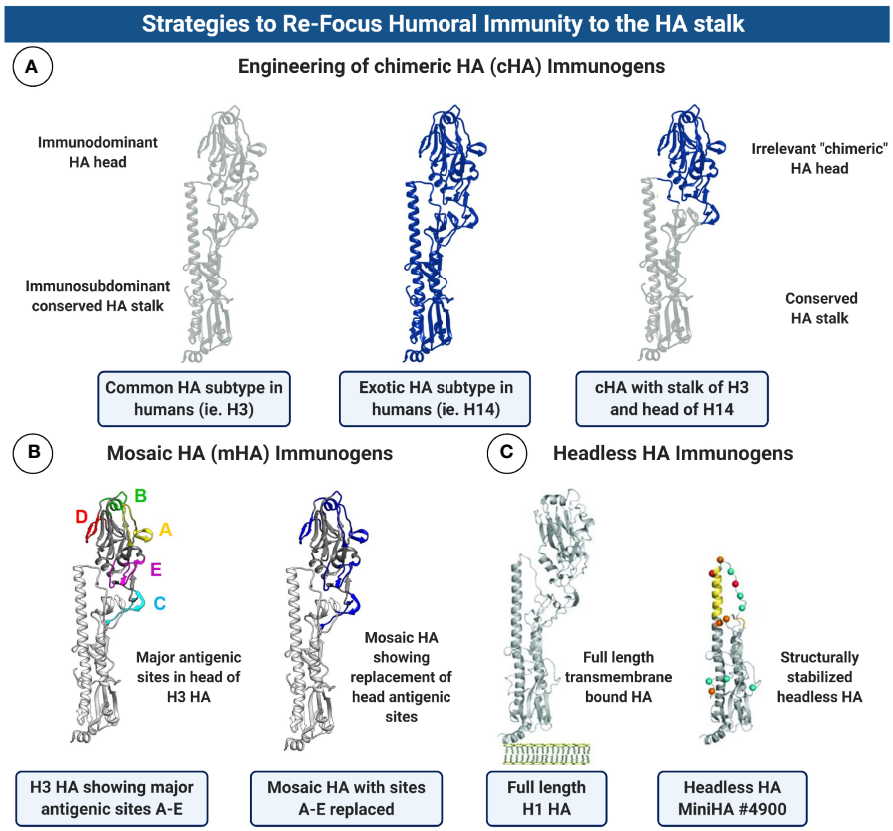

FIGURE 5 | Strategies to Re-focus Humoral Immunity to the HA stalk. **(A)** Schematic diagram showing the substitution of the HA head domain to make chimeric HA (cHA) immunogens. The concept behind this approach is to graft an exotic HA head, for which humans have no prior immunity, to the stalk of a HA subtype which is common in humans (ie. H1 or H3). Sequential immunization with cHA immunogens in which the exotic head is swapped with each boost can re-focus humoral immunity to the conserved HA stalk. *Note:* Structures are schematic and do not represent authentic junctions for substitution of the HA head region. **(B)** Mosaic HA (mHA) design is conceptually similar to cHAs but only the major antigenic sites in the HA head domain are swapped for comparable regions in an exotic HA. This can be used as an alternative approach to re-focus antibodies towards the HA stalk domain, with the added benefit of retaining possible conserved epitopes in the HA head. mHA structures kindly provided by Dr. Felix Broecker and Prof. Peter Palese, ISMMS. **(C)** Structurally stabilized headless HAs have been engineered which completely lack the immunodominant HA head domain, allowing boosting of immune responses towards the stalk only. HA structures in **(C)** are reproduced with permission from Impagliazzo et al. (147). Reprinted with permission from AAAS (License 4907650635299). Figure created with ©BioRender - Biorender.com.

(149, 150). Although disassociating the HA head from the HA stalk domain, through the use of headless HA-based immunogens, appears to improve the inherent immunogenicity of the stalk (151), sequential immunization approaches are still required to achieve broad protection. In addition, differences in the immunogenicity of the HA stalk as an immunogen when presented in different formulations exist. For example, use of recombinant protein-based HA stalk in a single shot is poorly immunogenic, although this can vary depending on the specific stalk construct used, its associated stability and structural integrity. The immunogenicity of the HA stalk can be improved by use of adjuvants, or by modification of HA stalk constructs through covalent coupling to immunogenic carrier proteins (152) or nanoparticles (94). Although the immunosubdominance of the HA stalk can likely be overcome with the right immunogen and vaccination regimen, the immunological factors which contribute to its subdominance are intriguing. Poor accessibility of stalk epitopes or steric hindrance imposed by the HA head (153), polyreactivity (154) and potentially counterselection of HA stalk Abs with low affinity for B cell receptors (155) and the paucity of MHC II epitopes in the stalk relative to the head which could affect Tfh responses (152), are all mechanisms which have been proposed as underlying factors. Regardless, in support of its potential as a universal vaccine target, numerous studies have demonstrated that sequential immunization with stalk-focused immunogens can confer heterosubtypic protection from lethal challenge in animals (94, 139, 140, 145, 147, 156). For example, sequential immunization with a headless or cHA immunogen with the H1 stalk, can confer protection against a distinct IAV subtype from the same phylogenetic group (i.e., G1 avian influenza H5N1). Importantly, cHA based vaccines have recently been evaluated in clinical trials and have shown that they can effectively boost stalk-reactive Abs capable of recognizing distinct G1 HAs including H1, H2, H9 and H18 in humans (157). In addition, headless HA vaccine candidates are also undergoing clinical evaluation (i.e., NCT03814720).

A major advance in the field was the discovery that broadly cross-reactive HA-stalk Abs can protect using a range of mechanisms which are independent of HAI activity (**Figure 3**). Anti-stalk Abs can be neutralizing, inhibiting the fusion activity of HA by preventing its structural rearrangement and exposure of the fusion peptide, thereby blocking viral entry. HA stalk Abs can also interfere with the enzymatic activity of the viral NA by steric hindrance, blocking viral egress (158). However, more recently we have begun to understand the contribution of *non-neutralizing*, but broadly cross-reactive stalk Abs in mediating protection *in vivo* (72). As the latter class of protective Ab does not always neutralize *in vitro* when using classical assays, such as HAI or MN, their importance was long under-appreciated. We now know that a large number of stalk-reactive Abs can protect *in vivo* by engaging FcγRs (159), and triggering Fc-mediated effector functions such as ADCC (34, 35) or ADCP (36). This is a very important consideration in the evaluation of novel vaccines designed to elicit heterosubtypic immunity, as the current correlates of protection for the licensure of influenza virus vaccines are based on the HAI assay, and stalk-reactive Abs with substantial breadth are HAI$^-$. To date, vaccines designed to elicit HA-stalk mediated protection have employed conventional IIV, LAIV or recombinant protein-based platforms, with or without adjuvants (138–140, 156). However, alternative approaches have used nanoparticles or VLPs bearing headless HA. Importantly, all of the innovative HA designs described above (i.e., headless, cHA or mHA expression cassettes) are well-suited for genetic incorporation into non-replicating Ad vaccines (**Figures 4**, **5**).

Some concerns regarding the use of HA stalk as a vaccine target are based on selected studies which have implicated stalk Ab responses in leading to vaccine-associated enhanced respiratory disease (VAERD). A study by Khurana and colleagues reported that pigs immunized with adjuvanted whole-inactivated influenza (WIV) vaccine based on H1, developed enhanced disease following viral challenge with an antigenically mismatched H1N1 virus. The authors attributed this to non-neutralizing stalk Abs which promoted virus membrane fusion activity (160). However, subsequent studies compared the adjuvant used in the latter study head-to-head with WIV immunization using different adjuvants and did not observe VAERD, suggesting that the immune-enhancement effects in the Khurana study were associated with that particular choice of adjuvant (161). Importantly, Braucher and colleagues compared adjuvanted-WIV with an Ad5-based vaccine encoding HA in pigs and showed that unlike WIV, the Ad vector did not induce VAERD, and elicited superior protection against heterologous challenge viruses (162). Antibody-mediated immune enhancement upon challenge with H3N2 viruses in mice has also previously been reported for mAbs which bind to the HA head, or base of the HA head (163). The mechanism was proposed to be destabilization of the HA stalk, resulting in increased viral fusion kinetics. Although experimental studies with epitope-specific mAbs are useful in better understanding mechanisms of protection or disease enhancement, information which will guide next-generation vaccine design, the physiological response to immunization results in a pool of Abs which recognize multiple epitopes, and/or multiple viral antigens present in the vaccine formulation. It is also important to note that stalk Abs are prevalent in humans, boosting of stalk Abs can occur in humans following immunization (164, 165) or natural infection (166, 167), and stalk Abs have recently been identified as a correlate of protection in a household cohort study of natural influenza virus infection (167).

NA

In addition to HA, the other major surface glycoprotein is the viral sialidase, NA. NA is responsible for cleaving SA from the surface of host cells and plays a role in viral entry (by facilitating movement through mucus in the respiratory tract) (168, 169), in allowing the release of budding virions from the surface of infected cells, as well as preventing the aggregation of released viruses (169–171) (**Figure 3**). In recent years NA has gained interest as a new universal vaccine target (171, 172). Although

Abs to NA do not provide sterilizing immunity, they can limit disease severity in animals (173–175), and have been shown to reduce viral release/shedding and symptomatic infection in human challenge studies (176–178). Unlike HA, breadth of reactivity to NA is usually across a particular subtype (i.e., N1) rather than between different NA subtypes (172). Unfortunately, seasonal vaccines do not contain standardized amounts of NA, its stability and abundance in these formulations is low relative to HA. In addition, issues related to antigenic competition between intravirionic HA (dominant) and NA (subdominant), when presented together on the same vaccine platform (i.e., IIV), can preclude the development of robust immunity to NA (179). Therefore, vaccines such as non-replicating Ad vectors, which are capable of driving high-level *in vivo* expression of NA under the control of exogenous promoters, might enable improved immune responses to NA (180). In addition, the *in vivo* expression of NA following immunization using a viral vectored vaccine could overcome issues related to the poor shelf-life stability of NA in current vaccine formulations.

NP

Influenza virus nucleoprotein (NP) is a structural protein which coats the viral RNA genome, forming the viral ribonucleoprotein complex (vRNP). Although NP has been implicated in mediating the switch from transcription to viral genome replication (181), recent data suggests that NP does not regulate this process (182). Nonetheless, NP is known to interact with other viral proteins, including components of the polymerase complex and M1 (183). With respect to its potential role in conferring heterosubtypic immunity, the high sequence conservation of NP (> 90%) (184) and its role in providing partial protection from influenza virus infection in mice (185), suggests it should be included in next-generation vaccines. In further support of this, NP-specific T cells have been correlated with limiting symptomatic infection and reducing shedding in humans during natural infection and influenza virus challenge studies (51–53), and NP-specific CTLs have been shown to cross-react with avian viruses (82, 186). Moreover, clinical studies have already shown that NP-specific T cell responses can be boosted in healthy adults and in the elderly when using Ad-based vaccines expressing NP as the transgene Ag (67, 68). Although CTL responses to NP cannot provide sterilizing immunity, the inclusion of NP in vaccines designed to protect against emerging avian viruses could help limit disease severity, or virus shedding and replication, both important considerations in mitigating the early impact of a pandemic.

In addition to eliciting T cell responses, Abs to NP have been reported to display ADCC activity (187, 188). Despite NP being an internal virus protein, it can be expressed on the surface of infected cells (189), providing an explanation for its role in triggering Fc-mediated effector functions. In addition, studies have described that human Abs to NP, elicited in response to seasonal influenza viruses, can cross-react with avian influenza strains from both phylogenetic groups, H5N1 and H7N9, and trigger ADCC (188). However, the contribution of NP Abs to heterosubtypic protection from influenza virus challenge in animals is not conclusive, with some reports of protection (190–192), and others describing minimal protection, depending on the challenge virus tested (93). This may be due in part to differences in the IgG subclass profile of the Ab response elicited by different vaccine platforms or in response to influenza virus infection. It is well-established that specific IgG subclasses have differential affinities for activating FcγRs, and as a result, this can have a different outcome on the induction of ADCC (193, 194). Therefore, NP may not be ideal as the primary Ag target but would be well-suited to vaccine platforms which can encode or express more than one Ag simultaneously.

M1

Matrix protein-1 is an internal Ag which forms a stabilized shell under the IAV envelope (**Figure 2**). M1 therefore plays an important structural role, engaging in interactions with the vRNP (195) and recruiting other viral components which facilitate virion assembly. In addition, M1 has been reported to interact with the cytoplasmic tails of both HA and NA (196, 197). Similar to NP, M1 exhibits a high degree of amino acid sequence identity (> 95%) amongst global IAV isolates (198), also making it an attractive target to elicit heterosubtypic cellular immune responses. Evidence in animal models suggests that M1 responses can confer a degree of protection (192). A dominant HLA-restricted epitope does exist in M1, and individuals with this high-population-frequency haplotype (HLA-A*02) have detectable M1-specific $CD8^+$ T cells (199, 200). Clinical trials using a heterologous prime:boost immunization regimen with a chimpanzee Ad vector and MVA expressing NP+M1 as a fusion Ag, demonstrated that although NP T cell responses were boosted significantly following vaccination, M1 boosting was minimal (68). However, in a previous study, volunteers who were HLA-A*02 (7/15) exhibited pronounced fold changes in T cell responses to this M1 epitope (67). Interestingly, modified vaccinia Ankara (MVA) vaccines expressing NP or M1 conferred protection from influenza virus challenge in HLA-A2 transgenic mice (201). However, a role for immune responses to M1 as a potential correlate of protection in humans is currently unclear.

M2

The M2 protein is an ion channel which is displayed on the virion surface in low abundance, although its density is increased on the surface of infected cells (202). It is involved in virus uncoating during entry, and in the formation of new virions and budding. The ectodomain of M2 (M2e) is highly conserved between all IAVs (203, 204), making it a good target for vaccines (205). Studies in animals first demonstrated that a monoclonal antibody against M2 could protect mice from challenge with influenza virus (206). Subsequent experiments using multimerized M2e fused to hepatitis B virus core (HBc) demonstrated significant protection in mice, which was Ab-mediated (204). In addition, other approaches have tested M2e presented on VLPs (207), or have supplemented conventional IIV-based vaccines with M2e presented on VLPs (208). It is considered that M2e induces protection in a manner similar to broadly reactive stalk-Abs, through engagement of Fc-mediated effector functions, with a crucial role for alveolar macrophages (36, 209). Some disadvantages of using M2e as a universal

immunogen include its low abundance on the virus, its small size and therefore restricted space for T cell epitopes, and the fact that despite its high sequence conservation, several phylogenetic lineages of M2e exist (203). Clinical trials based on M2e-fusion proteins with HBc formulations, such as ACAM FLU-A have been registered (NCT00819013 and NCT03789539) (204, 210). Despite reported safety and seroconversion for ACAM FLU-A, no further development of this vaccine has been reported. M2e-based vaccines have also advanced to clinical trials where the vaccine is formulated as a tandem immunogen linked to bacterial flagellin, a TLR5 ligand (NCT00603811) (211). Early studies using low doses of the latter immunogen (0.3 and 1.0 μg) were well-tolerated, and immunogenic, particularly following a second dose. However, higher doses (3.0 and 10.0 μg) of flagellin-M2e were associated with adverse events and reactogenicity. Recently, the use of a full length mutant M2 encoded within an mRNA-based vaccine, displayed promising efficacy against a range of influenza virus challenges, suggesting that alternative platforms for delivery of M2 may be a worthy pursuit (93).

PB

The viral polymerase complex of IAVs is composed of three subunits: PB1, PB2, and PA. Although not a major focus for universal influenza virus vaccine development, components of the polymerase, namely PB1, may be of interest for vaccine development, particularly from the standpoint of eliciting heterosubtypic CTLs. One study using non-replicating Ad vectors determined that PB1 was not as immunogenic as NP, but this could be overcome using a molecular adjuvanting approach in which PB1 is fused to the murine invariant chain (Ii), increasing its presentation (212). This genetic fusion strategy was originally designed to exploit the canonical role of Ii in MHC II presentation, with a view to augmenting Ag presentation to $CD4^+$ T cells, but unexpectedly led to increases in Ag-specific $CD8^+$ T cell responses (213, 214). However, despite improved T cell responses, fusion of PB1 to Ii did not translate into robust vaccine efficacy and protection from influenza virus challenge in mice. This is in agreement with another study which determined that PB1 was not effective as a sole Ag in providing protection from challenge (192). What is interesting is the recent implication that $CD8^+$ T cell responses to PB1 in humans exhibit unprecedented breadth (215). In particular, two conserved epitopes in PB1 were identified which are restricted by common HLA types, suggesting that the development of novel vaccines which are capable of boosting these responses could elicit broad cross-reactivity in a significant proportion of the human population (215). $CD8^+$ T cell responses to one of these epitopes cross-reacted against IAV, influenza B virus (IBV) and influenza C virus (ICV). Therefore, it may be that authentic evaluation of the contribution of PB1 to protection is complicated by differences between studies in mice and humans. However, as stated previously, it is also important to note that the presence of cross-reactive CTLs does not guarantee that they will contribute to increased viral clearance or protection. The phenotype, functional activity and protective capacity of PB1-specific T cells in humans remains to be investigated in more detail.

ADENOVIRAL VECTORS IN DEVELOPMENT AS VACCINES FOR AVIAN INFLUENZA

The unexpected pandemic caused by the introduction of SARS-CoV-2 in late 2019, highlighted the importance of accelerating the development of vaccine platforms which have the capacity for rapid scale-up, which already have a strong track-record for use in clinical trials and ideally, which would elicit broad protective immunity against antigenically distinct avian influenza viruses. The suitability of Ad vaccines for many aspects of this endeavor has ensured that they are now center-stage in this global effort, with Ad5, Ad26 and ChAdOx1 vaccines for SARS-CoV-2 already in clinical trials in humans (ie. NCT04324606, NCT04313127, NCT04436276, NCT04436471 and NCT04437875) (109, 110, 113).

Adenoviruses are dsDNA viruses which have three main structural proteins that play a role in their tropism and type-specificity: these include the fiber, which is involved in binding to receptors on the cell surface, the penton base which induces viral internalization and the hexon, the most abundant viral protein in the capsid (56, 98, 216). The flexibility of the Ad platform has facilitated its combination with many innovations in Ag design: such as the ability to encode multiple transgenes, computationally designed consensus Ags (125, 217) and the insertion of antigenic epitopes into the capsid of the Ad vector (218). We already outlined the main Ag targets for achieving broad immunity against influenza viruses. We will now summarize the status of Ad-based vaccines for avian influenza viruses from pre-clinical models, and vaccines which have already entered clinical trials in humans.

Pre-Clinical

Ad5 Vaccines

Several studies have reported the pre-clinical evaluation of Ad5-based vaccines against avian influenza viruses in mouse models (all studies described are in mice unless otherwise stated). In 2006, Hoelscher et al., described the successful construction of an Ad vaccine encoding H5 A/Hong Kong/156/197) which provided protection against antigenically distinct H5N1 influenza viruses (219). Hassan and colleagues described the construction of vaccines encoding full length sequences for H5 (H5N1: A/Vietnam/1203/2004), H7 (H7N7: A/Netherlands/219/2003) or H9 (H9N2: A/chicken/Hong Kong/G9/1997) (220). Interestingly, the authors also constructed a multi-epitope based vaccine (Ad-ME) encoding highly conserved domains, or regions from diverse viral proteins from H5N1: M2e, the fusion peptide of the HA stalk, an immunodominant T cell epitope in NP and the α-helix domain of the HA stalk (another highly conserved target on HA) (221). This vaccine elicited ELISA Ab responses to M2e and the HA fusion domain, but not to the α-helix. In addition, T cell responses to NP were detected by ELISpot. Challenge experiments were set up to test heterologous H5N2 virus, a distinct G1 challenge virus H9N2 and a G2 virus, H7N9. Although viral lung titers were reduced somewhat for

the Ad-ME vector upon challenge, these were still significantly higher than the matched HA control vaccines, suggesting that protection was only partial and the inclusion of structurally intact HA immunogens may be required to confer adequate Ab-mediated protection.

With this in mind, many investigators have designed Ad vaccines which express conformationally intact, full length HAs. An advance on this is computationally designed, centralized consensus HA sequences which have now also been applied in the development of Ad-based vaccines, with the aim of increasing the breadth of reactivity against diverse HAs (217). The concept behind this approach is that a computationally optimized broadly reactive antigen (COBRA) (222) sequence would represent the central node of a HA phylogenetic tree. In a study by Webby and Weaver, the authors engineered Ad5- and rare species Ad4 vectors encoding consensus H1 (H1-Con), H3 (H3-Con) and H5 (H5-Con) transgenes and tested them in a heterologous prime:boost regimen in mice (125). Vaccines encoding avian H5-Con induced HAI^+ Abs with titers ≥40 against two out of three H5 viruses tested. This prime:boost regimen conferred protection from mortality following challenge with divergent H5 viruses, but protection from morbidity (weight loss) was only observed following challenge with the H5N1 A/Vietnam/1203/04 virus, not two other strains.

In an effort to re-focus humoral immunity away from the HA head and towards the conserved HA stalk, Lin et al., designed Ad vaccines encoding H5 (A/Thailand/1(KAN-1)/2004) in which the immunodominant antigenic sites in the HA head were shielded from immune recognition by hyperglycosylation, *via* the introduction of specific glycosylation sites (223). Using a heterologous Ad prime:recombinant protein boost (both with hyperglycosylated H5), the authors demonstrated that these "masked" HAs elicited Abs with greater cross-clade HAI^+/NAb and anti-RBS ELISA Abs against diverse H5 viruses, as well as inducing Abs to the conserved HA stalk domain (223).

Another strategy to increase the breadth of protection is to target more than one Ag, or use co-administration or prime: boost vaccination regimens to elicit immunity towards multiple targets. In a study by Kim et al., the authors encoded H5 (A/Vietnam/1230/2004: H5N1), in addition to M2e as a potential pandemic vaccine candidate (224). Similar to using a wildtype PR8 infection, Ad5-H5/M2e protected mice from heterologous challenge with H5N2 virus, A/Aquaticbird/Korea/W81/2005. The vaccine elicited both HAI^+ and NAb responses against the H5N2 virus, but impressively, also induced high titer stalk Abs (ELISA) which cross-reacted with the H1 stalk of cHA, cH9/1. In addition, H1-stalk reactive Abs elicited following immunization with Ad5-H5/M2e were sustained for 12 months. Vaccines that elicit broadly reactive Abs which are also durable would be a desirable outcome for a pandemic vaccine. In a separate manuscript, the authors also demonstrated that this vaccine was superior to Ad vaccines expressing either Ag alone when administered *i.n.* (225). Again, the Ad5-H5/M2e vaccine elicited robust stalk-specific Abs and was capable of protecting from heterosubtypic H1N1 challenge following a single immunization.

Alternatively, the use of bivalent Ad vaccines encoding more than one avian HA, or combinations of Ads expressing different HAs, has been investigated as a strategy to induce protective immunity against multiple avian influenza viruses in mice (226). Vemula and colleagues engineered Ad5 vaccines encoding two H5 immunogens, or H7 and H9, and compared these head-to-head with matched monovalent Ad vaccines which expressed each HA individually, as well as NP derived from an avian H5 strain. The authors subsequently tested these vaccines in a multivalent formulation, whereby combinations of bivalent vaccines were evaluated (ie. Ad-H5+H5 with Ad-H7+H9). A monovalent vaccine expressing NP was also combined with these bivalent Ads on the basis that NP on its own can only confer partial protection, but could increase heterosubtypic protection from challenge with unrelated viruses. All vaccines elicited Ag-specific cellular (IFN-γ ELISpot and NP/HA pentamer detection of Ag-specific $CD8^+$ T cells) and humoral immune responses (HAI^+ NAb and ELISA binding Abs), with increased breadth of protection from challenge with distinct viruses observed for the bivalent or multivalent vaccine formulations. The authors also noted the presence of stalk Abs following Ad immunization with multi-subtype HAs (as measured by ELISA against stalk peptides, not intact protein) (226). They concluded that these Abs did not play a role in protection due to their inability to reduce viral lung titers. However, it is now well-established that stalk-reactive Abs can be non-neutralizing (36) and would therefore not always reduce early viral infection and replication the lung, but may contribute to viral clearance at later time-points through engagement of Fc-mediated effector functions.

Many published studies have focused on evaluating HA, NP or M2e as vaccine immunogens. However, PB1 has also been selected as an Ag. Despite its recent implications in broadly cross-reactive $CD8^+$ T cells in humans, its ability to confer protection in mouse models has been disappointing. Uddbäck and colleagues constructed an Ad5-based vaccine encoding PB1 and compared it to a similar vaccine encoding NP (212). The authors determined that PB1 was intrinsically less immunogenic than NP, and attempted to increase immune recognition of PB1 by combining it with an innovative genetic adjuvanting approach in which it was tethered to murine invariant chain (described in previous sections), resulting in increased frequencies of PB1-specific $CD8^+$ T cells, as detected by ICS flow cytometry. However, despite these high frequencies, the modified Ad-PB1 vaccine was less protective than Ad-NP in a H1N1 (A/Puerto Rico/8/34) challenge, due to reduced killing capacity by PB1-specific $CD8^+$ T cells. These data cast doubt on its potential as a broadly cross-protective immunogen, although future studies in humans may enable a more comprehensive understanding of the protective capacity of cross-reactive T cells which recognize PB1.

As outlined above, the concept of trying to increase the immune recognition and/or breadth of vaccine Ags encoded by Ad vectors by using molecular or genetic adjuvanting approaches (213, 227–229), has been applied to avian influenza viruses (230). Alternatively, vaccine administration using different routes of administration can elicit distinct immunogenicity profiles, with mucosal immunization being particularly attractive for strategies

aimed at protecting against pathogens with a tropism for mucosal sites (98). To overcome poor immunogenicity due to limited Ag recognition following oral delivery of Ad vaccines, Scallan and colleagues at Vaxart, Inc encoded H5 (A/Indo/05/2005), in addition to a dsRNA hairpin, which acts as a TLR3 stimulant to induce Type I IFNs and thereby adjuvant the immune response to H5 (vaccine known as ND1.1). They demonstrated that this approach did indeed improve humoral immune responses to H5 following oral immunization in mice, but the adjuvant did not offer further improvements following intramuscular (*i.m*) vaccination, which was already immunogenic (and far superior to oral administration of Ad-HA without the TLR3 adjuvant component). Protection from homologous challenge was confirmed in mice and in ferrets, although only partial survival (6/8) was observed in ferrets immunized with Ad-HA-dsRNA administered orally as compared with *i.m* immunization, which had 100% survival. It is well established that the route of vaccine administration can have an impact on the magnitude and phenotype of immune response. This is due to differences in the types of cells present at the immunization site, and subsequently the *in vivo* tropism of the Ad vector, along with numerous species-specific factors (98) which could have affected efficacy in the aforementioned ferret study. The ND1.1 vaccine candidate subsequently advanced into *Phase I* clinical trials (NCT01335347), and was reported to elicit some modest T cell responses to H5 (ELISpot), but no HAI active Abs were detected (231).

Rare Serotype Human Ad Vaccines

Although Ad5 is currently under evaluation in several clinical trials as a vaccine against SARS-CoV-2 (i.e., NCT04313127, NCT04436471, and NCT04437875), its widespread use in humans may be hampered by pre-existing immunity, which could negatively impact the magnitude of immunity directed towards the encoded transgene Ags. To overcome this issue, several alternative Ad vectors which are known to have low seroprevalence in humans have been evaluated pre-clinically. One such vector is a species E Ad, HAdV-E4 (Ad4) which has been used extensively by the US military in a replication-competent oral formulation to protect against Ad4 respiratory illness, suggesting that it may also be immunogenic when used as a vehicle to deliver avian influenza virus HAs. With this in mind, Alexander and colleagues tested a replication-competent Ad4 vector in mice (*human Ads do not replicate efficiently in mice due to host species restrictions*) encoding H5 from A/Vietnam/1194/2004 inserted into the E3 region of the Ad genome (126). When administered *i.n*, this vaccine elicited H5-specific cellular and humoral immune responses, even in the face of pre-existing immunity to Ad4. In support of this, altering the route of vaccine administration, or increasing the vector dose has previously been shown to help overcome pre-existing anti-vector immunity (232). Importantly, the vaccine was capable of conferring 100% protection from homologous challenge, with sterilizing protection in the lung at an Ad dose of 10^9 viral particles (vp).

Non-Human Ad Vaccines

AdC7

Similar to the rationale for investigating rare species human Ad vectors, many investigators have evaluated Ad vaccines derived from NHP as avian influenza vaccines, on the basis that these vectors have low seroprevalence in humans. Many promising vectors which have been developed were isolated from chimpanzees, which cluster phylogenetically with species E human Ads. A chimpanzee Ad vector AdC7 (*also known as ChAd7, SAd24, Pan7*) was engineered to encode NP from A/Puerto Rico/8/34 on the basis that the high conservation of NP could facilitate heterosubtypic protection from challenge with H5N1 avian influenza strains (127). When compared with Ad5-NP in mice, AdC7 elicited similar frequencies of IFN-γ^+ $CD8^+$ T cells, but the AdC7 vector appeared to elicit greater frequencies of polyfunctional $CD8^+$ T cells (i.e., double or triple positive cytokine secretion). However, Ad5-NP still elicited superior, albeit partial, protection from challenge with two distinct H5N1 viruses, A/Vietnam1203/04 and A/Hong Kong/483/97. Another study by Cheng and colleagues evaluated the AdC7 vaccine encoding full length H5 (A/Chicken/Henan/12/2004) as the encoded transgene in a homologous prime:boost immunization regimen in mice with a dose of $5x10^{10}$vp (233). Ag-specific $CD8^+$ T cell responses were detected following the prime (HA tetramer staining, and ICS), but these were not expanded upon boost, unlike HAI^+ Abs which were not detected following prime, but reached titers of >1:125 following boost immunization. This AdC7-H5 vaccine conferred 100% protection from homologous lethal challenge, with no morbidity (weight loss) and minimal lung pathology. The authors also demonstrated that protection was Ab-mediated by performing passive transfer with immune sera prior to challenge.

AdC68

Xie and colleagues used consensus-based sequence selection and prediction of $CD8^+$ T cell epitopes from six conserved IAV proteins, M1, M2, NP, PA, PB1, and PB2, to design a heterologous transgene Ag for incorporation into chimpanzee Ad vector, AdC68 (128) (*also known as ChAdV-68, ChAd68, SAd25, Pan9, ChAdOx2*) (98). When tested in a heterologous prime:boost regimen in mice with two DNA immunizations delivered *i.m*, AdC68 elicited robust cellular immune responses (IFN-γ ELISpot and ICS) and conferred complete protection from sub-lethal challenge with H7N9 A/Shanghai/4664T/2013. Although the authors also evaluated vaccine efficacy following *i.n* immunization with AdC68 and lethal H7N9 challenge, these results are complicated by the fact that they also used an additional vaccinia boost, making it difficult to evaluate the contribution of the Ad vector to protection. In a separate study, Zhou et al., inserted the conserved M2e epitope into hypervariable regions (HVR) within the AdC68 major capsid protein, the hexon (218). The hexon of Ads have a number of flexible loops (i.e., HVRs), which are exposed on the surface of the virion and have been shown previously to tolerate the insertion of targeting ligands or vaccine Ags (216, 234, 235).

Following a head-to-head comparison of different constructs, including hexon-modified Ads encoding M2e and NP, the authors determined that modification of HVR1 was optimal for eliciting M2e-specific Abs. The HVR1-modified Ad vectors with or without the transgene were superior to other constructs in conferring protection following challenge with H1N1. However, vaccine efficacy was not evaluated for avian influenza challenge viruses. A subsequent study developed AdC68 encoding H7 HA (A/Zhejiang/DTID-ZJU01/2013) and tested its immunogenicity and efficacy in mice (236). The authors demonstrated that a single-shot at a dose of $5x10^{10}$vp was sufficient to elicit virus-specific NAb and T cell responses (ICS), and provide 100% protection from challenge with a heterologous H7N9 virus (A/Shanghai/4664T/2013).

ChAdOx1

A chimpanzee Ad (ChAd) platform, ChAdOx1, has also been evaluated pre-clinically, and has advanced to clinical trials as a vaccine for IAV and SARS-CoV-2 (67, 68, 113, 237). Using ChAdOx1 encoding NP+M1 or H7 HA in a prime:boost, or co-administration approach in mice, Tully and colleagues detected Ag-specific immune responses (IFN-γ and IgG ELISpot), as well as NAbs against H7 (129). However, all challenge experiments included an MVA boost immunization, so it is difficult to ascertain the protective efficacy of the ChAdOx1 vaccine platform from these studies. Subsequently, a collaborative effort between investigators in the US and UK tested ChAdOx1 vectors encoding cHA immunogens, in which the HA stalk was derived from H3 but the HA head domain was from an exotic HA strain, in addition to the NP+M1 fusion Ag (131). Again, the ChAdOx1 vaccine was not evaluated as a standalone vaccine, but in a prime:boost regimen with a modified vaccinia Ankara (MVA) boost. Various regimens were capable of conferring protection against challenge with three different G2 viruses: H3N2, H10N8 and H7N9. However, three sequential immunizations using viral vectors encoding cHAs (ChAd-MVA-cHA protein+adjuvant) were required to elicit strong G2 cross-reactive Ab responses. This would not be ideal in a rapidly evolving pandemic situation, where a single-shot vaccine which elicits robust and rapid cross-reactive immunity would be preferable. However, a subsequent prime: boost study in ferrets using the ChAdOx1 and MVA vaccines encoding cHAs (i.e., cH14/e or cH15/3) as well as vectors containing NP+M1, elicited Abs which cross-reacted with H3, H7 and H10 HAs, including H3 stalk-reactive Abs. This translated to reductions in viral titers in the respiratory tract of ferrets including nasal turbinates, the olfactory bulb and trachea (130). As ferrets are a very relevant animal model for the study of influenza vaccine efficacy, these data suggest that cHA-based immunization approaches, in combination with conserved Ags such as NP+M1, may be a promising approach to elicit broad immunity to IAVs in humans.

PAV-3

A promising platform based on a porcine Ad vector, PAV3-H5, with low seroprevalence in humans has also been developed as a vaccine for H5 avian influenza virus, encoding H5 from A/Hanoi/30408/2005 (132). When tested in mice, this vector elicited equivalent, or in some cases superior, humoral immune responses to H5 when compared with an Ad5-H5 control, and HAI^+ Abs were sustained to higher levels one-year post-immunization. In addition, PAV3-H5 elicited more rapid cellular immunity (IFN-γ ELISpot and ICS) than Ad5-H5 prompting the investigators to assess virus challenge at early time-points post-immunization (D8 and D10), where they observed that survival following PAV3-H5 was superior to Ad5-H5. Vaccine efficacy with PAV3-H5 was also improved relative to Ad5-H5 when mice were challenged with homologous virus 28-day, or 1-year post-immunization.

BAdV-3

Similarly, another rare serotype vector based on a bovine Ad has been described by the Mittal laboratory (133). Again, the authors performed a head-to-head comparison with Ad5, known to be a potently immunogenic vector and as such represents a valuable benchmark for comparing the potency of novel Ad vaccine platforms (98). Ad5- and BAdV-3 vectors were engineered to encode H5 from A/Hong Kong/156/97, and vectors were evaluated in a dose de-escalation study in mice following *i.n* or *i.m* administration. Ab responses following *i.m* vaccination with BAdV-3 or Ad5 concluded that these platforms had similar immunogenicity. However, when vaccine was administered *i.n*, Ab responses to H5 were increased for the BAdV-3 vector, and this was particularly notable at low vector doses. Importantly, in addition to eliciting increased numbers of HA-specific $CD8^+$ T cells relative to Ad5 (IFN-γ ELISpot), the novel BAdV-3 vector also elicited higher levels of IgA in the lung and nasal washes, as measured by ELISA. Impressively, this translated into sterilizing protection from viral infection of the lung following heterologous challenge with A/Vietnam/1203/2004, even at vaccine doses as low as 10^6 plaque-forming-units (PFU). In contrast, the lowest dose of Ad5-H5 which conferred comparable protection in the lung was $3x10^7$ PFU. These data suggest that the BAdV-3 platform is a promising vaccine candidate for mucosal delivery, which would be well-suited to the development of vaccines for respiratory pathogens. In addition, the capacity to elicit robust immunogenicity, superior to that of Ad5, at low doses would make this platform very attractive for pandemic preparedness, potentially allowing for dose-sparing without loss of potency, as well as reducing the cost-per-dose for manufacturing.

Clinical Trials

In addition to extensive evaluation in pre-clinical animal models, Ad-based vaccines for avian influenza have advanced into clinical trials in humans, using oral, *i.n* or *i.m* administration. The justification for administration *via* oral or *i.n* vaccination, is to stimulate mucosal immunity in the respiratory tract, the natural site of influenza virus infection. The earliest studies included a randomized *Phase I* trial (NCT01335347), in which 54 healthy subjects were administered orally with a non-replicating Ad5 vaccine encoding H5 (A/Indo/05/2005) and a dsRNA TLR3 ligand as a molecular adjuvant, formulated in a hypromellose capsule (231). Vaccinees were assigned into 10^8,

10^9, and 10^{10} infectious units (IU) groups, and compared with the placebo group. Four weeks following the prime immunization, 12 of 18 subjects in the intermediate group were given 10^9 IU vaccine boost. Vaccines were well-tolerated with no adverse events reported over grade 1 severity. However, HAI^+ Abs, an important current correlate of protection for influenza vaccines, were not detected. In addition, although the authors reported dose-dependent increases in cellular immunity (IFN-γ ELISpot) when compared to placebo group or pre-vaccination levels, these were very low level, suggesting that oral administration of non-replicating Ad5-based vectors, is not optimal in inducing immunity against influenza virus HA.

Subsequent studies tested an alternative Ad vector species E, HAdV-E4, using a similar oral capsule administration (100). However, this study selected a replication-competent Ad4 vaccine, rather than non-replicating. The rationale for choosing replication-competent Ad4 is based on its exemplary safety profile and historical use in the US military as a vaccine against Ad4 respiratory illness. To evaluate its potential as a vaccine delivery platform for avian influenza virus, the authors designed a randomized, multicenter, *Phase I* clinical trial to test PaxVax Ad4-H5-VTN (encoding H5 from A/Vietnam/1194/2004) in 166 subjects, aged 18–40. Vaccinees were assigned to one of five cohorts, each receiving three immunizations of a set dosage. Vaccine groups were administered doses ranging from 10^7 to 10^{11}vp, compared to a placebo group. Although all vaccines were well-tolerated, similar to findings with the oral Ad5 vaccine, Ad4-H5-VTN HA seroconversion as determined by HAI, MN, and GMT was low and was similar to placebo group. The authors propose that the poor inherent immunogenicity of avian influenza virus HAs, or cellular tropism of the Ad4 vector (currently unknown) following oral delivery may have contributed to the sub-optimal Ab responses, as it is well established that the latter can impact on the potency of Ad vaccines (98).

Upon completion of the 3-vaccine regimen, 105 subjects elected to take part in a follow-up study. Sub-study subjects were then boosted with 90μg of inactivated parenteral H5N1 vaccine. Interestingly, following boosting with the inactivated H5N1 subvirion vaccine, seroconversion (as determined by a four-fold rise in baseline HAI^+ titer) and seroprotection (as measured by HAI titres ≥40) in vaccine groups were noticeably increased when compared to placebo group. This was best seen in the 10^{11}vp cohort which exhibited 100% seroconversion and 89% seroprotection by HAI, compared with placebo group displaying 33 and 14%, respectively. H5-specific cellular responses in this study were similar to those seen in *i.n* LAIV. From these findings, it is possible that using Ad vaccines as a prime may enable improvements in Ab and cellular immunity for Ags which are intrinsically poor in terms of immunogenicity.

Considering the poor humoral immunity following oral vaccination with either Ad5- or Ad4-based vaccines, and implications that the route of administration may have negatively impacted on the induction of robust humoral immunogenicity, *Phase I* clinical trials (NCT00755703 and NCT01806909) were initiated to evaluate *i.n* administration of the aforementioned Ad5 and Ad4 vectors for influenza. These studies collectively aimed to monitor safety and immunogenicity in healthy adults from ages 18–49, but formal published findings have not yet been reported. A more recent clinical study in humans (NCT01443936) tested the replication-competent PaxVax vaccine Ad4-H5-VTN (A/Vietnam/1194/2004) by tonsillar, *i.n*, or oral route (238). This vaccine was administered to 56 healthy individuals, aged 18–49, with doses ranging from 10^3 and 10^8vp for the tonsillar or *i.n* route, and a dose of 10^{10}vp administered orally. Ad4 seroconversion was seen in tonsillar and *i.n* groups at doses of $>10^4$vp. Although overall serum neutralization by MN assay against H5 was modest, Abs with broadly neutralizing activity and potency were detected in peripheral memory B cell populations. Importantly, although not reflected at the serum level, immunization with the replication-competent Ad4-H5 vector induced prolonged increases in somatic hypermutation (SHM), and subsequently increased Ab potency against H5 for several months. In addition, numerous novel mAbs were identified, including one stalk-reactive Ab belonging to a new multidonor class of Abs. Ad vaccines are well-established in animal models to facilitate sustained transgene Ag expression (98, 122, 239, 240), which, although not formally investigated in this study, may have contributed to the prolonged evolution of Ag-specific B cell responses. However, these data highlighted the importance of considering sustained B cell evolution as a valuable parameter when evaluating the success of various vaccine platforms. Better understanding the kinetics and significance of this process may enable the design of optimal vaccine platforms or immunization regimens designed to elicit broad and durable protective immunity. Although promising, some disadvantages in using replication-competent Ads is the induction of anti-vector immunity and its possible competition with transgene Ag. In addition, the use of replication-competent vaccines, including Ad4, are contraindicated for use in young children, pregnant women, the elderly and immunocompromised individuals, thereby limiting its widespread use as a pandemic vaccine to protect vulnerable, at-risk groups.

In addition to human Ads, vectors derived from NHPs have also been evaluated in humans. One candidate vaccine is a species E chimpanzee Ad vector encoding conserved IAV Ags NP and M1, with the aim of stimulating cross-reactive and heterosubtypic cellular immunity. A first-in-human *Phase I* dose-escalation trial to evaluate the immunogenicity of non-replicating ChAdOx1-NP+M1 (Ag sequence from H3 A/Panama/2007/99) following *i.m* immunization was carried out with 15 subjects in a 3 + 3 study model (67). Overall, the vaccine was well tolerated in volunteers, allowing the progression to the highest dose of $5x10^{10}$vp. However, two of the three vaccinees in this group experienced local and systemic reactions, concluding that this dose was not ideal for a prophylactic vaccine. All groups displayed increases in T cell responses, most peaking day 14 post-vaccination. In addition, a sub-set of participants in the $5x10^{10}$vp group received a heterologous boost with $1.5x10^8$ PFU of MVA-NP+M1, resulting in all three subjects displaying an increase in Ag-specific T cells (IFN-γ ELISpot); responses which

were elevated for 8 weeks post-boost. This first-in-human clinical trial was subsequently extended in a *Phase I*, randomized, multicenter study trial to evaluate a heterologous two-dose vaccination regimen using ChAdOx1-NP+M1 and MVA-NP+M1 *i.m* in 49 healthy subjects, aged 18–46, and 24 subjects, age 50 and over. The vaccine regimen proved safe and immunogenic for both younger and older adults. In young adults, the MVA/ChAdOx1 regimen, regardless of the time interval between first and second immunization, elicited T cell responses which remained elevated compared to the ChAdOx1/MVA regimen. In addition, a single vaccination with MVA displayed significantly higher fold-increase and peak immune responses compared to a single vaccination with ChAdOx1 vaccine in young adults. However, all groups following first vaccination in both younger and older adults, compared to baseline, displayed significantly elevated T cell responses to NP and M1 for up to 18 months (ELISpot and ICS). The two-dose heterologous vaccination regimens significantly increased the frequency of cross-reactive T cell responses to NP and M1 (ICS). This study confirmed the ability for viral vector vaccines to elicit sustained T cell responses to conserved Ags which have the potential for heterosubtypic cross-reactivity.

CHALLENGES FACING THE ADVANCEMENT OF ADENOVIRAL VACCINES

Pre-Existing Immunity

The development of vaccines based on Ad5 has waned in the last two decades, largely due to the seroprevalence of anti-Ad5 Abs in humans (which can differ geographically) and the potential for anti-vector Abs to limit vaccine efficacy (241). This topic has once again become the subject of debate, as a result of several Ad vectors undergoing evaluation as vaccines for SARS-CoV-2. Indeed, in clinical trials to evaluate an Ad5-based vaccine in humans, high-level (>1:200) pre-existing Abs to Ad5 (as well as increased age), compromised seroconversion to the encoded Ad5-delivered SARS-CoV-2 spike (109, 242). Interestingly, reactogenicity was reduced in older adults, and those with high pre-existing anti-vector Abs, suggesting that booster immunizations to overcome limited immunogenicity might be tolerated in these groups. However, in our opinion, the use of an alternative Ad serotype such as the Ad26 or ChAdOx1 vector, or a completely different vaccine platform as a booster would be preferable in eliciting an optimal immune response. Alternatively, increasing the interval between re-administration of the same Ad vector, or altering the route of administration may help to overcome the effects of anti-vector immunity, as demonstrated in mice (232, 243, 244).

Additionally, discouraging data from the Merck STEP vaccine trial: a HIV vaccine trial using Ad5 encoding *gag*, *pol* and *nef*, administered to men and women across the Americas, Caribbean and Australia (245), also dampened enthusiasm for Ad5-based vaccines. The STEP trial was halted after the first interim analysis, as the pre-determined, non-efficacy boundaries were reached. Post-hoc analyses revealed a trend towards increased HIV acquisition in vaccinated males (24/522 males), compared to the placebo group (20/536 males) (246). This was associated with an increased hazard ratio in men who were uncircumcised and had high pre-existing Ad5 Abs, in addition to being linked to specific sexual practices (245).

More recently, case-control study of two cohorts (n = 889 total) at elevated risk of HIV-1 infection showed no association between Ad5 seropositivity and incidence of HIV-1 infection (247). Furthermore, a larger case-control study (n = 1570) showed no association between pre-existing Abs to seven Ad serotypes (Ad1, -2, -6, -26, -35, and -48) and acquisition of HIV-1 infection across three HIV-1 vaccine efficacy trials: the VAX003 and VAX004 trials of non-adenoviral vectored vaccines, and the Merck Ad5 STEP study (248). With pre-existing Ad5 antibodies alone seemingly not a risk factor, it could be argued that the trend towards increased HIV acquisition in the STEP trial was a product of testing a non-efficacious vaccine (which lacked an Env antigen), in extremely specific cohorts with high HIV transmission. Indeed, as outlined above, pre-clinical and clinical vaccine development using Ad vectors has successfully continued. With this in mind, the possibility for repeated administration of Ad-based vaccines is supported by the large range of novel vectors to choose from, many of which have been, or are currently being vectorized (98). In addition, it is possible to make genetically chimeric Ad vectors, in which the major targets for type-specific anti-vector Abs (the fiber, or hexon) (249) are swapped for corresponding regions from rare Ad viruses (249).

Manufacturing Capacity

One further challenge, not limited to Ad vectors, is matching clinical grade vaccine production output with demand. Further to this, there can be differences in the manufacturing characteristics of distinct Ad serotypes (i.e., growth to high titers, genetic stability). The SARS-CoV-2 pandemic and urgent need for rapid production of a safe and effective vaccine has highlighted the importance of investing in scalable vaccines which are well-suited to stockpiling. As previously stated, Ad vectors in general have a number of beneficial attributes in terms of their potential for thermostabilization and cold-chain free storage requirements (98). However, outside of SARS-CoV-2 investment, traditional manufacturing processes for Ad vectors have not previously been considered cost-effective or commercially viable for global scale production (250). Vellinga and colleagues have summarized these issues and have highlighted strategies to improve this in an excellent review from 2014 (250). In terms of pandemic preparedness and the development of vaccines with immunological breadth to protect against antigenically drifted, or shifted viruses such as avian influenza virus, Ad vectors are ideal and are therefore a worthy investment as a tool to combat emerging viral infections (241).

Summary

Advances in innovative immunogen design will undoubtedly enable the development of optimized vaccine platforms capable

of eliciting breadth of reactivity, in addition to durability. For example, the use of computationally designed immunogens such as COBRA (251) or immunogens designed using Epigraph algorithms (252) may help to increase breadth across multiple Ags, against T cell epitopes and/or discontinuous B cell epitopes. Pre-clinical validation of these immunogens could also help to identify as-yet-undefined epitopes which play important roles in heterosubtypic protection against novel influenza subtypes

Significant advances have been made in developing novel HA immunogens with the aim of boosting immune responses against the highly conserved but immunosubdominant HA stalk. The HA head is immunodominant and antigenically variable, and in general, Abs elicited against this domain are strain-specific. This is a major factor contributing to the requirement to reformulate conventional IIV-based vaccines on an annual basis. However, highly conserved, broadly neutralizing epitopes do also exist in the HA head domain (253–255). Although Abs to these epitopes are rare and appear to be poorly elicited by seasonal vaccines (256), head-specific mAbs capable of neutralizing multiple IAV subtypes have been isolated from animals (257, 258) and humans (256, 259). Many of the target epitopes are located proximal to the RBS, and in some cases the mAbs use molecular mimicry of the HA surface receptor SA (256). However, anti-HA head mAbs with broad reactivity have also been identified which recognize epitopes distinct from the RBS (260–262). Therefore, conserved HA head epitopes could represent a novel target for next-generation influenza virus vaccine design. The use of structural biology techniques and alternative vaccine platforms may provide additional insight, and enable the improved induction of responses directed towards these unusual, or occluded epitopes, in a manner superior to conventional vaccine platforms.

In recent years, the influenza vaccine field has invested a significant amount of time in trying to better understand the differences between immunity elicited through immunization with different platforms, and natural infection. Evidence is growing that the primary influenza virus exposure in early life, or "*immunological imprinting*", can have a major impact on subsequent responses and susceptibility to infection with G1 or G2 IAVs in later life (263, 264). This is an important consideration when developing broad, or universal influenza virus vaccines, as it is unclear if such a vaccine should elicit equivalent immunity to G1 and G2 HAs simultaneously, and if immunization should ideally be prior to primary natural infection, in very early childhood. The foundations to support these major questions are currently being addressed by large cohort studies in humans, comparing populations with low vaccine coverage versus those with annual seasonal influenza vaccine programs (167, 265). Substantial funding investment to support the development of a universal influenza virus vaccine in recent years will undoubtedly have a positive impact on vaccines which elicit protective immunity, extending to the design of vaccines to protect against emerging avian influenza viruses.

AUTHOR CONTRIBUTIONS

SB and LK—wrote initial draft. LC and CB—wrote manuscript and oversaw final edits. LC—supervision. LC—funding. All authors contributed to the article and approved the submitted version.

FUNDING

LC is funded in part by National Institute of Allergy and Infectious Diseases (NIAID) R21AI146529, by Centers of Excellence for Influenza Research and Surveillance (CEIRS) contract HHSN272201400008C and by a small grant awarded by the Royal Society for Tropical Medicine and Hygiene (GR000550). CB is funded by a Wellcome Trust ISSF fellowship.

ACKNOWLEDGMENTS

We thank Prof. Peter Palese and Dr. Felix Broecker for providing mHA structures shown in Figure.5B, and Dr. Florian Krammer, Icahn School of Medicine at Mount Sinai, for helpful comments on the figures. Figures were created with ©BioRender - Biorender.com.

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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.

frontiers in Immunology

REVIEW
published: 19 February 2021
doi: 10.3389/fimmu.2020.599568

Ebolavirus: Comparison of Survivor Immunology and Animal Models in the Search for a Correlate of Protection

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Ebola viruses are enveloped, single-stranded RNA viruses belonging to the *Filoviridae* family and can cause Ebola virus disease (EVD), a serious haemorrhagic illness with up to 90% mortality. The disease was first detected in Zaire (currently the Democratic Republic of Congo) in 1976. Since its discovery, Ebola virus has caused sporadic outbreaks in Africa and was responsible for the largest 2013–2016 EVD epidemic in West Africa, which resulted in more than 28,600 cases and over 11,300 deaths. This epidemic strengthened international scientific efforts to contain the virus and develop therapeutics and vaccines. Immunology studies in animal models and survivors, as well as clinical trials have been crucial to understand Ebola virus pathogenesis and host immune responses, which has supported vaccine development. This review discusses the major findings that have emerged from animal models, studies in survivors and vaccine clinical trials and explains how these investigations have helped in the search for a correlate of protection.

Keywords: Ebolavirus, correlate of protection, animal models, survivors, vaccine

OPEN ACCESS

Edited by:
Neeltje van Doremalen, Rocky Mountain Laboratories (NIAID), United States

Reviewed by:
Selidji Todagbe Agnandji, Centre de Recherche Médicales de Lambaréné, Gabon
Rupsa Basu, TechnoVax Inc, United States

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Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: *27 August 2020*
Accepted: *29 December 2020*
Published: *19 February 2021*

Citation:
Longet S, Mellors J, Carroll MW and Tipton T (2021) Ebolavirus: Comparison of Survivor Immunology and Animal Models in the Search for a Correlate of Protection. Front. Immunol. 11:599568. doi: 10.3389/fimmu.2020.599568

INTRODUCTION

Ebolavirus belongs to the family *Filoviridae* and consists of six characterized species; Bundibugyo (BDBV), Reston (RESTV), Sudan (SUDV), Taï Forest (TAFV), Zaire (EBOV) and the recently described Bombali (BOMV) (1) (2). The EBOV species is commonly regarded as being the most pathogenic (3) and is the focus of this review. EBOV consists of 7 proteins; L-protein (L), Virion protein (VP) 40, VP24, VP30, VP35, Glycoprotein (GP), and Nucleoprotein (NP) (4) as shown in **Figure 1A**. The virion is enveloped by membrane GP and the genome is non-segmented, with proteins encoded for by negative single-stranded RNA (-ssRNA) (4). The GP, which resides in the viral envelope, is the primary focus of much vaccine research as it is responsible for binding to the host cell and mediating cell entry. Therefore, antibodies to the GP are critical to antibody mediated neutralisation (1). NP is the major component of the nucleocapsid along with VP35 and VP24. VP40 is the matrix protein essential for budding of new virions. The RNA-dependent RNA polymerase L and the polymerase cofactor VP35 facilitate genome replication and transcription. VP30 is a component of the nucleocapsid and a transcription factor (5, 6). Typically, following attachment, EBOV will be macropinocytosed by the host cell and will escape the resulting lysosome *via* binding to Niemen Pick C1 receptor (NPC1) on the endosome membrane. This interaction

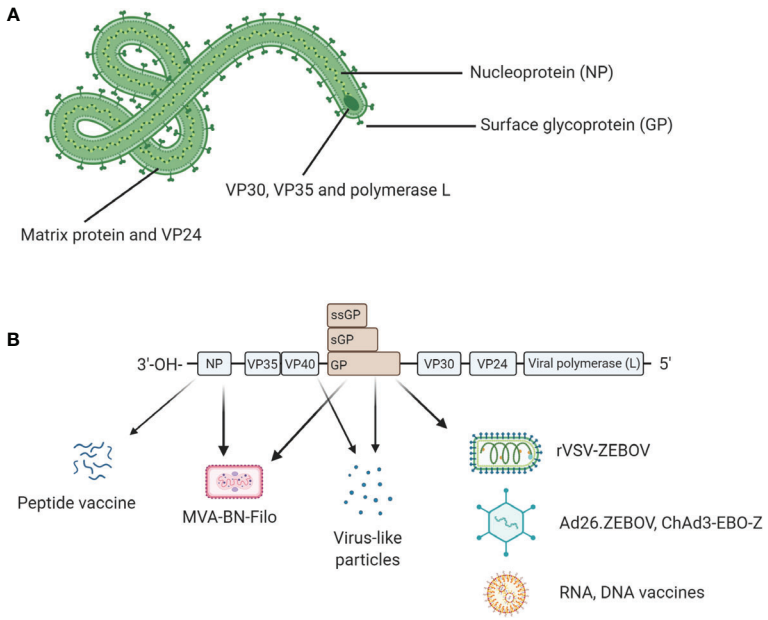

FIGURE 1 | **(A)** Structural proteins of EBOV. EBOV is composed of seven structural proteins: L-protein (L), Virion protein (VP) 40, VP24, VP30, VP35, Glycoprotein (GP), and Nucleoprotein (NP). GP is exposed on the viral envelope. It mediates host cell attachment and cell entry. The nucleocapsid is composed of NP, VP35, and VP24. NP binds the viral genome. The polymerase L and its cofactor VP35 drive genome replication and transcription. VP30 is a transcription factor which is also a component of the nucleocapsid. **(B)** Genomic structure of EBOV and genetic segments targeted for vaccines. Genes encoding for each structural protein are shown. Interestingly, the GP gene also encodes for soluble GP (sGP) and small soluble GP (ssGP). Licensed vaccines (rVSV-ZEBOV, Ad26.ZEBOV) and most candidate vaccines (ChAd3-EBO-Z, RNA and DNA vaccines) use GP as antigen because antibody responses following a natural infection mainly target GP. Other antigens such as NP or VP40 have been also evaluated in the licensed MVA-BN-Filo and some candidate peptide or virus-like particle (VLP)-based vaccines. Figure created with BioRender.com.

results in the release of viral product into the host cell cytoplasm (7). The genome is then transcribed into seven mRNAs by the viral polymerase which consists of L protein and VP35; these products are then translated by host cell machinery. The increase in viral protein eventually results in a switch to produce and package -ssRNA. Viral proteins converge at the host cell surface where they are packaged and bud from the cell resulting in viral progeny and ultimately cell death (7).

EBOV was first classified in 1976 following an outbreak of viral haemorrhagic disease in the Democratic Republic of Congo (DRC), formally Zaire. This outbreak is thought to have originated in a missionary hospital in the village of Yambuku (8–10). Initial blood samples were sent to the Institute of Tropical Medicine (ITM; Antwerp, Belgium) where a Marburg-like virus was identified by electron microscopy (10). Samples were then sent to the Microbiological Research Establishment (Porton Down, UK) and Centres for Disease Control and Prevention (CDC; Atlanta, Georgia) where it was shown that this was a new and separate species to Marburg virus (11). This new virus was named Ebola after the local river which translates as white or clear water in the local language (8, 12). This specific species of Ebola was named Zaire after the country of origin and the archetype variant is EBOV Mayinga, named after Mayinga N`seka, a 22-year-old nurse working in Kinshasa who contracted and died from the virus. By the end of this first outbreak a total of 318 cases were recorded along with a case fatality rate of 88%. Although searched for, no animal reservoir was found from this initial outbreak and it was not until more recently that bat species were identified as a likely reservoir for the virus (13). Since 1976, there have been a number of EBOV outbreaks, one notable outbreak in 1995 occurred in Kikwit, DRC and resulted in 316 cases with a case fatality rate of 82% (14, 15). From 1996–2013, there have been a number of sporadic outbreaks of EBOV in the DRC, Republic of Congo and Gabon totalling ~700 cases and a case fatality rate of ~75% (15).

During 2014, EBOV was brought to the forefront following a large epidemic in West Africa (16, 17). During late 2013 and early 2014, a number of haemorrhagic fever cases were reported in Guékédou, eastern Guinea (16). Samples were sent to Germany & France for analysis where it was confirmed that the causative agent was EBOV (16). Unfortunately, this initial outbreak spilt over into the neighbouring countries of Liberia and Sierra Leone and by 2016, when the epidemic was declared over, had resulted in 28,646 cases and over 11,323 deaths (18). The initial spillover event is thought to have taken place in the village of Meliandou, near Guékédou where interaction with the local wildlife (e.g., bats) is thought to have resulted in transmission to humans (19). The EBOV strain responsible for this outbreak was found to have been a separate clade to those known to be circulating in the DRC or central Africa and was named Makona after the local river (20). Persistence of the

virus amongst survivors and the transient but reoccurring nature of the animal reservoirs would suggest that this virus is likely still circulating within West Africa (21), therefore, future outbreaks cannot be ruled out and much research is currently ongoing to determine the prevalence of EBOV Makona amongst West African countries.

During August 2018, the world's second largest outbreak of EBOV occurred in eastern DRC resulting in 3481 cases and 2299 deaths (22). The heightened attention these outbreaks received and the continued outbreaks in the DRC have resulted in a rapid scientific effort to both contain the virus and develop therapeutics and vaccines. The rapid research seen during the 2013–16 epidemic has similarities to the current situation that we are facing with regards to the ongoing COVID-19 pandemic, and so there are many parallels that can be drawn in terms of therapeutic and vaccine research which may help inform on the course of this and future outbreaks.

To date both the Food and Drug Administration (FDA) (23) and European Medicines Agency (EMA) have licensed the Ebola vaccine Ervebo® which is based on the Vesicular Stomatitis Indiana virus (VSV) vaccine platform and manufactured by Merck. This is a viral vaccine vector that displays the EBOV Kikwit 1995 GP on the VSV capsid surface (24). Another lead candidate vaccine uses the Vaccitech Chimpanzee Adenovirus Oxford platform (ChAdOx), whereby, the EBOV Mayinga GP is incorporated into a Chimpanzee Adenovirus subgroup 3 virus; this vaccine is known as ChAd3-EBO-Z. This vaccine has been trialled as a single dose or as part of a prime-boost method with Modified Vaccinia Ankara (MVA). Due to its larger genome, the MVA boost encodes for the same EBOV Mayinga GP with additional SUDV GP, Marburg GP and TAFV NP (MVA-BN-Filo) (25). In July 2020, the EMA granted marketing authorisation for a prime-boost approach manufactured by Janssen (Johnson & Johnson) based on the recombinant Adenovirus type-26 expressing EBOV Mayinga GP (Ad26.ZEBOV) (Zabdeno®) and the previously mentioned MVA-BN-Filo (Mvabea®) in the European Union for persons greater than one year of age (26). The application of animal models in pre-clinical vaccine development and the characterisation of vaccine induced and naturally acquired immunity in humans has enhanced our understanding of potential correlates of protection for EBOV. Aspects of survivor immunology and what has been learned from animal models will now be discussed in more detail.

EBOV ANIMAL MODELS OVERVIEW

Due to the pathogenic nature of EBOV there has been a need to develop suitable animal models to study pathogenesis and vaccine efficacy. The main challenge was to develop animal models that recapitulate EVD observed in humans. The most obvious model to develop, due to cost, versatility and ethical considerations is the mouse model. However wild-type EBOV does not cause pathology in mice (27, 28). To address this problem, Bray et al. developed a susceptible mouse model to EBOV by sequentially passaging EBOV Mayinga in suckling BALB/c mice (29). The resulting mouse-adapted EBOV (MA-EBOV) now caused lethal disease in both C57B6 and BALB/c mice with death typically occurring between 4 and 6 days post-infection due to massive cytokine release followed by systemic organ damage, paralleling what is seen in humans. However, unlike human disease no haemorrhaging is seen when using MA-EBOV. Therefore, wild-type (WT) mice challenged with MA-EBOV allow for a more ethically acceptable and cost-effective way to study EVD but it does not fully recapitulate human disease (30). To address this issue, researchers have looked into using humanised mouse models which enable adoptive transfer of human peripheral blood mononuclear cells (PBMC) or $CD34^+$ haematopoietic stem cells (HSC) into immune deficient mice, such as the Severe Combined ImmunoDeficient (SCID), Non-Obese Diabetic (NOD) or NOD SCID gamma (NSG) mice (31). The major benefit of these humanised models is that you are providing human target cells and probing the human immune response in an *in vivo* setting.

Additional models which have been used to investigate EVD are Guinea pigs and ferrets (32, 33). Guinea pigs have been used for filovirus research and although susceptible to infection, will only show mild-moderate clinical symptoms in response to wild-type EBOV (30, 32). Therefore, similar to the mouse, a Guinea pig-adapted EBOV strain was generated, in this case the disease course is closer to that seen in humans as haemorrhaging occurs (30). The ferret is a relatively new and promising animal model for EBOV. It has been demonstrated that ferrets infected intranasally with wild-type EBOV do succumb to the disease and show uncontrolled virus replications, as well as the characteristic haemorrhagic fever (34, 35). Ferrets do seem a promising model as they are susceptible to wild-type virus and show important characteristics of human disease, however an important limitation is the reduced numbers of ferret-specific reagents available. The above small animal models have been very important in pre-screening potential drugs and therapies, as well as investigating various aspects of EBOV pathogenesis, however, the gold standard animal model is the Non-Human Primate (NHP). Typically, African green monkeys, Rhesus macaques or Cynomolgus macaques have been used to study EBOV pathogenesis and the various aspects of this research are summarized below. The major benefit of NHP models is that they will more faithfully mimic the course of human disease. However, it is important to highlight the ethical concerns with regards to using NHPs, as well as the increased complexities of working at high containment and these associated costs (30). **Figure 2** illustrates pros and cons of the animal models used in EBOV research compared to human studies.

These animal models continue to have a key role in better elucidating the pathology associated with EVD, the future licensure of second generation vaccines, and in supporting general filoviruses research. We will now discuss the role these animal models in EBOV research and how these results compare with our understanding of naturally acquired immunity in EVD survivors.

Species	Features	Pro	Con
	Survivor studies have proved invaluable to our understanding of the immune response directed towards EBOV	Natural host	Controlled challenge experiments are not possible
	Recapitulates human disease and is susceptible to EBOV in the wild, many vaccine and challenge studies have been performed using NHP and this data has been invaluable to supporting vaccine licensure	Gold standard, recapitulates human disease	Ethical issues, study expense and facilities expertise
	Relatively recent model that is susceptible to EBOV and shows many aspects of human disease	Challenge with WT EBOV	Lack of reagents
	A Guinea pig-adapted strain of EBOV is needed to (GA-EBOV) to see severe disease in these animals	Established	Fails to fully recapitulate human disease
	A mouse-adapted strain of EBOV is needed to (MA-EBOV) to see severe disease in these animals. The use of transgenic mice and adoptive transfer techniques allows for the study of human cell subsets in an *in vivo* setting	Cost-effective and versatile	Fails to fully recapitulate human disease

FIGURE 2 | Pros and cons of animal models used in EBOV research compared to human studies. Studies in humans are invaluable to understand immunity. However, challenge experiments are only possible in animal models including non-human primates (NHPs), ferrets, Guinea pigs and mice. NHP is the gold standard as this model can be infected with WT EBOV and recapitulates EVD observed in humans. But this model is expensive and ethical and welfare concerns limit its broad use. It is the reason why other animal models have been developed even though they do not fully recapitulate human disease. Mouse models remain the most cost-effective and versatile model, but some adaptations are necessary to observe severe disease. Figure created with BioRender.

ANIMAL MODELS IN EBOV RESEARCH AND COMPARISON TO NATURALLY ACQUIRED IMMUNITY IN EVD SURVIVORS

Naturally Acquired Infection

The EBOV virions major route of transmission is *via* bodily fluids and once it infects an individual it will attach and enter the host cell *via* GP binding. The GP is heavily glycosylated and has been shown to bind to a number of C-type lectins found on granulocytes, particularly DC-SIGN located on dendritic cells (DC) (36). DC and macrophages are thought to be the initial cells of infection and disruption in the normal process of both these cell types will have a serious impact on both innate and adaptive immune responses (37). In 2002, it was shown that NHPs showed a prolific release of pro-inflammatory cytokines in response to EBOV infection. It was also suggested that infection of granulocytes resulted in bystander apoptosis in a non-virus dependent manor *via* Tumor Necrosis Factor (TNF) superfamily-mediated apoptosis, further hindering the ability of the host immune system to generate an effective response (38). In addition, it has been suggested that macrophages and monocytes aid the virus by migration through the lymphatics resulting in systemic infection (39). Interestingly, human DC infected with live EBOV have been shown to have abrogated Major Histocompatibility Complex (MHC) presentation and are unable to activate T cells, whereas stimulation of immature DC with EBOV virus-like particles resulted a robust pro-inflammatory response (39). We will now discuss the adaptive immune response seen amongst survivors and various animal models.

Prior to the West African epidemic of 2013–16 there was limited information on the survivor immune response to EVD. What is known, is that studies investigating fatalities and survivors of two large EBOV outbreaks in Gabon (1996) found that survivors tended to show early and sustained levels of IgG, followed by activation of cytotoxic T cells. Whereas fatalities tended to show early T cell activation and impaired humoral responses, this was followed by a collapse in the T cell population, likely due to virus-associated apoptosis (40, 41). Wauquier et al. collected human blood samples from EVD survivors and non-survivors from EBOV outbreaks in the DRC between 1996–2003 and found that fatal outcome was associated with hypersecretion of numerous pro-inflammatory cytokines, chemokines and growth factors, however, T cell-associated cytokines appeared to have been abrogated (42). Studies on human PBMC show apoptosis of lymphocytes and death of $CD8^+$ subsets and the loss of $CD4^+$ subsets which was predicted to impact on the ability of the infected individuals to make a robust IgG response (43). This observation in the depletion of lymphocytes was later experimentally verified in NHP models of EVD (44). Likewise, EBOV challenge studies using Cynomolgus

macaques showed, using flow cytometry, that within the PBMC compartment $CD4^+$, $CD8^+$, and NK cell counts decreased dramatically following the first few days of infection, in contrast $CD20^+$ B cell counts remained stable. Evidence for apoptosis was seen amongst $CD8^+$ and NK cell populations and it was concluded that EBOV likely blocks DC maturation thereby preventing the activation of EBOV antigen-specific T cell subsets and elimination of these subsets *via* FAS/FAS Ligand interactions (45). Similar loss of lymphocytes due to apoptosis was again later seen when using mouse models to recapitulate EVD (46). However, work has shown that adoptive transfer of moribund day 7 splenocytes into naïve mice protected these animals from MA-EBOV challenge therefore despite the commonly observed apoptosis and T cell dysregulation seen amongst fatalities, it seems that there is a functional T cell aspect which when transferred to naïve mice provides protection against challenge (47).

The comparative role of $CD4^+$ or $CD8^+$ T cells in protection against developing EVD has been debated. Interesting work from Gupta et al., 2004 showed that $CD8^+$ deficient mice would readily succumb to non-lethal MA-EBOV infection, whereas if $CD4^+$ T cells or B cell were depleted prior to challenge then mice would survive. Furthermore, using the B cell-depleted mice that survived infection, if $CD8^+$ T cells were then depleted prior to re-infection then the mice would succumb to disease. This was not the case if $CD4^+$ T cells were depleted prior to re-infection and suggests that memory $CD8^+$ T cells alone are capable to protecting against re-infection with EBOV. Interestingly, it was also found that viral antigen could persist between 120–150 days in the tissues of mice that had their B cells depleted prior to challenge and that those mice who additionally had their $CD4^+$ T cells depleted the magnitude of viral antigen that persisted was increased (48). This suggests that the humoral response is important in eliminating virus persistence, something which has been seen during the course of human disease.

Amongst human populations affected by EVD, the presence of asymptomatic individuals has been noted. Work by Leroy et al. was the first to demonstrate the potential for EBOV asymptomatic infection. Indeed, they identified individuals who mounted a strong and effective pro-inflammatory response to the virus. Furthermore, they demonstrated that following this early response there is cytotoxic T cell activation followed by an EBOV-specific IgG response (49, 50). Serological surveys have also played an important role in the understanding of EVD amongst human populations. One such survey using an IgG ELISA to measure the GP-specific response amongst rural villages in Gabon measured >4000 individuals over 3 years and found that 15.3% of participants showed prior exposure to EBOV; this was higher amongst the forested regions where ~20% of people showed exposure to EBOV (51). This finding that the seroprevalence amongst the rural populations is greater that the urban area has again been shown in the DRC (52).

Following a large outbreak of EBOV in 1995, located primarily in Kikwit, DRC, a number of studies investigated the humoral response to EVD amongst survivor cohorts. Maruyama et al., 1999 generated a panel of human monoclonal antibodies using survivor PBMC samples from the Kikwit outbreak and phage display technology (53, 54). This resulted in the generation of the widely used human Monoclonal antibody (MAb) KZ52 which was subsequently shown to protect Guinea pigs from challenge with EBOV (55).

The West African epidemic of 2013–16 fuelled a significant EBOV research programme. It led to the largest cohorts of survivors, which strengthened international efforts to understand host immune responses following naturally acquired infection. Several longitudinal studies were initiated at that time. Adaptive immune responses were dissected in survivors in the field and in western repatriated patients. In addition, animal models enabled the assessment of the protective role of specific immune components.

The antibody response following EBOV infections in humans has been described in several studies. MAbs isolated from EVD survivors of previous outbreaks in Africa as previously mentioned (53–57) or the 2013–2016 West African epidemic (58–60) were shown to be neutralizing and protective in animal EBOV challenge models. Bornholdt and colleagues isolated 349 GP-specific MAbs from the peripheral B cells of a convalescent donor who survived the 2014 EBOV epidemic. They found that 77% of MAbs were able to neutralize live EBOV. They also showed that MAbs which targeted the GP stalk region proximal to the viral membrane, inhibiting cleaved virus in endosomes, were particularly efficient to protect mice against lethal EBOV challenge (59). Interestingly, some MAbs isolated from this library showed pan-neutralizing and protective capacities in mice and ferrets (58). Additional MAbs isolated from 2013–2016 West African epidemic and 2018 outbreak in the DRC targeting epitopes at the base region of the GP also demonstrated pan-neutralizing and protective capacities in mice, Guinea pigs and ferrets (60). Similar findings were observed in some survivors from the 2014 Boende EVD outbreak who mounted pan-ebolavirus responses but also pan-filovirus neutralizing responses (61). Recently, Dowall et al. investigated whether the established human serological reference standard (the 1st WHO International Standard) for Ebola virus antibody, could be used to provide a quantifiable correlate of immune protection *in vivo*. Dilutions of the serological standard were administered to groups of Guinea pigs through intraperitoneal route in comparison with one control group. One day later, all animals were challenged with a lethal dose of EBOV *via* subcutaneous route. They observed that only animals receiving the highest dose of the serological Standard exhibited evidence of delayed progression of disease (62). This standard may be very valuable for evaluation and prediction of protective humoral responses in vaccination studies.

Some studies have analyzed antibody isotypes generated post-EBOV infection and dissected the role of B cells following acute infection. Gunn et al. showed that GP- and secreted GP (sGP)-specific antibody responses were mounted in 14 survivors from Sierra Leone. Interestingly, most survivors developed neutralizing EBOV-specific IgG1 and IgA with innate immune effector functions (63). The role of antibodies and B cell responses in the initial control of infection was also studied in

western repatriated patients even though intensive and/or experimental care used in high-income countries might influence their immune responses. Four acute *Ebolavirus*-infected patients during the 2014 West Africa epidemic and repatriated to Emory University hospital (USA) were enrolled for a longitudinal study analysing B cell responses to Ebola virus infection. Davis et al. found that IgG1 persisted over time, IgG3 declined early and IgG4 appeared late. Following the recruitment and activation of B cells, they observed that EBOV infection induced changes in the antibody repertoire. Only a small subset of antibodies was able to recognise cell-surface GP but this subset contained all identified neutralizing antibodies. Interestingly, they also reported conserved neutralizing antibody rearrangement across donors (64). Williamson et al. demonstrated that human B cell responses between 1–3 months post-recovery were characterized by a low frequency of EBOV-specific B cells encoding for antibodies displaying low neutralizing activity even though one neutralizing antibody human antibodies isolated in this study led to protection in a mouse EBOV challenge model (65). Khurana et al. analyzed longitudinal human antibody repertoire against viral proteome from an Ebola virus survivor from Sierra Leone evacuated to USA and treated on a randomised controlled clinical trial comparing an immunotherapy plus standard of care to standard of care alone. This patient had randomised to receive standard of care treatment alone. They found long-lasting IgG/IgM/IgA epitope diversity. During the acute phase, antibodies predominate to VP40 and GP. One year post-onset of symptoms and despite undetectable virus, a diverse IgM repertoire against VP40 and GP epitopes was observed suggesting occult viral persistence. Finally, they described specific sites in C terminus of GP1 and GP2 which were immunogenic following immunisation in rabbits leading to neutralizing antibody induction which were protective in a lethal EBOV challenge mouse model (66).

Following the West African epidemic of 2013–16, cellular responses have also been analyzed in more detail amongst survivor cohorts and among repatriated western survivors. Ruibal and colleagues evaluated T cell immune responses in EVD patients at the time of admission to the Ebola Treatment Centre in Guinea and longitudinally until discharge or death. They found that patients with elevated levels of T cell inhibitory molecules PD-1 and CTLA-4 expressed on $CD4^+$ and $CD8^+$ T cells were more likely to succumb to disease. These parameters correlated with elevated inflammatory markers and high virus load (67). In another study, the same team demonstrated that T cell responses in fatal patients were oligoclonal and did not result in viral clearance. Contrary to fatal cases, survivors developed highly diverse T cell responses and maintained low levels of T cell inhibitors, which led to viral clearance (68). Other studies analyzed EBOV antigens leading to cellular responses. In 2018, work by Sakabe et al. was focused on the Ebola-specific $CD8^+$ T cell responses in individuals infected during the 2013–2016 epidemic in Sierra Leone. They examined T cell memory responses to GP, sGP, NP, VP24, VP30, VP35, and VP40. They found that $CD8^+$ responses to the NP were immunodominant (69). Herrera et al. developed an anthrax toxin-based Enzyme-Linked ImmunoSpot (ELISPOT) assay to analyze T cells responses in 19 EVD survivors, 10 asymptomatic individuals who were known to be closed contacts with symptomatic EVD patients and six control healthcare workers in Nigeria during 2017. They found that seropositive asymptomatic individuals mounted stronger IFNγ and TNFα responses to NP, VP40, and GP1 EBOV fusion proteins compared to the EVD survivors (70). Consistent with Sakabe and colleagues' study (69), cellular responses directed to the EBOV NP were strongest in comparison with other EBOV antigens in survivors and asymptomatic individuals (70). Interestingly, Lavergne et al. made a connection between post-Ebola syndromes observed in patients in the field and T cell responses. The impact of the persistence of T cell responses on post-Ebola syndromes in 37 survivors in Sierra Leone was analyzed and they demonstrated that survivors with arthralgia and ocular symptoms had a significantly higher EBOV-specific $CD8^+$ and $CD4^+$ T cell responses compared to survivors without any sequelae (71). Recently, we reported in a longitudinal study that T cell and neutralizing antibody responses were long lived in EVD survivors in Guinea. We determined that the dominant $CD8^+$ polyfunctional T cell phenotype was $IFN\gamma^+$, TNF^+, $IL\text{-}2^-$ (72). We further characterized T cell epitopes to the EBOV GP and we found that survivors generally responded to a portion of the receptor-binding domain. We found that both $CD4^+$ and $CD8^+$ T cells contributed to specific T cell memory but with a different cytokine profile. $CD4^+$ T cells produced IFNγ, TNFα, and IL-2 whereas $CD8^+$ T cells only produced IFNγ and TNFα (73). Longitudinal analyses in western repatriated patients infected during the 2013–2016 West Africa epidemic also gave new insights about cellular immune responses. Agrati and colleagues studied immune responses in two western patients repatriated to Italy. They showed a reduction in $CD4^+$ T cell frequency and an increase of $CD8^+$ T cell frequency during the vireamic period (74). This inversion of CD4/CD8 ratio was reverted during the recovery period of these patients and interestingly, this inversion had not previously been observed in mouse models (46). They also found a significant T cell activation and an enhanced PD-1 expression, as well as an impaired IFNγ production which was associated with virus reactivation (74). McElroy et al. investigated the cellular response to four previously-mentioned acute *Ebolavirus* infected patients at Emory University hospital, where they noted a robust T and B cell activation in these four patients. A high percentage of activated $CD4^+$ and $CD8^+$ was also observed up to 60 days after symptom onset. They found activation of both $CD4^+$ and $CD8^+$ T cells to several Ebolavirus proteins. They observed consistent IFNγ responses following stimulation with NP peptide pools. The strongest responses were $CD8^+$ T cell-mediated and directed against the Ebola virus NP (75); this correlates with Sakabe and colleagues' findings in survivors in Sierra Leone (69). Dahlke et al. described more specifically the persistence of T cell activation beyond viral clearance in a western repatriated patient at the University Medical Centre Hamburg-Eppendorf. At days 37 and 46 after illness onset, GP-

specific T cells were especially found in the $CD8^+$ T cell population and were detectable at low magnitude. The largest fraction of EBOV GP-specific T cells revealed an overall low IFNγ, IL-2, and MIP-1β responses upon stimulation with GP peptide pools. However, they observed that the largest fraction of this cell population produced TNFα and expressed the degranulation marker CD107a (76). These findings contrast with previous findings found in NHPs showing a peak of IFNγ and IL-2 expression by $CD8^+$ T cells at day 14 post-infection (77).

Finally, key transcriptomic studies informed on correlates of protection and gave new insights about predictors to patient outcomes. Transcriptomic immune signature was analyzed by Liu et al. in blood samples from 112 infected Guinean patients in 2014 and 2015 who survived or succumbed to EVD. These samples were taken during EBOV diagnosis where blood was analyzed by RT-PCR in the Ebola Treatment Centre. Subsequent transcriptomic analysis showed that fatal patients displayed significant elevated levels of IFN and acute phase response signalling compared to survivors during the acute phase of infection. An upregulation of albumin and fibrinogen genes was also observed in fatal cases suggesting significant liver pathology. Interestingly, this study demonstrated that there was an increase of NK cell populations in survivors (78). A similar gene expression profile study was performed with 44 survivors or fatal EVD patients in Sierra Leone between 2014 and 2016. They observed a dysregulation in inflammatory responses in fatal cases compared to survivors in the late phase of disease. In addition, a strong positive correlation between the upregulation of inflammatory mediated and EBOV viremia was observed. They also found that survivors developed anti-IgM and anti-IgG responses earlier and to a greater extent than fatal patients (79). Similar transcriptional analysis can be performed in animal models like NHPs and ferrets whose sequence genome was published in 2014 (80). Cross et al. compared transcriptomics in Ebola Makona-infected ferrets, NHPs and humans showing strong similarities between pro-inflammatory and pro-thrombotic programs induced in the species analyzed in this study (81). RNAseq technologies were rapidly developed and diversified in the last decade. It is now an essential tool to pursue dissection of immune responses in the context of Ebola virus infections in humans and animal models.

Collectively, the data generated in survivors and animal models for more than two decades have significantly helped in identification of host immune responses following Ebola virus infection. However, a better link should be made between these numerous findings to understand the complex interplay between innate and adaptive immunity. NK cell subsets, T and B cell memory responses could be dissected in more detail. RNAseq technologies will also be crucial to analyze T cell receptor and B cell receptor repertoires. Humanised mice recently developed are also an efficient tool in the pursuit of a search for correlates of protection. Finally, most studies of adaptive immunity have been largely performed in the context of survival during the immediate aftermath of acute infection. However, it was observed that hundreds of long-term survivors experience a range of chronic symptoms which are still poorly understood (82).

The sum of these results also significantly helped in therapeutics and vaccine development. The 2013–16 West African epidemic highlighted the urgent need to produce and assess a safe and effective Ebola vaccine for humans (83).

Immune responses to EBOV infection in animal models and human survivors detailed in this section are summarized in the **Table 1**.

Vaccination

On August 8, 2014, the WHO declared the epidemic in West Africa a global health emergency which coincided with rapid development of an effective Ebola vaccine which built on efforts initiated in the 1990s. Vaccines which were successfully licensed are based on viral vectors. However, vaccine candidates based on other strategies have been tested in preclinical trials or even in early stage of clinical trials. As shown in **Figure 1B**, most Ebola vaccine strategies are based on GP as antigen given it has been shown that neutralizing antibodies produced after a natural infection mainly targeted EBOV GP in humans and animal models. However, antibody responses and cellular responses specific to NP and VPs have been also described in some studies. Thus, NP or VP40 have been also evaluated in some vaccine formulations.

The next section will discuss vaccination studies in animal models and humans which provided key information on the role of the cellular and humoral immune responses with regards to EBOV pathogenesis and protection.

Animal Studies

Early animal studies performed by Olinger et al. showed the importance of the cellular immune response by vaccinating WT C57B6 or BALB/c mice with Venezuelan equine encephalitis virus replicons (VRP) which had been genetically modified to express GP, NP, VP24, VP30, VP35, or VP40 proteins. They found that murine antigen-specific T cells to NP and GP were readily generated and if these antigen-specific T cells were expanded *in vitro* they could protect naïve mice from EBOV challenge when adoptively transferred (91). A potentially important role for $CD4^+$ T cells was demonstrated in 2002 when mice and NHPs were vaccinated against EVD using liposome-encapsulated irradiated EBOV. It was found that if during or prior to vaccination mice had their $CD4^+$ T cell compartment depleted using antibodies, then the protective effect of vaccination was abrogated and mice succumbed to infection with MA-EBOV (100). The finding, that $CD4^+$ T cells play a key role in protection against EVD following vaccination, was more recently shown by Marzi et al. In a similar fashion, they depleted the $CD4^+$ population from NHPs prior to vaccination with rVSV and concluded that the $CD4^+$ population was critical for establishing an IgG specific response (101). Seminal evidence for the importance of T cells to EVD survival comes from the work of Sullivan et al. who vaccinated NHPs with human recombinant adenovirus serotype 5 (rAdHu5) which encoded for EBOV GP. Cynomolgus macaques were vaccinated then exposed to EBOV. Interestingly, if post-vaccinated animals underwent T

TABLE 1 | Immune responses to EBOV infection in animal models and human survivors.

Model	Responses to EBOV infection				
	Clinical Presentation	**Antibody production and protection**	**$CD4^+/CD8^+$**	**B cells**	**NK cells**
Human survivors	Range from asymptomatic to flu-like symptoms with continued progression to: vomiting, diarrhoea, rash, kidney and liver damage, haemorrhaging and low white blood cell (84).	Strong and early humoral response is associated with survival (78) (72).	Strong and sustained T cell responses, with reduced expression of inhibitory molecules and a diverse T cell repertoire, are associated with survival (67) (68). Persistent T cell responses are associated with sequalae (71) (72) (73).	Extensive activation during acute infection. Subclass composition changes over time with persistent IgG1, rapid decline of IgG3 and late increase in IgG4 (64).	Increase in survivors and decrease in fatalities (78).
Macaques (cynomolgus and rhesus)	Identical to human EVD (85).	Antibodies produced in response to infection contribute to survival (86).	Both $CD4^+$ and $CD8^+$ counts decrease during acute infection stage (45).	Levels in blood remain stable (45).	Decline during acute phase (45) (87).
African green monkeys	Identical to human EVD. Rash is less frequent (85).	Protection from natural infection is difficult to determine due to lack of NHP model for mild EVD (88).	Both $CD4^+$ and $CD8^+$ cells show signs of apoptosis and depleted levels in lymph nodes. $CD4^+$ cells increased in germinal centres (44).	Lymphocyte depletion in B cell follicles (44).	ND
Mice (mouse-adapted model)	Mouse-adapted virus and intraperitoneal injection required for infection. Unlike humans, EVD does not manifest with rash, coagulopathy or haemorrhagic symptoms. However they can succumb to disease (85).	Protection from antibody transfer has been reported (59) (60) (65).	T cells show drastic depletion during the acute phase (46) but are still important for protection. $CD8^+$ T cells provide protection against acute infection (47) whilst $CD4^+$ T cells confer long-term protection and challenge viral persistence (48).	Important for fighting viral persistence (48).	Drastic depletion in blood during acute phase (46) but accumulation in EBOV infected tissues (89). Differential effects depending on viral load (89).
Naïve or immunocompromised mice	Unlike the mouse-adapted model, EVD in this model can manifest with haemorrhagic symptoms (85).	Acquire protection from antibody transfer (90).	Adoptive T cell transfer from vaccinated or infected mice can confer protection against MA-EBOV (91) (47).	ND	NK cells are required for protection against infection, even if they have received prior protection from VLPs (92).
Humanised mouse models	Various models capable of recapitulating human EVD (85). Can mimic human EVD, including symptoms of hepatic pathology, lymphocyte apoptosis, haemorrhaging, and lethal outcome when infected with MA-EBOV (88) (93) (94).	ND	T cell environment varies depending on model. Restricted $CD8^+$ responses reported, with limited MHC interactions (95). $CD8^+$ (and presumed $CD4^+$) T cell activation observed 8 days after inoculation with EBOV (96).	Can show measurable B cell responses (88) (88) but the B cell compartment is short-lived (95).	Important in early immune response and shows a significant decrease 5 and 8 days after inoculation (96).
Ferret	Can be infected with wild-type EBOV and demonstrate human EVD symptoms including rash, coagulation abnormalities and haemorrhaging, not seen with some other models (34) (35).	Acquire protection from antibody transfer (58) (60).	ND	ND	ND
Guinea pig	Require Guinea pig-adapted virus. Does not recapitulate rash or haemorrhagic symptoms but EVD does manifest with platelet reduction, liver pathology, and lymphocyte apoptosis (85).	Acquire protection from antibody transfer (55).	ND	ND	ND

(Continued)

TABLE 1 | Continued

Model	Responses to EBOV infection				
	Clinical Presentation	Antibody production and protection	$CD4^+/CD8^+$	B cells	NK cells
Hamster Model	Requires adapted EBOV. Recapitulates key features of EVD including coagulopathy and haemorrhaging, absent in many rodent models (97) (98) (85).	Acquire protection from antibody transfer (99).	CD4-dependent antibody responses (99).	ND	ND

Clinical presentation and adaptive immune responses observed in each species. NA, Not applicable; ND, Not determined.

cell depletion using an anti-CD3 MAb, they lost their ability to control disease and succumbed to infection. Furthermore, if prior to challenge primates were $CD8^+$ T cell depleted using a MAb then, again, they were unable to control disease, this was not the case for $CD4^+$ T cell depletion prior to challenge (102). Additionally, using NHPs, it has been suggested that protection resulting from Adenoviral vectors requires the generation of effector memory $CD8^+$ T cells that produce both IFNγ and TNFα and that durable protection, as shown in NHPs, require $CD8^+$ memory T cells that are polyfunctional for IFNγ, TNFα, and IL-2 (103).

Using Guinea pigs and mice to investigate various vaccination routes, it was shown that vaccination *via* the sublingual route with an adenoviral (Ad5) vector provided a cellular immune response that was unaffected by pre-existing immunity to the viral vector. Therefore, making it more likely that this vector and delivery route could be used multiple times for the same host. Additionally, both mice and Guinea pigs were protected from lethal challenge (104).

Pre-existing immunity to the viral vector is a concern for successful vaccination and the implications of this have been modelled using animal studies. For example, Choi et al. found that adoptive transfer of splenocytes from animals previously vaccinated with the empty viral vector (Ad5) followed by immunisation with the Ad5-EBOV vaccine showed abrogation in $CD8^+$ T cells responses and reduced GP specific IgG1 responses. However, if vaccination was given *via* a different route to that of the empty vector, $CD8^+$ T cell responses were not abrogated. This importantly highlights the role of pre-existing immunity to eliciting the optimum immune response to EBOV (105). Early use of adenovirus vectors for vaccination against EVD came from Roy et al, who used simian Adenovirus type-22 and -21 and compared the responses with the human Ad5 platform in an attempt to circumvent any issues with vaccinating human populations already exposed to human Ad5. It was found that both mice and NHPs mounted B and T cell responses and that mice were protected from MA-EBOV challenge following vaccination (106).

Initial preclinical work using VSV as a vaccine platform demonstrated that antibodies were sufficient to protect mice from infection after immunisation with VSV expressing Zaire EBOV Kikwit 1995 GP (rVSV-ZEBOV), while the depletion of $CD8^+$ T cells did not compromise protection (107). Feldmann et al. tested the efficacy of rVSV-ZEBOV vaccine in post-exposure treatment in Rhesus macaques and found that 4/8 macaques were protected if treated up to 30 min following a lethal infection. They compared immune responses between the macaques which survived and those ones which succumbed. They observed that neutralizing antibodies were detected on days 14–36 after challenge in animals that survived the challenge, while humoral response was not detected in animals that succumbed to the challenge, suggesting a major role of the humoral response. They also observed a decline of $CD4^+$ and $CD8^+$ in most macaques regardless of treatment and outcome. The only difference noticed in cellular responses was the percentage of NK cells which did not decrease but actually increased in most macaques treated with the vaccine (86). Geisbert et al. analyzed rVSV-ZEBOV immunogenicity and protective efficacy in a macaque model challenged with ZEBOV by aerosol. All vaccinated primates were protected from challenge, and they found that immunisation induced ZEBOV-specific IgG responses which increased following the challenge but there was no evidence of IFNγ or TNFα production in $CD4^+$ or $CD8^+$ before or after the challenge (108). The same team also showed that in immunocompromised NHPs the vaccine was protective and well tolerated. Here, Rhesus macaques previously infected with simian-human immunodeficiency virus were vaccinated with rVSV-ZEBOV. The vaccine produced no serious side effects. However, when challenged with EBOV, 2/6 of these vaccinated animals succumbed to disease; interestingly those animals that died had lower $CD4^+$ counts (109). Investigations into the mucosal response to vaccination against EVD found that NHPs vaccinated intranasally with rVSV-ZEBOV were protected against challenge, and this route seemed to be more potent than intramuscular injection. These findings will have important implications to vaccination amongst human populations (110). Finally, Konduru et al. made an Fc fusion protein consisting of the extracellular domain of EBOV GP and human IgG1 (ZEBOVGP-Fc). Mice vaccinated with ZEBOVGP-Fc showed both cellular and humoral responses and were protected against challenge with MA-EBOV (111). Demonstrating that vaccine platforms do not necessarily need to be viral vector based. Some studies have attempted to compare the immune correlates elicited in both murine and primate experiments. Here, Wong et al. vaccinated immunocompromised mice and found that vaccine induced protection was primarily B and $CD4^+$ T cell mediated. They also demonstrated that Guinea pig and NHP showed a strong correlation between survival and GP-specific IgG. This suggests that the magnitude of the GP-specific IgG response is a meaningful correlate of protection (112). A study

also analyzed the long-term protective efficacy of rVSV-ZEBOV in murine and Guinea pig models. While they observed 100% survival of Guinea pigs challenged with a lethal dose of adapted EBOV 12 and 18 months post-vaccination, only 80% of mice survived at 12 months post-vaccination. Interestingly, they measured higher EBOV GP-specific IgG responses in mice which survived up to 12 months post-vaccination, suggesting a major role of antibody responses in protection (113).

Clinical Trials

Preclinical studies were the foundation of the world's first Zaire Ebola vaccine rVSV-ZEBOV (brand name Ervebo®) approved by the EMA and FDA in 2019. Following animal studies, between 2015–2017, the rVSV-ZEBOV vaccine was also studied in Phase 1 clinical trials (114–117) and a Phase 3 safety and manufacturing-consistency clinical trial (118) in North America, Europe, and Africa. Even though the rVSV-ZEBOV has the potential for some adverse effects (119, 120), the sum of these data showed an acceptable immunogenicity and safety profile. Antibody response was measured in 89%–100% of vaccinated individuals for at least 24 months (121). One study was able to provide data on clinical efficacy. This Phase III study was conducted in Guinea using the approach of ring vaccination and in place of a placebo control, the rings were randomly assigned to either 'immediate' vaccination (in a defined timeframe of 24 h), or vaccination after a 21-day delay. In this study, Henao-Restrepo et al. reported 100% efficacy of the vaccine. Among 2,119 people who received the vaccine immediately, no cases were reported in a period of 11 days, whereas 16 cases were identified within the same time frame among 2,041 people in the delayed vaccination group (122). However, the protocol revealed there may be a bias with respect to the intervention of medical team in the immediate or delayed vaccination groups. The presence or absence of the medical team was not identical in the vaccinated groups and may have potentially influenced outcomes (123). Furthermore, indirect evidence from a randomised controlled trial of EVD therapeutics in DRC showed that a portion of clinical EVD cases had received vaccination. 25.0% reported that they had received the vaccine; of these, 38.7% reported that they had received the vaccine at least 10 days before the onset of clinical symptoms (124). Safety and immunogenicity of rVSV-ZEBOV were also studied following high-risk exposure. In 2015, rVSV-ZEBOV was used on a laboratory worker, 43 h after a high-titre needlestick injury. The physician developed fever and moderate-to-severe symptoms 12 h post-vaccination, but symptoms disappeared over 3 to 4 days. Activation of T cells and plasmablasts were detected early post-vaccination. The peaks of EBOV GP-specific IgG and IgM, as well as an increase of cytokine-producing $CD4^+$ and $CD8^+$ T cells were observed on day 17. Antibodies against VP40 (not in the vaccine) were not detected, suggesting that the immune responses are not due to natural EBOV infection (125). For 1 year, Davis et al. followed 45 people who came into direct contact with a healthcare worker presenting a late reactivation of EVD and who were elected to receive rVSV-ZEBOV. Three months following vaccination, 100% of individuals had seroconverted. In addition, neutralizing antibodies were detected in 73% of volunteers 12 months post-vaccination. Nobody exposed to the virus became infected. No severe vaccine-related adverse events were reported. However, common side effects associated with vaccination were characterized by fatigue, myalgia, headache, and arthralgia. Interestingly, arthralgia, myalgia and fatigue occurring at the time of study were associated with a higher proportion of $CD8^+$ IFNγ and $CD4^+$ IL-2-secreting cells, while headache was associated with higher $CD4^+$ IL-2 and IFNγ ELISpot responses (126). The rVSV-ZEBOV vaccine has been the most studied in animal models and clinical trials as prophylaxis or following high-risk exposure. Even though this one-dose vaccine was shown to be successful in the field, its level of efficacy in humans remains to be confirmed and correlates of protection to be clarified. **Table 2** summarizes protection and adaptive immune responses observed in humans and animal models following rVSV-ZEBOV vaccination.

In July 2020, the European Commission granted Marketing Authorisation for the two-dose vaccine regimen Ad26.ZEBOV (Zabdeno®) and MVA-BN-Filo (Mvabea®). This is a multivalent prime-boost vaccine based on an Adenovirus type 26-vectored vaccine encoding Ebola virus GP boosted by a MVA-vectored vaccine encoding GP from Ebola, Sudan and Marburg viruses, as well as the NP of Tai Forest virus. In a Phase I study of healthy volunteers in the UK, vaccination with Ad26.ZEBOV and MVA-BN-Filo did not result in any vaccine-related serious adverse events. All vaccine recipients had EBOV GP-specific IgG detectable 21 days post-boost and at 8-month follow-up. Within randomised groups, at 7 days post-boost, at least 86% of vaccine recipients showed Ebola-specific T cell responses (132). Another Phase I trial performed in Oxford showed that this two-dose vaccine could confer immunity for at least 360 days and was well tolerated (133). In 2019, Anywaine et al. and Mutua et al. published the results of two Phase I trials performed in Uganda and Tanzania, as well as in Kenya, respectively. Both trials showed good immunogenicity and safety profiles in healthy volunteers (134, 135). Phase II and III clinical trials in various cohorts of participants (e.g., healthy, elderly, HIV, children) have been recently completed or are currently in progress in Africa and Western countries (**NCT02564523, NCT04228783, NCT03929757, NCT02598388, NCT04028349, NCT02509494, NCT02661464, NCT02543268**). To assess immunogenicity of the vaccine, most clinical trials analyzed EBOV GP-specific IgG and sometimes neutralizing antibody levels. Based on current information available on ClinicalTrials.gov, only one clinical trial is evaluating plasma cytokines/chemokines and T cell responses (**NCT04028349**). Even though the efficacy remains to be determined in humans, this Ebola vaccine has been deployed in the North Kivu region of the DRC, following recommendation from the WHO's Strategic Advisory Group of Experts (SAGE), and in Rwanda, following conditional approval in 2019 under an "exceptional emergency", as part of outbreak containment efforts in the region (136). However, a two-dose regimen is less suitable for an outbreak response where immediate immune protection is essential. This

TABLE 2 | Responses to rVSV-ZEBOV.

Model	Responses to rVSV-ZEBOV				
	Protection?	**Antibody production?**	**$CD4^+/CD8^+$**	**B cells**	**NK cells**
Humans	Yes (127).	Yes. Antibodies also cross-react with other EBOV species (127).	Increased activation of both $CD4^+$ and $CD8^+$ T cells (76).	Polyclonal, convergent B cell responses observed in a trial of 4 vaccinees (127).	Modulation of NK cells contributes to early efficacy of the vaccine (128).
Macaques (cynomolgus and rhesus)	Yes (86) (108) (113).	Yes. Strong correlation observed between GP-specific IgG and survival (86) (108) (113).	Similar to natural infection, circulating $CD4^+$ and $CD8^+$ T cells declined during infection, regardless of prior treatment or vaccination (86). $CD4^+$ T cells likely contribute to establishing protection from vaccination (109) whilst $CD8^+$ T cells are not required for vaccine protection (101).	Required for production of antibodies which mediate protection. Although $CD20^-$ depleted macaques were shown to have detectable EBOV-specific antibodies, this is likely due to persistence in lymphoid organs (101).	Increased in response to vaccine (86).
African green monkeys	ND	ND	ND	ND	ND
Mice (mouse-adapted model)	Yes (129) (113).	Yes. Strong correlation observed between GP-specific IgG and survival (113).	$CD8^+$ T cell depletion does not compromise protection (107). $CD4^+$ T cells (and B cells) are main mediators of protection (112).	B cells (and $CD4^+$ T cells) are the main mediators of protection (112).	NK-cell population is enhanced by vaccine and increases survival (130).
Naïve or immunocompromised mice	Yes (107).	Able to tolerate vaccine (107).	NA	NA	NA
HSC-transfer mice	ND	ND	ND	ND	ND
Ferret	Yes (131).	Yes (131).	ND	ND	ND
Guinea pig (guinea-pig adapted model)	Yes (112).	Yes. Strong correlation observed between GP-specific IgG and survival (112).	ND	ND	ND
Hamster	Yes (24).	Yes (24).	ND	ND	ND

Protection and adaptive immune responses observed in humans and animal models. NA, Not applicable; ND, Not determined.

two-dose vaccine maybe a more suitable strategy for healthcare professionals, frontline workers or visitors who plan to go to areas with an ongoing EVD outbreak.

Another lead candidate is a vaccine named ChAd3-EBO-Z. This vaccine is based on chimpanzee Adenovirus subgroup 3 (ChAd3) vaccine encoding EBOV Mayinga GP. Stanley et al. demonstrated that a chimpanzee-derived replication-defective adenovirus vaccine was able to generate protection against acute lethal EBOV challenge in macaques and after a boost with MVA, they observed a durable protection against lethal EBOV challenge (137). Following this success in the NHP model, the vaccine evaluation advanced into clinical trials. Phase I and II trials investigated this vaccine on its own (138, 139) or in combination with an MVA boost (25, 140) or MVA-BN-Filo (141). The main specificity of this chimpanzee adenovirus platform is the induction of exceptional antigen-specific T cell responses previously observed in animal models (142) and humans (143) in the context of the development of vaccine against malaria. Similar results were shown in the context of Ebola vaccine. The ChAd3-EBO-Z vaccine boosted with MVA was shown to be safe (141) and to elicit EBOV-specific antibody and T cell immune responses superior to those induced by the ChAd3 vaccine alone (25). The single-dose ChAd3-EBO-Z was tested side-by-side with the single-dose rVSV-ZEBOV in Phase II trial in Liberia. In this study, antibody responses were slightly lower with ChAd3-EBO-Z vaccine compared to rVSV-ZEBOV. However, post-vaccination symptoms like headache, muscle pain or feverishness were less frequent with ChAd3-EBO-Z than post-vaccination with rVSV-ZEBOV. Unfortunately, T cell responses were not evaluated in this study (144). The efficacy of ChAd3-EBO-Z alone or in combination with MVA boost remains to be confirmed in humans.

Human and animal research demonstrate the importance of both B and T cell immunity in protection from EBOV infection. The current lead vaccines demonstrated that they were able to induce EBOV-specific antibody responses. However, the duration of antibody responses, as well as the generation of memory B cell responses remain to be confirmed. Lead candidate

vaccines previously described have also been shown to induce T cell responses. It is likely that the chimpanzee adenovirus platform is more suitable to induce strong antigen-specific T cell responses. However, as described in this review, the level of T cell responses might play a role in vaccine-associated side effects. Another crucial point is the presence of EBOV-NP specific T cell responses, especially $CD8^+$ T cell responses detected in survivors and even asymptomatic individuals, which could suggest an important role of NP antigen in protection. Currently, the lead Ebola vaccines are focused on EBOV GP as the antigen. Thus, NP should be included in vaccine formulation along with the EBOV GP as it might boost cell-mediated immunity. Finally, even though rVSV-ZEBOV, Ad26.ZEBOV- MVA-BN-Filo, ChAd3-EBO-Z generated strong immunogenicity and showed success in the field, to what extent B and T cell immunity is required for protection is still unclear. Consequently, immunogenicity and protection provided by vaccines must be compared to immunity in survivors. Some recent studies compared side-by-side immune responses following a natural infection versus vaccination. Fuentes et al. compared antibody responses in plasma from three Western survivors from the 2014 West Africa epidemic (2-6 months post-infection) who received experimental treatments, as well as a pool of 6 plasma from Sierra Leonean convalescents who did not receive experimental treatment versus pools of plasma from ChAd3-MVA vaccinees in the UK (2-12 months post-vaccination). One pool had low and the other one had high neutralization titres. They found higher antibody responses and stronger antibody affinity in survivors, as well as high neutralization titres compared to vaccinees. Survivors demonstrated IgG-dominant antibody responses whereas a predominant IgM response was detected after vaccination. Natural EBOV infection generated a more diverse antibody epitope repertoire compared to vaccination. They also observed that antibodies preferentially recognised antigenic sites in specific GP2 domains (the fusion peptide and HR2) in survivors than in vaccinees and that this was associated with neutralization titres (145). Another study compared GP epitopes bound by anti-EBOV GP antibodies following a natural EBOV infection versus vaccination with rVSV-ZEBOV. They analyzed the sera from seven vaccinees with rVSV-ZEBOV and one western EVD survivor who contracted an Ebola virus infection in Sierra Leone in 2014 and was repatriated to Germany. An epitope mapping approach showed that IgG and IgM antibodies from the survivor or the vaccinees bound different epitopes in the EBOV GP (146). Koch et al. compared the functionality of anti-EBOV GP antibodies between 10 rVSV-ZEBOV vaccinees from the Phase I clinical trial in Hamburg 6 months post-vaccination and 25 EVD survivors 12 months after discharge. They did not find any significant differences between the levels of circulating Ig subclasses. However, they observed a higher neutralization capacity of plasma samples from survivors than that of vaccinee samples, as well as a higher capacity to induce cellular responses. They also determined that the levels of IgG1 positively correlated with virus neutralization in survivors but not in vaccinees (147). The sum of these studies suggest that vaccines may induce different immune responses in vaccinees from those observed in survivors. However, it is essential to keep in mind that the quality of immunity might be also affected by the vaccine vector. Meyer et al. tested various human and avian paramyxoviruses expressing EBOV GP and demonstrated different serum antibody profiles in a Guinea pig model according to the vector they used even though the same antigen was used (148). To conclude, comparison of immune responses between survivors and vaccinees are crucial to improve the existing vaccines and develop the next-generation vaccines. One major evolution might be to design more targeted-vaccines using some specific EBOV GP and NP epitopes shown to be very immunogenic and to develop efficient vaccines against several Ebolavirus species.

Other Candidate Vaccines

Even though vaccines which were licensed or moved to late-stage clinical trials are based on viral vectors, other vaccine strategies have been investigated. Work using C57B6 or BALB/c mice vaccinated with EBOV Virus-like Particles (VLP) composed of GP and VP40, showed that mice survived primary challenge with MA-EBOV and that only if you adoptively transferred both serum and splenocytes into naïve mice would it rescue the mouse from MA-EBOV challenge. This confirms a role for both the humoral and cellular response in preventing fatal EVD following VLP vaccination (149). Furthermore, the same VLP were shown to protect NHPs against challenge with EBOV (150). DNA vaccination was also shown to induce EBOV-specific protective immune responses in Guinea pigs. In this case, immunisation with plasmids encoding for viral proteins such as EBOV GP, sGP, and NP generated antibody and T cell responses. Protection correlated with antibody titres and T cell responses to sGP or GP (151). Another study demonstrated that DNA vaccination boosted with recombinant adenoviral vectors encoding Ebola viral proteins could protect cynomolgus macaques against a challenge with a lethal dose of 1976 Mayinga strain of Zaire EBOV. Animals which were not vaccinated progressed to a moribund state and death in less than one week, while vaccinated animals were asymptomatic following this challenge for more than six months. The virus was not detected after the challenge and protection correlated with Ebola virus-specific $CD8^+$ T cell and antibody responses (152, 153). In 2006, a three-plasmid DNA vaccine encoding GP and NP from EBOV as well as GP from SUDV was evaluated in a Phase I clinical trial in the US. This vaccine was well-tolerated and induced specific antibody responses to at least one of three antigens, especially GP from EBOV or SUDV, four weeks following the third vaccine dose. In addition, $CD4^+$ T cell responses for GP (EBOV/SUDV) were detected in all vaccinees 2 weeks after the third vaccination (154). In 2011, Konduru et al. demonstrated that an EBOV GP fused to the Fc fragment of human IgG1 was able to generate neutralizing antibodies against rVSV-ZEBOV and T cell immunity against EBOV GP in mice. Here, seven/eight vaccinated mice were protected against challenge with MA-EBOV (111). Recently, a peptide vaccine based on a predominant NP epitope (NP44-52) found to induce $CD8^+$ T cell response in EVD survivors was tested in mice. A single

intradermal vaccination using an adjuvanted microsphere peptide vaccine formulation containing NP44-52 was shown to confer protective immunity against a MA-EBOV (155). Finally, mRNA vaccines based on EBOV GP and formulated with nanoparticles were demonstrated to induce GP-specific IgG and neutralizing antibodies in Guinea pigs. All vaccinated animals survived following an infection with a Guinea pig-adapted EBOV strain (156).

CORRELATES OF PROTECTION

Immunology studies in survivors and animal models, as well as human clinical trials as shown in **Figure 3** have had a major impact on our understanding of EBOV pathogenesis, as well as therapeutics and vaccine development. These studies also informed on correlates of protection, which can be defined as an immune response that is responsible for protection. Following a natural infection, the antibody response, especially GP-specific neutralizing IgG in serum, is considered as a major correlate of protection in survivors and animal models, however it is also evident that those that succumb to infection will also have detectable levels of IgG in their serum. Therefore, it is not clear what titre is needed and whether antibodies to particular antigens/epitopes are needed to provide protection. It is likely that antibody as a correlate of protection will not only be defined as a quantifiable titre but also by the kinetics of such a response and that these combined factors should define antibody as a correlate of protection. In addition, the role of IgG Fc receptors in potential antibody-dependent enhancement of disease is not clear in EVD disease outcomes. The important role of frequency, activation, and phenotype of T cells in survival has been clearly demonstrated in humans and animal models. GP- but also NP-specific T cell responses have been shown to be involved in protection. However, the predominant role of either $CD4^+$ or $CD8^+$ T cells may vary according to animal models or human studies and like with the antibodies timing of these responses will be critical. Following vaccination with GP-based antigen, specific IgG responses are seen as the main correlate of protection in animal studies and clinical trials. Cellular mediated responses

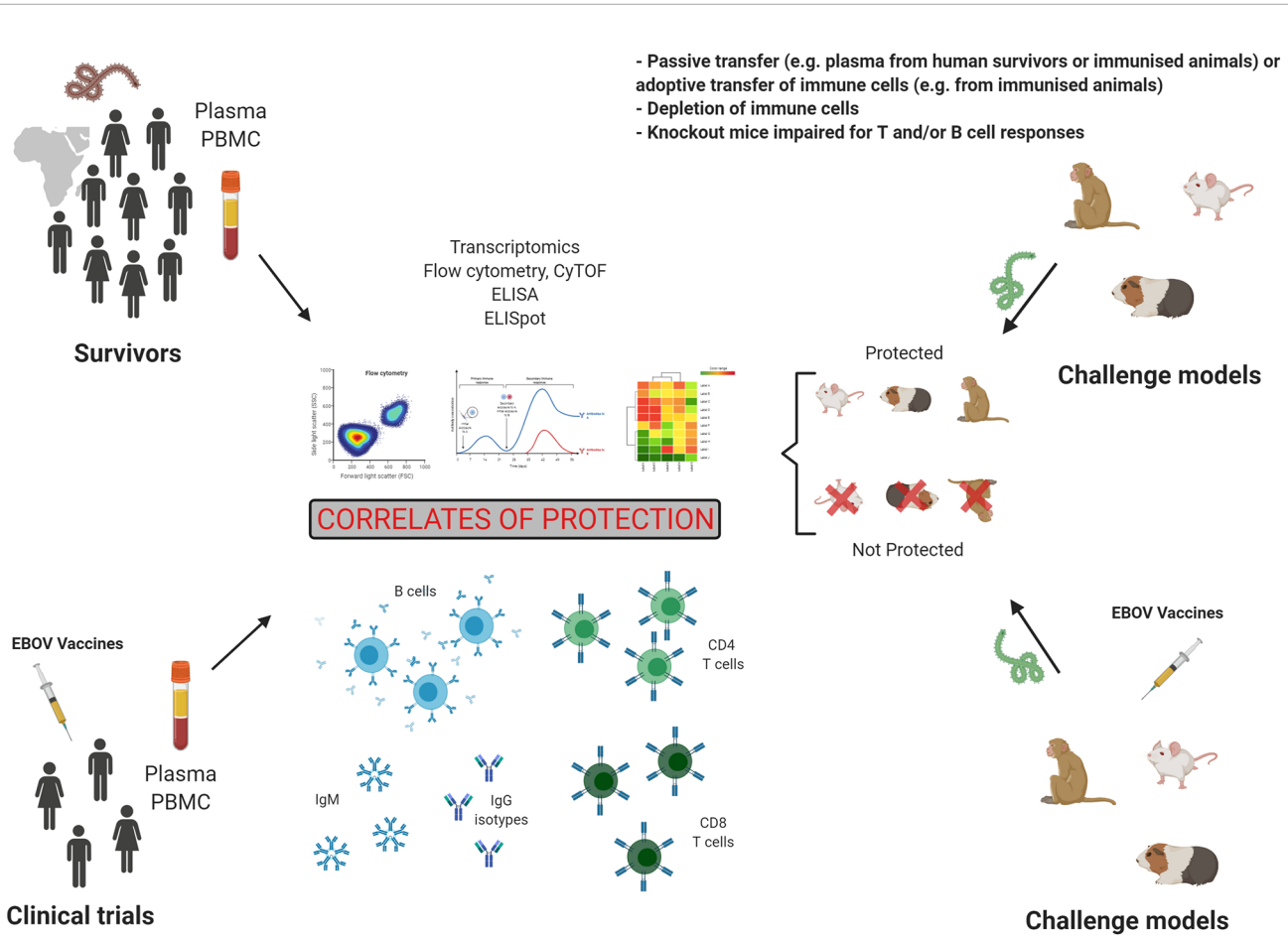

FIGURE 3 | Preclinical and clinical studies in the search for correlates of protection. Studies of humoral and cellular responses in Evola virus disease (EVD) survivors and in vaccinated individuals is one strategy to establish correlates of protection. However, controlled challenge studies cannot be performed in humans. The second strategy to identify correlates of protection is the analysis of immune responses in challenge animal models, which allows to compare immunity in protected and not protected animals. Figure created with BioRender.

have been especially assessed in animal models. The frequency and activation of $CD4^+$ and $CD8^+$ T cells play a role in protection. However, the cytokine phenotype leading to protection varies between the studies and remains unclear. Finally, the role of innate immunity in survival is yet to be fully elucidated, and this will likely play a key role considering what is known about asymptomatic and mild disease and that these innate responses will inform on the adaptive ones. Therefore, protection may also correlate with innate immune signatures.

LESSONS LEARNED FROM THE 2013–16 WEST AFRICA EPIDEMIC

Interestingly, some similarities can be observed between the rapid EBOV research following the 2013–2016 West Africa epidemic and the current international research to control the COVID-19 pandemic. The last section discusses the lessons learned from the 2013–16 West Africa epidemic and the strategies used to tackle EBOV epidemics which could be applied for the COVID-19 pandemic.

A novel acute respiratory syndrome, now called Coronavirus disease-19 (COVID-19), was first identified in Wuhan (China) in December 2019. The genetic sequence of the causative agent was found to have similarity with two highly pathogenic respiratory Betacoronaviruses, SARS-CoV (157) and MERS-CoV (158) and was called SARS-CoV-2 (159). This virus has currently infected more than 71 million individuals resulting in >1.5 million deaths based on Johns Hopkins University's live platform (160). Among the clinical signs of SARS-CoV-2 infection in humans, pneumonia was described in Chinese patients at the beginning of epidemic (161) (162). Later, a wider range of symptoms related to COVID-19 were described from mild-to-moderate symptoms including fever, cough, myalgia or loss of taste or smell to severe acute respiratory distress syndrome and sometimes multiorgan involvement, as well as shock. In severe cases, a cytokine storm (163) was shown to lead to systemic inflammatory response and endothelial damage, which may result in venous and arterial thrombotic events (164). The severity of disease was shown to be linked to advanced age and underlying conditions including hypertension, diabetes, cardiovascular disease, chronic respiratory disease and cancer (165). Even though information is still limited, post-COVID-19 symptoms have been observed in a significant number of patients at least for 4–8 weeks post-discharge from hospital (166). Interestingly, it was reported that some individuals were RT-PCR positive but were either asymptomatic or minimally symptomatic. Increasing evidences are showing that asymptomatic individuals can efficiently spread the virus (167).

To effectively tackle the COVID-19 ongoing pandemic, it is essential to come back to lessons learned from previous EVD epidemics, as well as to understand the similarities and differences between EVD epidemics and COVID-19 pandemic. It is crucial to evaluate the potential of strategies used to tackle EVD epidemics, especially the 2013–2016 West Africa epidemic, and to see if similar strategies could be applied for the current COVID-19 pandemic.

As in the context of EVD, the development of animal models that replicate human disease is a crucial step to study pathogenesis, establish correlates of protection, as well as assess the safety and efficacy of candidate vaccines and therapeutics. Some transgenic mouse (168–172), ferret (173–175), and NHP (176–178) models have been already developed to understand SARS-CoV-2 transmission, infection, as well as the development of local and systemic disease. Even though some asymptomatic cases have been suggested for EVD (70), the range of COVID-19 diseases seems to be wider from asymptomatic people (179) to mild-to-moderate disease in major cases and severe diseases in some individuals sometimes leading to death. Consequently, it becomes obvious that different types of animal models are necessary to replicate the range of illness severity and the variability of symptoms observed in humans. Animal models associated with studies in COVID-19 convalescents have a crucial role in dissection of protective immune responses and searching for correlates of protection.

During 2013–2016 and 2018 EVD epidemics in West Africa and DRC, clinical trials have been conducted in Ebola affected countries. Convalescent plasma (180, 181), monoclonal antibody (182, 183) and antiviral (184) therapies have been evaluated in the field. In the COVID-19 pandemic, the compassionate access to treatments and the implementation of clinical trials were rapidly adopted. Some drugs like hydroxychloroquine, remdesivir, lopinavir/ritonavir ± interferon beta-1A have been or are currently being evaluated in clinical trials including the Solidarity (185) and DisCoVeRy trials (**NCT04315948**), while the Recovery trial is also testing the efficacy of anti-inflammatory drugs such as dexamethasone or Tocilizumab, the antibiotics Azythromycin, as well as COVID-19 convalescent plasma (186). In addition, the lead Ebola vaccines such as rVSV-ZEBOV, Ad26.ZEBOV-MVA-BN-Filo, ChAd3-EBO-Z were also successfully evaluated in the field (187). Currently, 163 COVID-19 vaccines are in preclinical evaluation and 51 are in clinical trials including 6 in Phase III based on WHO communication on the 2nd of December 2020 (188). These candidate vaccines include replicating or non-replicating viral vectored vaccines, DNA vaccines, mRNA vaccines, autologous dendritic cell-based vaccine, and inactive virus vaccines (189). Some of them are currently in clinical trials overseas where the viral circulation is high. For instance, the efficacy of ChAdOx1 nCoV-19 also using a chimpanzee adenovirus vector similarly to ChAd3-EBO-Z, is currently under evaluation in Phase III trials in diverse population cohorts in the UK but also in Brazil and South Africa (190). The safety and immunogenicity of this vaccine in a prime-boost regimen was shown to be safe in young and old adults (191) and a Phase III interim analysis indicated that the vaccine was 70.4% effective when combining data from two dosing regimens but up to 90% efficacy in one regimen (192). The mRNA-based vaccine candidates BNT162b2 and mRNA-1273 announced to be 95% (193) and 94.5% effective

(194), respectively. The mRNA-based vaccine BNT162b2 was approved by the UK regulators for use on the 2nd of December 2020 (195), by Health Canada on the 9th of December 2020 (196) and the FDA granted emergency use authorisation on the 11th of December 2020 (197). Consequently, the integration of treatment and vaccine clinical trials into epidemic response is considered as one of best strategies to tackle epidemics and learn about immune responses in humans.

As in the context of EVD epidemics, international efforts from public health groups, universities, vaccine developers, regulators and funders are the key to progress in understanding SARS-CoV-2 infections, search for correlates of protection in COVID-19 convalescents and animal models, as well as to develop efficient therapeutics and vaccines.

AUTHOR CONTRIBUTIONS

SL and TT wrote the manuscript and designed the figures. JM designed the tables and revised the manuscript. MC revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

SL, TT, and MC are supported by the US Food and Drug Administration (grant number: HHSF223201510104C). JM is funded *via* PHE PhD studentship program.

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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.

frontiers in Immunology

ORIGINAL RESEARCH
published: 26 February 2021
doi: 10.3389/fimmu.2021.598778

A Proteome-Wide Immunoinformatics Tool to Accelerate T-Cell Epitope Discovery and Vaccine Design in the Context of Emerging Infectious Diseases: An Ethnicity-Oriented Approach

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Edited by:
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Reviewed by:
Julio Alonso Padilla, Instituto Salud Global Barcelona (ISGlobal), Spain
Srinivasa Reddy Bonam, Institut National de la Santé et de la Recherche Médicale (INSERM), France

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Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: *25 August 2020*
Accepted: *11 January 2021*
Published: *26 February 2021*

Citation:
Oyarzun P, Kashyap M, Fica V, Salas-Burgos A, Gonzalez-Galarza FF, McCabe A, Jones AR, Middleton D and Kobe B (2021) A Proteome-Wide Immunoinformatics Tool to Accelerate T-Cell Epitope Discovery and Vaccine Design in the Context of Emerging Infectious Diseases: An Ethnicity-Oriented Approach. Front. Immunol. 12:598778. doi: 10.3389/fimmu.2021.598778

Emerging infectious diseases (EIDs) caused by viruses are increasing in frequency, causing a high disease burden and mortality world-wide. The COVID-19 pandemic caused by the novel SARS-like coronavirus (SARS-CoV-2) underscores the need to innovate and accelerate the development of effective vaccination strategies against EIDs. Human leukocyte antigen (HLA) molecules play a central role in the immune system by determining the peptide repertoire displayed to the T-cell compartment. Genetic polymorphisms of the HLA system thus confer a strong variability in vaccine-induced immune responses and may complicate the selection of vaccine candidates, because the distribution and frequencies of HLA alleles are highly variable among different ethnic groups. Herein, we build on the emerging paradigm of rational epitope-based vaccine design, by describing an immunoinformatics tool (Predivac-3.0) for proteome-wide T-cell epitope discovery that accounts for ethnic-level variations in immune responsiveness. Predivac-3.0 implements both CD8+ and CD4+ T-cell epitope predictions based on HLA allele frequencies retrieved from the Allele Frequency Net Database. The tool was thoroughly assessed, proving comparable performances (AUC ~0.9) against four state-of-the-art pan-specific immunoinformatics methods capable of population-level analysis (NetMHCPan-4.0, Pickpocket, PSSMHCPan and SMM), as well as a strong accuracy on proteome-wide T-cell epitope predictions for HIV-specific immune responses in the Japanese population. The utility of the method was investigated for the COVID-19 pandemic, by performing *in silico* T-cell epitope mapping of the SARS-CoV-2 spike glycoprotein according to the ethnic context of the countries where the ChAdOx1 vaccine

is currently initiating phase III clinical trials. Potentially immunodominant CD8+ and CD4+ T-cell epitopes and population coverages were predicted for each population (the Epitope Discovery mode), along with optimized sets of broadly recognized (promiscuous) T-cell epitopes maximizing coverage in the target populations (the Epitope Optimization mode). Population-specific epitope-rich regions (T-cell epitope clusters) were further predicted in protein antigens based on combined criteria of epitope density and population coverage. Overall, we conclude that Predivac-3.0 holds potential to contribute in the understanding of ethnic-level variations of vaccine-induced immune responsiveness and to guide the development of epitope-based next-generation vaccines against emerging pathogens, whose geographic distributions and populations in need of vaccinations are often well-defined for regional epidemics.

Keywords: immunoinformatics, T-cell epitope, ethnicity, emerging-infectious disease, epitope discovery, vaccine design, SARS-CoV-2

INTRODUCTION

Emerging infectious diseases (EIDs) are defined as infections whose incidence or geographic range is rapidly increasing or threatens to increase in the near future. EIDs have emerged at an unprecedented rate due to a plethora of factors driven by globalization and climate change, posing serious threats to public health and economies (1). Wildlife is considered to be the major source of viral pathogens causing emerging zoonotic outbreaks (2), including mosquito-borne diseases (e.g., dengue, Zika fever) (3), rodent-borne hantaviruses (4) and bat-borne diseases (5), such as Ebola hemorrhagic fever, Nipah virus encephalitis and severe acute respiratory syndrome (SARS). According to the World Health Organization (WHO), disease outbreaks and epidemics caused by emerging pathogens are increasing in frequency over the past decades (6). In late 2019, the novel SARS-like CoV designated as 2019-nCoV (SARS-CoV-2) emerged in the city of Wuhan, China, causing a global pandemic with high morbidity and mortality (7). As of August 23nd 2020, SARS-CoV-2 has caused ~23 million cases of the disease (COVID-19) and ~800,000 deaths across the world.

Vaccination is a critical tool in the response to unpredictable outbreaks of EIDs, but the complete process for bringing a vaccine from the research laboratory to the market is long, complex, and expensive (8). Traditional live-attenuated or whole-inactivated viral vaccines are slow to develop and have biosafety issues that make them poorly suited to respond to a rapidly evolving pandemic crisis, especially without the advantage of time and prior knowledge or experience with viral growth or pathogenesis mechanisms (1). Vaccine development for emerging pathogens is thus moving onto faster and more advanced recombinant and nucleic acid-based (DNA/RNA-based) approaches that address these issues by incorporating modern technologies and a rational design basis (9, 10). Accordingly, among the most advanced COVID-19 vaccine candidates are those encoding the SARS-CoV-2 spike (S) protein, which have proved to be safe and immunogenic over clinical development stages (11–13). These type of vaccines has recently initiated phase III clinical trials to evaluate protective efficacy at population level, including a recombinant adenovirus-vectored vaccine (ChAdOx1; NCT04400838) and a lipid nanoparticle-encapsulated mRNA-based vaccine (mRNA-1273; NCT04470427).

HLA class I and class II molecules play a central role in the immune response by presenting peptide antigens to CD8+ cytotoxic T-cells (CD8+ T-cell epitopes) and to CD4+ helper T-cells (CD4+ T-cell epitopes). However, the huge variability of the HLA system is a major issue for epitope-based vaccine design, since individuals display different sets of HLA alleles with variable ligand specificities (HLA-epitope restriction) and expression frequencies that substantially differ among ethnicities (14). Careful consideration of the HLA genetic background is thus paramount to ensure effectiveness and ethnically unbiased population coverage during vaccine development, especially considering variations in T-cell responses across multiple ethnicities (15). This problem is underscored by a significant body of evidence accounting for population-level associations of HLA polymorphisms with vaccine-induced immune responses (16) and also with vaccine failure (17, 18). Likewise, COVID-19 has been associated with disproportionate mortality amongst world populations (19, 20) and recent literature indicates that individuals from minority ethnic communities are at increased risk of infection from SARS-CoV-2 and subsequently adverse clinical outcome (21, 22). Individual genetic variations of the HLA system (different genotypes) may help explain differential T-cell mediated immune responses to the virus and could potentially alter the course of this disease (23), which has been well-described for the closely related SARS-CoV (24, 25).

Epitope-based vaccination is gaining interest in the scientific community, which allows for rational design of the immunogens based on short protein regions or peptides that avoid non-essential viral components and potentially toxic or immunosuppressive protein fragments (26, 27). These vaccines offer the prospect for a more prominent role of HLA-restricted T-cell immune responses ("T-cell vaccines"), by inducing large repertoires of T-cell specificities and further enabling rapid and economic large-scale production through recombinant DNA technology (28). Predicting the specificity of HLA class

I-restricted CD8+ T-cell epitopes and HLA class II-restricted CD4+ T-cell epitopes is also a major consideration for epitope-based vaccine design, due to the influence of the HLA phenotype in the ability to mount effective immune responses (16). Therefore, immunoinformatics tools play a key role in this arena, as they allow to accelerate epitope discovery and vaccine design through *in silico* mapping of thousands of peptides (proteome-wide analysis) and by helping reduce the time and cost involved in experimental testing (21). In addition, these tools offer a framework to rationally deal with the enormous diversity HLA proteins, which reached 27,599 HLA alleles as of July 2020 (20,192 HLA class I and 7,407 HLA class II alleles), according to the IMGT/HLA Database (Release 3.41.0) (29).

A few immunoinformatics methods have been developed to aid the selection of T-cell epitopes by considering the fraction of individuals potentially covered by epitope-based vaccines (30, 31). Our previously reported method Predivac-2.0 optimizes the selection of HLA class II-restricted CD4+ T-cell epitopes predicted for specific target populations (32, 33). Herein, we extended our specificity-determining residues (SDRs) approach to CD8+ T-cell epitope prediction and subsequently describe a substantial enhancement of the method to build on the emerging paradigm of rational epitope-based vaccine design. The new Predivac-3.0 tool was successfully cross-validated and benchmarked against state-of-the-art pan-specific methods suited for population level analyses [NetMHCPan 4.0 (34), Pickpocket (35), PSSMHCPan (36) and SMM (37)], which are capable of using available experimental MHC binding data to infer binding preferences toward uncharacterized MHC molecules (38).

Predivac-3.0 was investigated for proteome-wide ethnicity-driven predictions to guide the discovery and selection of immunodominant HIV-1 specific T-cell epitopes, as well as the identification of epitope-dense regions (clusters) of CD8+ and CD4+ T-cell epitopes associated with high-population coverages (hotspots), in agreement with previous work showing the utility of *in silico* tools to identify epitope hotspots in the sequence of protein immunogens tested in subjects from different ethnic backgrounds (39, 40). We finally demonstrate the utility of the tool in the context of vaccine development for COVID-19 pandemic, by providing insight into putative T-cell epitopes and hotspots in the SARS-CoV-2 spike glycoprotein that are potentially immunodominant for the countries where the ChAdOx1 vaccine (University of Oxford/AstraZeneca) is currently carrying out phase III clinical trials (The United Kingdom, South Africa and Brazil).

To the best of our knowledge this is the first computational approach for ethnicity-driven proteome-wide discovery of T-cell epitopes and hotspots capable of inducing large repertoires of immune specificities in populations at risk of emerging pathogens, especially because the geographic distributions of the zoonotic viruses and populations in need of vaccinations are often well-defined for regional epidemics. Therefore, Predivac-3.0 holds potential to contribute in the understanding of vaccine-induced immune responsiveness in population contexts and to aid the rational design of epitope-based next-generation immunogens considering ethnic-level variations of vaccine induced immune responses for EIDs.

MATERIALS AND METHODS

Semi-Automated Identification of SDR Positions

The identification of specificity-determining residues (SDRs) involved in peptide ligand-protein recognition events has been described previously for protein kinases (the Predikin tool) (41, 42) and for HLA class II proteins (the Predivac tool) (33), based on the inspection of crystal structures. Herein we introduce an improvement to the method for SDR determination, by implementing a Python-based semi-automated workflow to assist and simplify the identification of SDR positions in the peptide-HLA protein interface. We first constructed a dataset comprising 57 peptide-HLA class I complex structures (19 unique allotypes) available at the Protein Data Bank (PDB) (43) (**Table S1**). The structures were manually processed to select only the α chain with the corresponding bound peptide, focusing the analysis on the recognition region (groove) formed by the floor (eight antiparallel β-sheet folds) flanked by two polymorphic helical regions (α1 and α2 domains). The interaction interfaces were analyzed with the standalone version of the Arpeggio tool (44), which uses geometrical and biochemical features to automatically calculate and classify interatomic interactions between each pairs of atoms for a wide range of contact types (hydrogen bonds, halogen bonds, carbonyl interactions, hydrophobic interactions, among others). We assessed the role of each α-chain residues in contributing to peptide binding by considering all possible non-covalent pairwise interactions to extract nearest-neighbor atoms at each peptide position (p1 to p9), using Arpeggio´s default cut-off distance (5 Å). A consensus list of positions mainly involved in determining the interactions was built with a threshold of 30% occurrence, i.e. the residue in the interaction was present in 30% or more of the structures. Subsequently, analysis of conservation/variability and identification of polymorphic positions were carried out by means of two metrics: (i) Shannon entropy (45) and (ii) conservation score metrics described by Valdar in 2002 (46), which is implemented in the AACon tool[1]. AACon receives as input a list of aligned sequences in Clustal format, which was performed using MAFFT with default parameters (47) over a dataset of 10,089 HLA class I protein sequences (allotypes) from the Immuno Polymorphism Database (IPD)-IMGT/HLA Database release 3.37 (29). Finally, a small set of critical and polymorphic residue positions from the consensus list was selected as those dictating specific interactions in HLA class I proteins.

Software Implementation

The new Predivac-3.0 method was re-written in Python 3.7 It consists of a main module that queries a purposed-built database of HLA class I and II specificity-determining residues (SDRs) that are associated with HLA protein sequences with high-affinity peptide binders (PredivacDB) and a database of HLA allele frequencies

[1] http://www.compbio.dundee.ac.uk/aacon/

available at the Allele Frequency Net Database[2] (AFND) (48). The PredivacDB was updated to include both experimentally validated high-affinity peptide ligands for HLA class I and class II proteins (**Table S2**). In total, the database contains 26,068 peptides, accounting for 77 HLA class I alleles (23,373 peptides) and 29 HLA class II alleles (2,695 peptides) that were exported and filtered from the Immune Epitope Database (IEDB) (49). HLA class I peptides with sequence length of 9 residues and experimentally determined binding affinity ($K_D/IC_{50}/EC_{50}$) < 500 nM were considered, while sequences were removed if their binding affinity was determined by whole-cell based assays, non-natural atoms were present or Ala percentage > 50%. The method implemented by Predivac-3.0 requires from the user to provide the query proteome (Fasta file with multiple sequences) and to select the target population (country/region). The tool then fetches HLA allele data for this population (from the AFND) and automatically extracts SDRs information from the HLA query proteins to perform *in silico* T-cell epitope mapping. This is carried out by implementing the Predivac scoring scheme based on peptide ligands available in the PredivacDB. The whole procedure allows to predict ethnicity-driven CD8+ and CD4+ T-cell epitopes along with performing population coverage analysis, through a workflow that is subsequently explained.

Scoring Scheme

The Predivac binding score is calculated by establishing a predictive correlation between the SDRs in the HLA query protein(s) and the SDRs associated with HLA class I or class II proteins of known specificity from a pre-generated database (PredivacDB), by following the next steps: (i) SDRs are identified in the HLA protein sequence; (ii) PredivacDB is queried with the SDRs to retrieve peptide ligands associated with HLA proteins sharing similar residues in these positions (SDRs are considered similar if their sequence comparison using the BLOSUM62 substitution matrix returns a positive score); (iii) amino acid frequencies and weights are calculated from the binding data and (iv) *in silico* T-cell epitope mapping is carried out by parsing the protein sequence (query) into overlapping 9-mer segments (peptides), which are recursively assigned a binding score with the SDR-derived position weight matrix (sliding window technique). Predivac-3.0 selects by default T-cell epitopes scoring in the top 1% of the full set of peptides for a given protein, i.e. it employs a Peptide Percentile Rank (PPR) of binding scores of 1 (PPR = 1). However, the user is allowed to retrieve a greater number of putative T-cell epitopes by setting higher stringencies of 1, 2 or 3.

Ethnicity-Driven T-Cell Epitope Mapping of Viral Proteomes

A workflow of the algorithm is presented in **Figure 1**, showing that Predivac-3.0 accepts as input both protein sequences (Fasta file) and full proteome sequences (multi-Fasta file). The method runs for single HLA alleles (allele-specific T-cell epitope prediction) and for specific target populations (ethnicity-driven T-cell epitope prediction), by fetching HLA allele frequency distributions from ethnic populations available at the AFND. Ethnicity-driven T-cell epitope mapping follows a five-step process: (i) the user sets a target geographic region or country; (ii) the program retrieves from the AFND all available HLA class I and class II allele frequencies for population samples occurring in this country/region; (iii) the program applies the Predivac scoring scheme to predict T-cell epitopes for each HLA allele and then it searches for promiscuous epitopes restricted to as many alleles as possible in the target population; (iv) population coverage is calculated for each promiscuous T-cell epitope as the fraction of individuals that would be potentially covered in the selected target population, by implementing a previously reported algorithm (50); and (v) two alternative methods are implemented to select T-cell epitopes based on population coverage: (a) Epitope Discovery and (b) Epitope Optimization.

Epitope Discovery

This method outputs a full list of single putative T-cell epitopes with their corresponding positions in the query proteins sorted by population coverage, providing thus a top-down peptide ranking from the highest to minimal coverage calculated for the target population. In addition, the user is allowed to set a particular Population Coverage Threshold (PCT) to filter the report for T-cell epitopes delivering population coverage values higher than a given threshold (%). By default, the method retrieves all predicted T-cell epitopes (PCT = 0%).

Epitope Optimization

Predivac-3.0 implements a genetic algorithm (GA) that explores numerous combinations of putative epitopes to find a combination of *l* epitopes that maximizes the target population coverage. The pseudocode of the GA is shown below:

Algorithm 1 Genetic Algorithm

Input: Initial parameter for GA

```
PopSize ← 100
MaxIteration ← 50
Stopcicle ← False
l ← 1
PopFitness ← Genetic.fitness(epitopeHits)
MaxFitness←max(PopFitness)
BestIndividual ← GetIndividual(PopFitness)
while MaxFitness ≤ 99 and not stopcicle do
stopcond ← False
i ← 1
Population ← InitPopulation(epitopesHits,PopSize)
Population ← PairwiseComb(Population,BestIndividual)
while i < MaxIteration and not stopcond do
PopFitness ← Genetic.fitness(Population)
Parents ← Genetics.selection(Population,PopFitness,0.2)
O f f spring ← Genetic.crossover(Parents,Population)
Population ← Genetic.mutatePopulation(O f f spring,mutation Rate = 0.2)
NewPopFitness ← Genetic.Fitness(Population)
MaxFitness ← max(NewPopFitness)
BestIndividual ← GetIndividual(NewPopFitness)
if StopCondition() then
stopcond ← True
end if
i ← i+ 1
end while
i ← i + 1
```

(Continued)

[2] http://www.allelefrequencies.net/

Continued

Algorithm 1 Genetic Algorithm

Input: Initial parameter for GA

23: **If StopCicle**() **then**
24: *stopcicle* ← *True*
25: **end if**
16: **end while**
27: **return** the best solution

Predivac-3.0 seeds a first epitope (l = 1) to start the iterative process, which corresponds to the epitope (or individual) delivering the highest population coverage in the target population (*BestIndividual*). Each individual represents an epitope/HLA restricted alleles predicted by Predivac-3.0 (*EpitopesHits*). Then, a random population of 100 individuals is generated at each GA cycle (loop in lines 4-26) and subsequently paired with the previous *BestIndividual*. At each GA iteration (inner loop in lines 9-21), each individual is assigned a fitness score equal to the population coverage calculated for the target population (region/country). The top-20 individuals are selected to breed a new set of individuals by random, pairwise crossover, i.e. the top quintile of epitope combinations that retrieve the highest population coverage. Inner iterations are run until *MaxIteration* is achieved or until fitness score does not change during last 10 iterations. The GA runs until population coverage reaches a *MaxFitness* ≥ 99% or until the *MaxFitness* value does not change in two consecutive cycles by considering 3 significant figures (*StopCycle*), upon which the list of epitopes (best solution) is returned.

Immunodominant T-Cell Epitope Clusters and Hotspots

For proteome-wide analysis, the program scans the protein sequences to detect regions with high-epitope density that are associated with a high population coverage in the target country/region. This process is performed through the Epitope Discovery mode of Predivac-3.0, by detecting clusters of epitope overlaps (in 9-mer regions) or by detecting epitope-rich regions in windows-frames of user-defined length (in 30-mer regions, by default). In 9-mer regions, epitope density was determined by considering both partially and completely overlapping epitopes, while in 30-mer regions only completely (full-

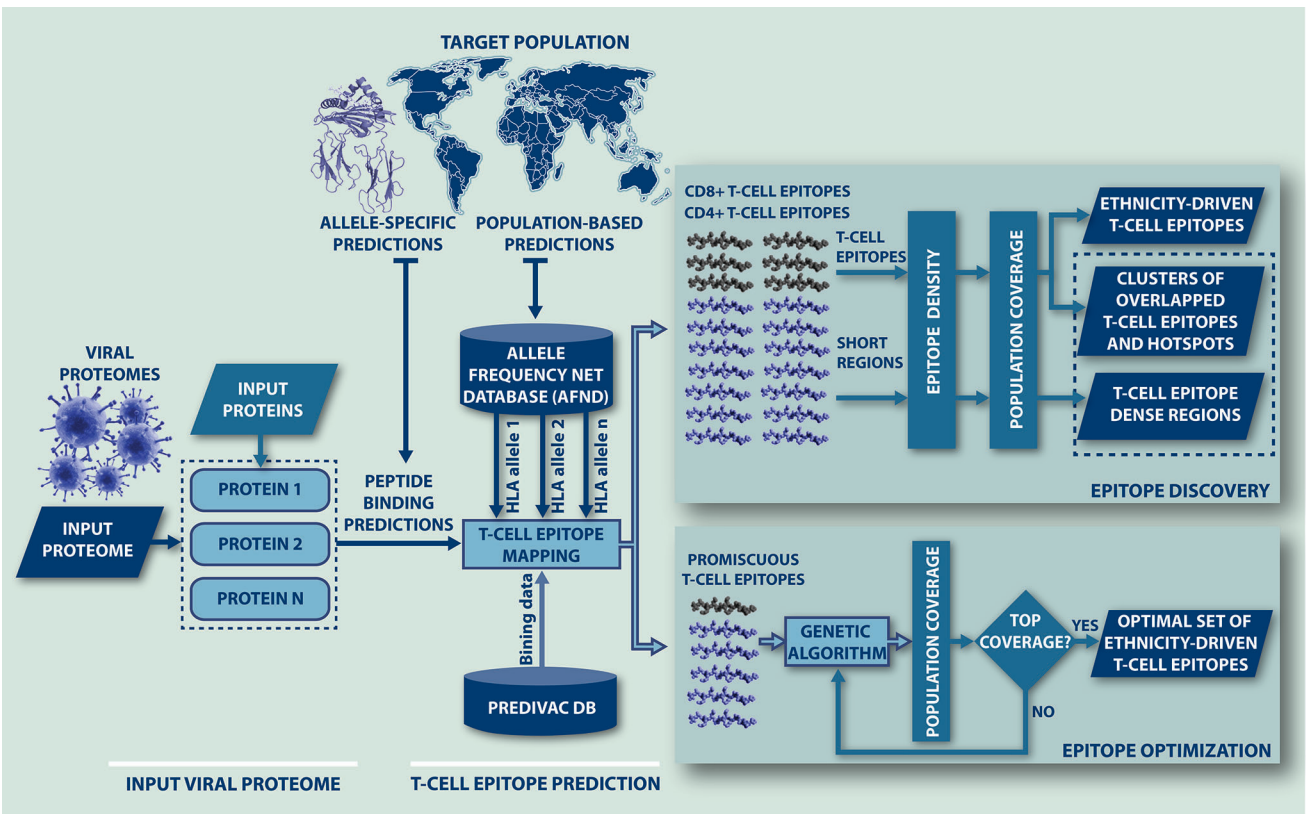

FIGURE 1 | Flow-chart representing the steps followed by Predivac-3.0 to perform *in silico* ethnicity-driven T-cell epitope mapping over viral proteomes. The user must input the sequence of the query proteome (or individual proteins) and set the target population (country or geographic region), upon which the program retrieves from the AFND the HLA class I and class II allele frequency data available for population samples occurring in this country/region. Then, it searches the input proteome/proteins for putative CD8+ and CD4+ T-cell epitopes and epitope-rich regions (clusters/hotspots) by applying the SDR-based approach and querying the PredivacDB (Epitope Discovery mode). Predivac-3.0 also implements a genetic algorithm that explores and optimizes T-cell epitope combinations maximizing population coverage (Epitope Optimization mode).

length) overlapping epitopes were employed. Clusters meeting the following criteria were selected for proteome-wide analysis: (i) epitope density ≥ 90% of the top amount of T-cell epitope overlaps (in a proteome basis) and (ii) population coverage ≥ 20% in the target population. The statistical significance of these clusters was determined through a simulation procedure consisting of randomly selecting (1000 times) 10% of same-length regions, using the average epitope density of each simulation as the epitope distribution to calculate p-values. Those regions having p-value < 0.001 were considered as clusters. Further, overlapping clusters were merged together and potentially the most reactive (immunodominant) regions with population coverages ≥ 80% were denoted as hotspots.

Validation of Allele-Specific Predictions

The predictive performance in identification of CD8+ T-cell epitopes was measured in terms of the area under the receiver operating characteristic curve (AUC), which is a graphical plot of the sensitivity versus the false positive rate (1 - specificity) as the discrimination threshold is varied. The AUC provides an indication of the accuracy of a prediction method, where an AUC = 1 corresponds to perfect predictions and AUC = 0.5 reflects random predictions. The method was assessed by leave-one-allele-out cross-validation (LOOCV) using a dataset of 17,425 high-affinity peptide binders restricted by 46 HLA class I alleles with 25 or more peptide ligands in PredivacDB, as previously reported (33). In addition, the method was benchmarked against the pan-specific methods NetMHCpan 4.0 (34), PickPocket (35), PSSMHCpan (36), and SMM (37). Two datasets were employed: (i) the IEDB-dataset, 5750 experimentally determined CD8+ T-cell epitopes (restricted by 47 HLA class I alleles) selected from the IEDB database and (ii) the DFRMLI-dataset, 887 high-affinity viral peptide ligands (tumor antigens were excluded) restricted by 7 HLA class I alleles (HLA-A*01:01, A*02:01, A*03:01, A*11:01, A*24:02, B*07:02, B*08:01, and B*15:01) (dataset available in **Supplementary Data Sheet 1**). The DFRMLI-dataset was built from high-throughput binding affinity data available at the Dana-Farber Repository for Machine Learning in Immunology[3], which accounts for the cytomegalovirus (CMV) matrix protein pp65 (51), the human respiratory syncytial virus (RSV) and the human metapneumovirus (MPV) (52).

Assessment of Population-Based Predictions

The ability of Predivac-3.0 to identify ethnicity-driven T-cell epitopes was tested on the HIV-1 proteome, by comparing CD8+ T-cell epitope predictions against a validation dataset derived from Los Alamos HIV Molecular Immunology Database[4] (here referred as the HIV-dataset), which consists of 103 unique CD8+ T-cell epitopes that were experimentally determined from *in vivo/in vitro* studies carried out in Japan (53) (dataset available in **Supplementary Data Sheet 2**). This dataset includes immunodominant T-cell epitopes from the following HIV-1 proteins, using the reference strain HXB2 (GenBank K03455): Integrase (Pol; UniProt ID: P04585), envelope glycoprotein (gp160; UniProt ID: P04578), Gag polyprotein (Gag; UniProt ID: P04591), Nef protein (Nef; UniProt ID: P04601), viral protein R (Vpr; UniProt ID: P69726), and viral infectivity factor (Vif; UniProt ID: P69723). The predictive accuracy and efficiency were calculated by the following equations:

$$Accuracy\ (\%) = \left(\frac{number\ of\ correct\ matches}{total\ number\ of\ validation\ epitopes}\right) \times 100 \quad 1$$

$$Efficiency\ (\%) = \left(\frac{number\ of\ correct\ matches}{total\ number\ of\ predicted\ epitopes}\right) \times 100 \quad 2$$

A correct match means that a predicted 9-mer T-cell epitope is equal to or it is contained in the sequence of an experimentally determined T-cell epitope from the validation dataset. Several analyses were further carried out regarding the capability of Predivac-3.0 to identify well-described immunodominant T-cell epitopes, including Japanese-specific protective epitopes from Gag and Pol protein regions included in the T-cell mosaic vaccine tHIVconsvX (**Table S3**) (44) and a number of T-cell epitopes recognized across multiple ethnicities (**Table S4**) (15).

Proteome-Wide Visualization

Circular representations of the viral proteomes were generated to visualize ethnicity-driven T-cell epitope distributions, population coverage and immunodominant clusters (hotspots) across the viral proteins. Proteome maps were constructed using the Circos package (54), which renders concentric layers of information in the following data dimensions (from outside inward): (i) location of CD8+ and CD4+ T-cell epitopes relative to the reference strain HXB2 (epitope mapping); (ii) number of T-cell epitopes spanning each amino acid position (epitope density maps); (iii) percentage of individuals potentially covered by predicted T-cell epitopes in user-defined target populations (population coverage), both at each amino acid position (in nonameric clusters) and for windows frames of user-defined amino acid length (epitope-rich regions); and (iv) short epitope-rich regions associated with high-population coverages in the target population (hospots).

SARS-CoV-2 Case Study

The spike glycoprotein of SARS-CoV-2 (UniProt ID: P0DTC2) was investigated with the Predivac-3.0 tools (using the Epitope Discovery and Epitope Optimization modes). The goal of this analysis was to identify immunodominant CD8+ and CD4+ T-cell epitopes and putative clusters/hotspots that are potentially specific or common to the populations of the three countries (The United Kingdom, South Africa and Brazil) where phase III clinical trials are currently underway for the ChAdOx1 adenovirus-vectored vaccine (University of Oxford/AstraZeneca). The Japanese population was additionally considered for comparison purposes to include an Asian ethnic background. HLA allele frequency distributions in the four target populations are illustrated in **Figure 2** (AFND data), including HLA class I (loci A and B) and HLA class II alleles (locus DRB).

[3] http://projects.met-hilab.org/DFRMLI/
[4] http://www.hiv.lanl.gov/content/immunology/

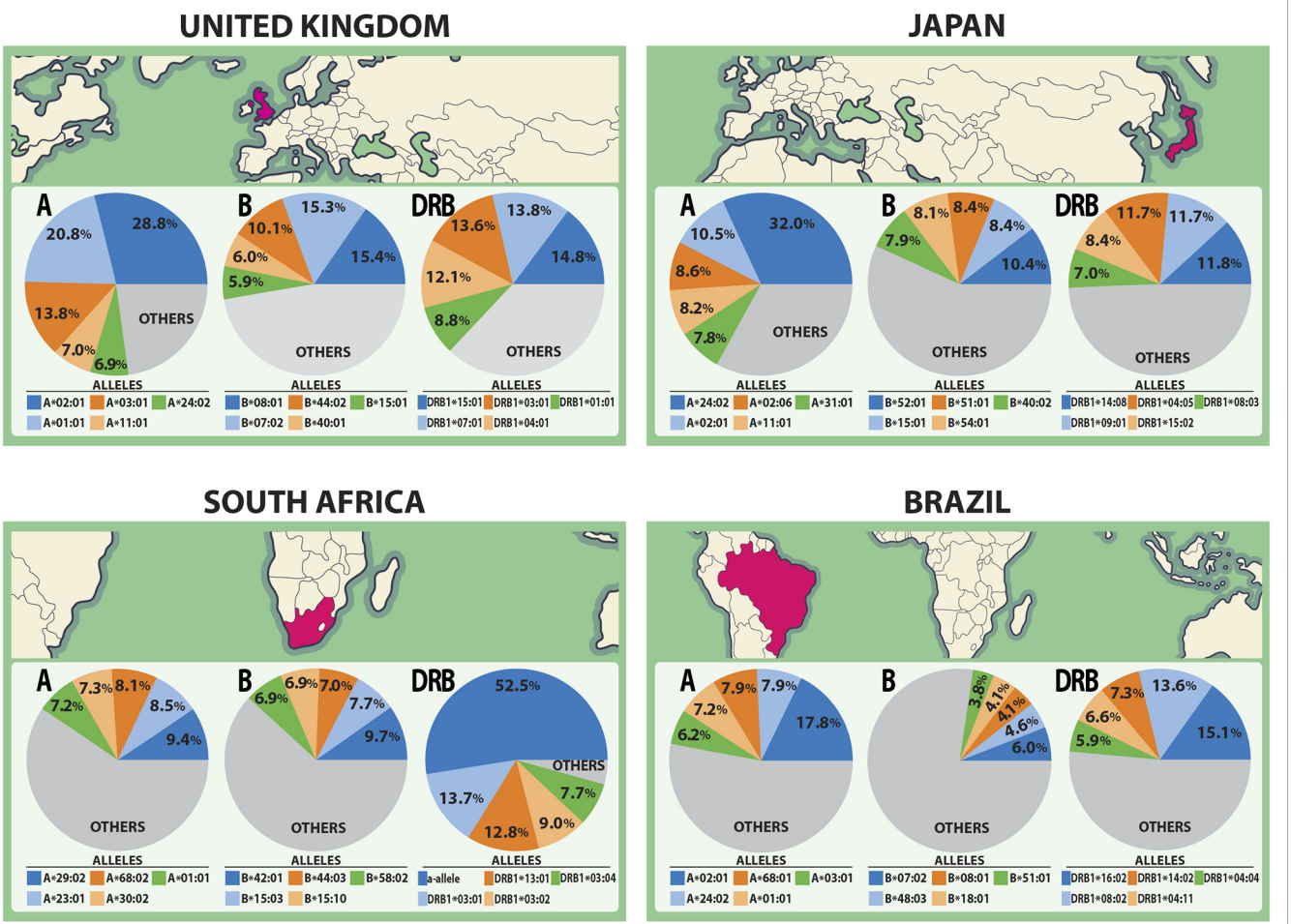

FIGURE 2 | HLA class I and class II allele distributions in Japan and the countries where the ChAdOx1 vaccine (University of Oxford/AstraZeneca) is currently developing phase III clinical trials (The United Kingdom, South Africa and Brazil). The pie charts highlight the five most frequent HLA class I alleles (A and B loci) and class II alleles (DRB locus) in each population according to data from the AFND.

RESULTS

Allele-Specific T-Cell Epitope Predictions

Predivac-3.0 was implemented and assessed for its new capability of CD8+ T-cell epitope prediction, based on SDR positions that were determined in the peptide-HLA (pHLA) class I interaction interface through a combination of structural analysis of pHLA complex crystal structures and sequence analysis of HLA polymorphisms (see *Materials and Methods*). SDRs in the HLA protein sequence that were selected and implemented in the software are the following positions (for each P1-P9 peptide ligand position): P1 (62, 163), P2 (7, 9, 62, 99), P3 (66, 156, 159), P6 (70, 73, 156), P7 (152, 155, 156), P8 (76, 77) and P9 (77, 97, 116). Interactions for P4 and P5 were not considered, since the side-chains of amino acid residues in the middle of the peptides protrude out of the binding groove, delivering only marginal contributions to specificity.

The LOOCV procedure to determine the accuracy on HLA class I alleles involves the exclusion of a single allele from the database and then assessing the performance using the binding data associated with that particular excluded allele. To build balanced datasets for AUC calculation, we followed an established validation strategy based on splitting the source protein of each epitope (positive) into overlapping peptides of the same length, and all peptides except the annotated peptide were taken as negatives. The predictive performance is shown in **Figure 3A**, proving a strong capability to predict high-affinity peptide ligands (overall AUC = 0.8). Predivac-3.0 was also benchmarked against state-of-the-art pan-specific methods for 8 HLA class I alleles using the DFRMLI-dataset (high-affinity peptide binders), yielding AUC values of 0.909 (Predivac-3.0), 0.900 (NetMHCpan-4.0), 0.914 (Pickpocket), 0.905 (PSSMHCpan) and 0.928 (SMM-align) (**Figure 3B**). Finally, performance comparison for CD8+ T-cell epitope

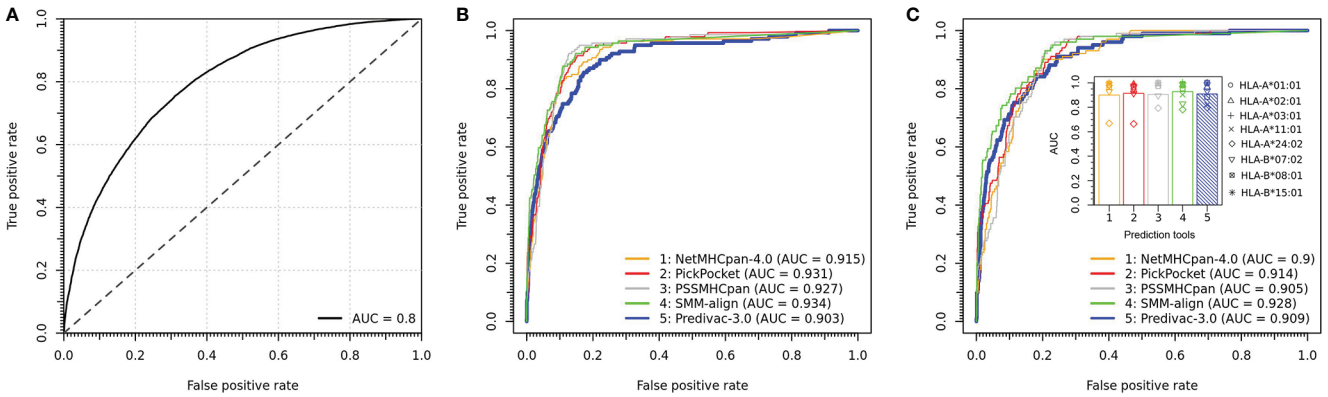

FIGURE 3 | Validation of CD8+ T-cell epitope predictions. **(A)** Overall predictive performance of Predivac-3.0 measured by leave-one-out cross-validation over 46 HLA class I alleles having more than 25 associated peptide ligands in PredivacDB. The benchmark against pan-specific methods (NetMHCpan 4.0, Pickpocket, PSSMHCpan and SMM-align) using **(B)** a dataset of experimentally validated CD8+ T-cell epitopes derived from the IEDB Analysis Resource (IEDB-dataset) and **(C)** a dataset of high-affinity peptide ligands derived from the Dana-Farber Repository for Machine Learning in Immunology (DFRMLI-dataset) (See *Material and Methods*).

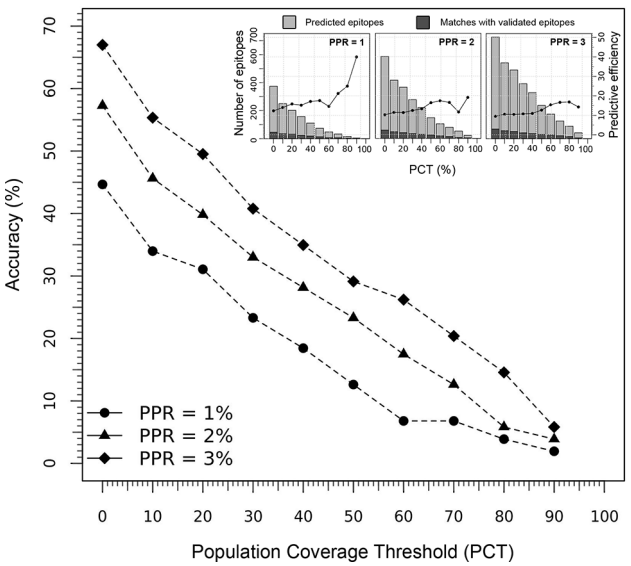

FIGURE 4 | Predictive performance of Predivac-3.0 in identification of HIV-1 CD8+ T-cell epitopes specific for the Japanese population. The main figure illustrates the accuracy delivered by the tool at Peptide Percentile Rank (PPR) values of 1, 2 and 3, while the threshold of individuals potentially covered by the epitopes in this population was varied between 0-100% (PCT). The inset figures show the predictive efficiency of the tool at each PCT (for PPR 1, 2 and 3), while stacked bars represent the proportion of positive predictions or matches (dark grey) with respect to the total number of predictions (light grey).

predictions (IEDB-dataset) resulted in AUC values of 0.903 (Predivac-3.0), 0.915 (NetMHCpan4.0), 0.931 (PickPocket), 0.927 (PSSMHCpan) and 0.934 (SMM-align) (**Figure 3C**).

Ethnicity-Driven T-Cell Epitope Prediction

The performance of the tool to deliver correct predictions of CD8+ T-cell epitopes and immunodominant hotspot was evaluated for the specific ethnic context of the Japanese population (using the Epitope Discovery and Epitope Optimized modes), by determining the accuracy and efficiency of the T-cell epitope mapping algorithm on the HIV-1 proteome (**Figure 4**). Using default parameters (PPR = 1; PCT = 0%), Predivac-3.0 predicted 374 putative CD8+ T-cell epitopes and detected 46 epitopes out of a top number of 103 T-cell epitopes in the HIV-dataset (accuracy = 44.7%; efficiency = 12.3%). The accuracy curves followed a comparable declination slope for the three PPR values (1, 2 and 3) and behaved similarly in the identification of CD8+ T-cell epitopes as the PCT was varied from 0 to 100%, with top accuracies (at PCT = 0%) of 44.7% (PPR = 1), 57.3% (PPR = 2) and 67% (PPR = 3). The search reduced up to 202 peptides by increasing the PCT to 20%, reaching a slightly lower accuracy of 31.1% (32 correct matches) with an increase in the predictive efficiency up to 15.8%. The predictive efficiency for PPR 1 continues to rise as the PCT increases, unlike for PPR 2 and PPR 3 that tend to maintain around average values. For PPR 1, the average efficiency (19.3%) proved statistically higher ($p<0.05$) than that for PPR 2 (13.9%) and PPR 3 (12.8%). For details, see **Table S5**.

Proteome-Wide Analysis

Circular maps for visualization of ethnicity-driven CD8+ and CD4+ T-cell epitopes are presented in **Figure 5** for the Japanese population, which provide information on the distribution of T-cell epitopes density across the HIV-1 proteome (rings 1–4), population coverage potentially afforded in the Japanese population (rings 5–7) and putative T-cell epitope clusters and hotspots (ring 8), both for nonameric T-cell epitope

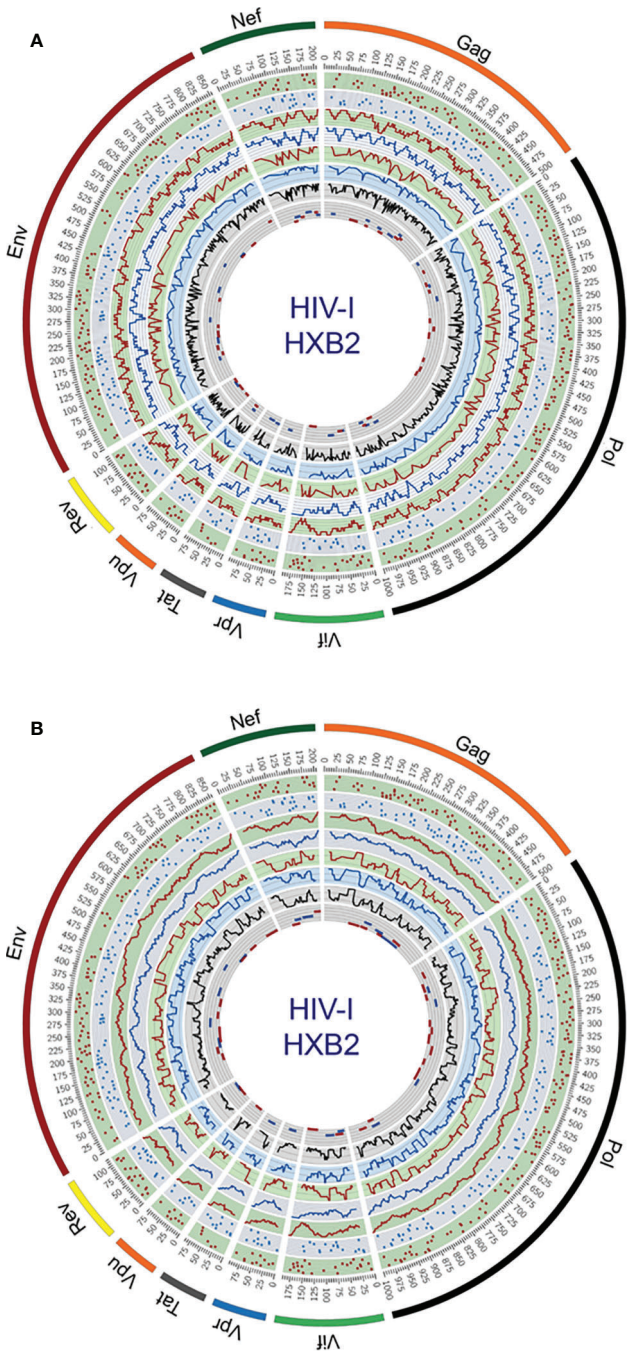

FIGURE 5 | Circular plots showing the distribution of putative T-cell epitope clusters (hotspots) mapped onto the HIV-1 proteome and their corresponding population coverages predicted for the Japanese population, either for **(A)** nonameric windows frames of overlapped T-cell epitopes and **(B)** 30 residues long window frames of epitope-rich regions. Circles display the following features from the outside inward, based on the numbering standard of the reference strain HXB2 (GenBank K03455) for amino acid coordinates: ring 1, CD8+ T-cell epitope map (magenta dots), ring 2, CD4+ T-cell epitope map (blue dots); ring 3, CD8+ T-cell epitope density plot (magenta plot); ring 4, CD4+ T-cell epitope density map (blue plot); ring 5, population coverage calculated for CD8+ T-cell epitope clusters (magenta plot); ring 6, population coverage calculated for CD4+ T-cell epitope clusters (blue plot); ring 7, population coverage calculated for the combined set of CD8+ and CD4+ T-cell epitope clusters (black plot) and ring 8, putative hotpots for CD8+ T-cell epitope clusters (magenta lines) and CD4+ T-cell epitope clusters (blue lines). The innermost ring is divided by five parallel lines delimiting segments of population coverage ranges between 0-19%, 20-39%, 40-59%, 60-79% and 80-100%.

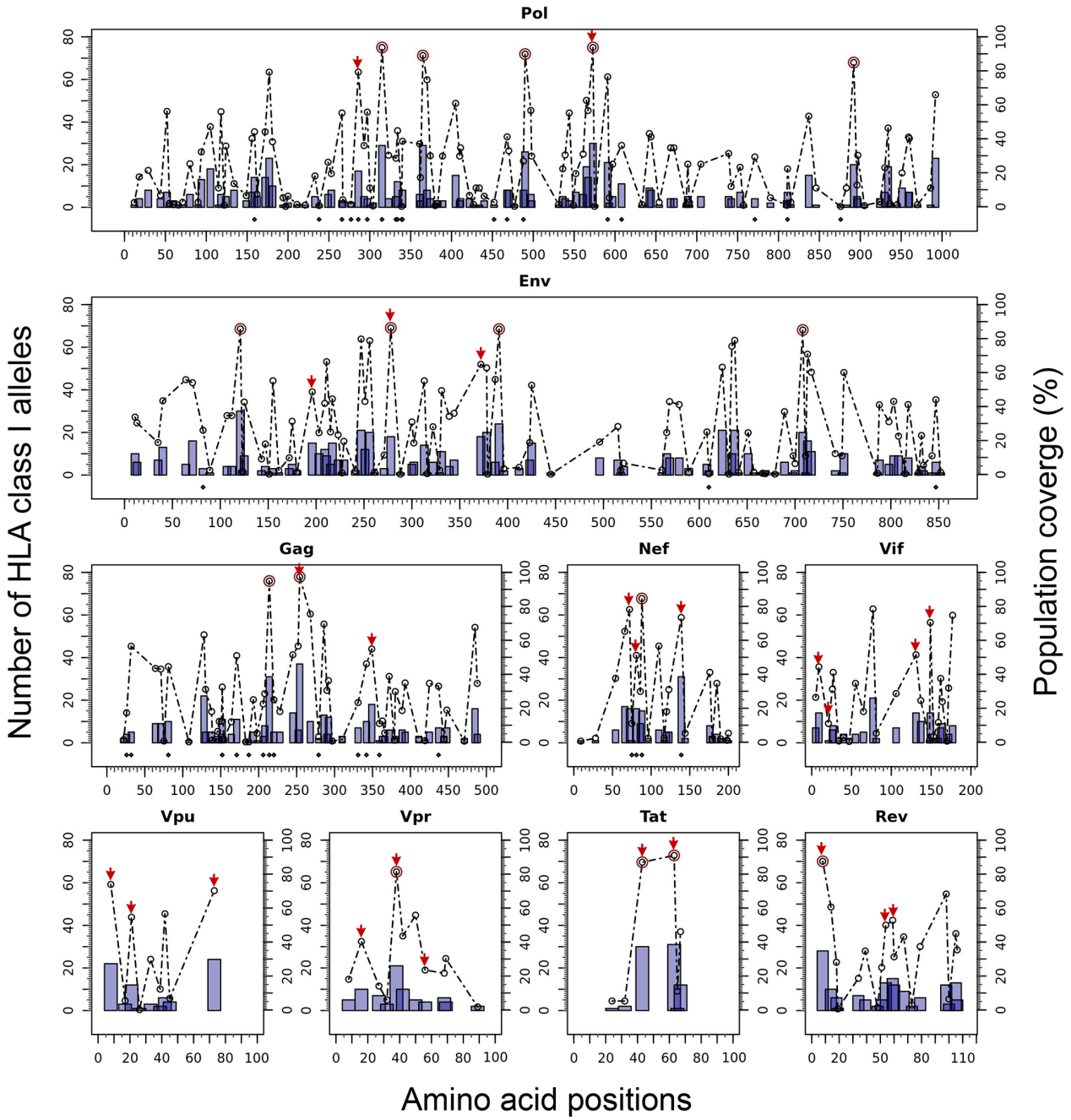

FIGURE 6 | *In silico* HLA class I-restricted T-cell epitope mapping of the HIV-1 proteome (isolate HXB2) targeting the Japanese population, with the Epitope Discovery and Epitope Optimization modes of Predivac-3.0. Bars represent the position of putative CD8+ T-cell epitopes in Pol, Env, Gag, Nef, Vif, Vpu, Vpr, Tat and Rev proteins, whose height accounts for the number of HLA class I alleles restricted by each epitope and black diamonds denote matches with immunodominant CD8+ T-cell epitopes from the HIV-dataset. Circles correspond to the population coverage potentially afforded by each epitope, using red color to highlight specific epitopes delivering coverages ≥ 80% in the target population. Red arrows correspond to CD8+ T-cell epitopes predicted by Predivac-3.0 with the optimization algorithm.

overlaps (**Figure 5A**) and for short regions (30 residues long) with high-concentration of putative T-cell epitopes (**Figure 5B**). As shown in proteome-wide plots, T-cell epitopes are concentrated in epitope-dense regions across the HIV-1 proteome, allowing the detection of relevant interactions between CD8+ and CD4+ T-cell epitope clusters located in Pol (111–141, 879–950), Env (619–646), Gag (267–301), Nef (68–109, 172–202), Vif (11–44), Vpu (26–49), and Rev (44–97).

These regions are potential immunodominant hotspots delivering high population coverages in the Japanese population.

Epitope Discovery and Epitope Optimization

The Predivac-3.0 output for the Epitope Discovery mode is presented in **Figure 6**, showing the position of all predicted CD8+ T-cell epitopes in the HIV-1 proteome (PPR = 1; PCT = 0), as well as the putative number of HLA class I alleles restricted by each epitope and the corresponding population coverages in the Japanese population. Promiscuous CD8+ T-cell epitopes predicted to cover ≥ 80% of this population are highlighted with red circles and listed in **Table 1** for the proteins Env (4 epitopes), Gag (2), Nef (1), Pol (5), Rev (1), Tat (2) and Vpr (1). The total number of CD8+ T-cell epitopes matching epitopes in the HIV-dataset (46 epitopes) is presented in **Table S6**, including Pol (21), Env (3), Gag (17) and Nef (5). Reactive T-cell epitopes predicted to cover ≥20% of the Japanese population are listed and described in **Tables S7** (CD8+ T-cell epitopes) and **Table S8** (CD4+ T-cell epitopes). The most reactive CD8+ T-cell epitopes (and population coverages) predicted in Gag are MTNNPPIPV (97%), AEWDRVHPV (94.9%), and ILDIRQGPK (69.5%); in Nef AVDLSHFLK (84.6%), FPVRPQVPL (78.1%), YPLTFGWCF (73.4%) and EEEEVGFPV (62.5%); and in Vpr FPRIWLHSL (81.3%). Likewise, a putative CD4+ T-cell epitope predicted inside a Gag cluster (RWIILGLNK) is predicted to cover 75.5% of the Japanese population. Interestingly, the top epitope predicted by Predivac-3.0 in the Nef protein (AVDLSHFLK) is also located within a peptide sequence (TYKA**AVDLSHFLK**EK) that was reported as the most frequently targeted (47%) from a cohort of HIV-1 infected US individuals (n=47) and also found within the predicted epitope cluster FPVTPQVPLRPMTYKA**AVDL SHFLK**EKGGLEGLIHSQRRQDI. Finally, **Table 2** describes the optimal set of promiscuous CD8+ T-cell epitopes predicted with the Epitope Optimization mode to maximize the population coverage in this country.

T-Cell Epitope Clusters and Immunodominant Hotspots

Figure 7 illustrates the position, number of associated T-cell epitopes and predicted population coverage (color scale 0-100%) of 66 HIV-1 specific T-cell epitope clusters spanning Env (14 epitopes), Gag (13), Nef (7), Pol (17), Rev (4), Tat (2), Vif (5), Vpr (1) and Vpu (3), both for CD8+ and CD4+ T-cell epitopes (magenta and blue bars, respectively). Detailed information about these T-cell epitope clusters is provided in **Supplementary Material** (**Table S9**). In addition, 48 epitope-rich regions are highlighted along with the nonameric T-cell epitope overlaps, according to information provided in the inner ring of Circos plots (see *Materials and Methods*). Potentially most reactive T-cell epitope clusters (hotspots) are highlighted inside dotted-line rectangles, which are regions predicted to deliver ≥ 80% of population coverage in Japan (**Table 3**). This figure also shows the colocalization of 42 T-cell epitopes from the HIV-dataset with several putative clusters (**Table S10**), which predictively would represent an accuracy of 40.8% and an efficiency of 63.6%. Detailed information on the position and statistical significance of CD8+ and CD4+ T-cell epitopes in each cluster predicted in the HIV-1 proteome are presented in **Tables S11** and **S12**. In addition, **Figure 7** highlights the position of 11 Japanese-specific vaccine-induced CD8+ T-cell epitopes (5 from Gag and 6 from Pol) that have proved protective in this population in response to the mosaic bivalent T-cell vaccine tHIVconsvX (55). While the four T-cell epitopes directly predicted by Predivac-3.0 were RMYSPTSIL, IYQEPFKNL, ELKKIIGQVR and TAFTIPSI, T-cell epitope clusters were capable of capturing knowledge on the position of 7 out of 11 epitopes. As shown for Pol, five epitopes were colocalized with putative clusters predicted in this protein: YTAFTIPSI (**YTAFTIPSI**NNETPGIRYQYNVLPQGW), IYQEPFKNL (IQKQGQGQWTYQ**IYQEPFK**), ELKKIIGQVR (VVESMNK**ELKKIIGQVR**DQA), GERIVDII and GERIVDIIA (YSA**GERIVDIIA**TDIQTKE) and two additional epitopes were found within Gag clusters: RMYSPTSI

TABLE 1 | Putative CD8+ T-cell epitopes from the HIV-1 proteome (HXB2 isolate) predicted by Predivac-3.0 to cover over 80% of the Japanese population (Epitope Discovery mode).

Protein	Peptide	Protein amino acid position	No of HLA class I alleles	Coverage (%)
Env	SVNFTDNAK	274–282	18	86.3
Env	KPCVKLTPL	117–125	30	85.7
Env	STQLFNSTW	387–395	24	85.6
Env	IVNRVRQGY	704–712	20	85.6
Gag	MTNNPPIPV	250–258	37	97.4
Gag	AEWDRVHPV	210–218	31	94.9
Nef	AVDLSHFLK	84–92	15	84.6
Pol	WEFVNTPPL	569–577	30	93.7
Pol	SPAIFQSSM	311–319	29	93.7
Pol	KQGQGQWTY	486–494	26	89.9
Pol	RQHLLRWGL	361–369	29	89.0
Pol	KTAVQMAVF	888–896	20	84.9
Rev	RSGDSDEEL	4–12	28	87.5
Tat	HQNSQTHQA	59–67	31	91.0
Tat	ITKALGISY	39–47	30	87.0
Vpr	FPRIWLHGL	34–42	21	81.3

TABLE 2 | Optimal combinations of CD8+ T-cell epitopes predicted by Predivac-3.0 in each HIV-1 protein to maximize population coverage (≥ 99%) in the Japanese population (Epitope Optimization mode).

GA generation	T-cell epitope	Protein amino acid position	Individual population coverage	Combined population coverage
Env				
3	SVNFTDNAK	274–282	0.863	1.0
	KLTSCNTSV	192–200	0.486	
	DPEIVTHSF	368–376	0.648	
Gag				
2	MTNNPPIPV	250–258	0.974	0.996
	GPAATLEEM	338–346	0.462	
Nef				
3	FPVTPQVPL	68–76	0.781	1.0
	YPLTFGWCY	135–143	0.734	
	RPMTYKAAV	77–85	0.511	
Pol				
2	WEFVNTPPL	569–577	0.937	0.997
	YTAFTIPSI	282–290	0.793	
Rev				
3	RSGDSDEEL	4–12	0.875	1.0
	RQIHSISER	50–58	0.499	
	YLGRSAEPV	63–71	0.43	
Tat				
2	HQNSQTHQA	59–67	0.91	1.0
	ITKALGISY	39–47	0.87	
Vif				
4	WQVMIVWQV	5–13	0.444	1.0
	HIVSPRCEY	127–135	0.515	
	LQYLALAAL	145–153	0.705	
	RIRTWKSLV	17–25	0.11	
Vpu				
3	IPIVAIVAL	4–12	0.739	0.999
	VEMGHHAPW	69–77	0.702	
	IIAIVVWSI	17–25	0.546	
Vpr				
3	FPRIWLHGL	34–42	0.813	0.992
	REPHNEWTL	12–20	0.403	
	DTWAGVEAI	52–60	0.235	

(ILGLNKIV**RMYSPTSI**LDIRQGPKEPFRDYVDRFY) and A T L E E M M T A (L L V Q N A N P D C K T I L K A L GPA**ATLEEMMTA**CQGVGG), providing insight into the validity of these broadly protective clusters and T-cell epitopes for the Japanese population. In addition, the position of several putative T-cell epitope clusters (in Gag and Nef proteins) overlapped with previously identified regions that were frequently recognized in HIV-tested subjects from four ethnicities (African-Americans, Caucasians, Hispanics, and West Indians) (15). **Figure 8** depicts the sequence and position of putative CD8+ and CD4+ T-cell epitope clusters overlapping these immunodominant regions, showing their colocalization with 4 protein regions (2 in Gag and 2 in Nef), as well as several putative CD8+ T-cell epitopes with high population coverages predicted for the Japanese population, such as AEWDRVHPV (Gag 210–218; 94.9%), MTNNPPIPV (Gag 250–258; 97%) and AVDLSHFLK (Nef 84–92; 84.6%).

Application to SARS-CoV-2

Finally, to test the utility of the method we performed *in silico* mapping on the spike glycoprotein of SARS-CoV-2. **Figure 9** presents the output for the Epitope Discovery mode by targeting the United Kingdom, South Africa, Brazil and Japan, showing the position of CD8+ and CD4+ T-cell epitopes and clusters potentially immunodominant for these populations, as well as the number of restricting HLAs and predicted population coverages. Most reactive T-cell epitopes in each country (population coverage ≥ 80%) are highlighted in red circles and listed in **Table 4**, showing the presence of two CD8+ T-cell epitopes that would afford high coverages for all the populations (ESNKKFLPF and KQIYKTPPI) and the epitope GTITSGWTF would be highly promiscuous for the South African, Brazilian and Japanese populations. **Figure 9** also highlights with red arrows the set of T-cell epitopes that was selected through the genetic algorithm (the Epitope Optimization mode) in order to maximize the coverage in each target population (**Table 5**). Top coverages (≥ 99%) could potentially be reached with 2 to 4 T-cell epitopes, with the exception of CD4+ T-cell epitopes for the South African population (72.4% coverage). **Table S13** shows with more detail the combination of epitopes selected by the algorithm at each generation. Finally, **Table 6** summarizes T-cell epitope clusters potentially delivering population coverages

TABLE 3 | Immunodominant T-cell epitope clusters (hotspots) predicted by Predivac-3.0 in the HIV-1 proteome (HXB2 isolate) that are associated with population coverages ≥ 80% in the Japanese population.

Prot	Cluster		T-cell epitopes		Population coverage (%)
	Sequence	Position (start-end)	CD8+ T-cell epitopes	CD4+ T-cell epitopes	
Env	IISLWD**QSLKPCVKLT**PLCVSL	108–129 (114–123)	4	–	92.6
Env	YA**FFYKLDIIPIDNDTTSYKLTSCN**TSVI	173–201 (175–197)	–	7	81.9
Env	SVITQ**ACPKVSFEPIPIHYCAPAGFAILKC**NNKTF	199–233 (204–228)	8	–	88.5
Env	STVQCTH**GIRPVVSTQ**LLLNGSL	243–265 (250–258)	4	–	92.0
Env	HSFNCG**GEFFYCNSTQ**LFNSTW	374–395 (380–389)	4	–	85.7
Env	LEQIW**NHTTWMEWDREINNYTSLIH**SLI	619–646 (624–643)	5	5	99.9
Env	GLRIVF**AVLSIVNRVRQGYSP LSFQT**HL	694–721 (700–719)	6	–	97.1
Gag	HSNQ**VSQNYPIVQNIQGQMVHQAISPRTLNAWVKVVEEK**AFSPEV	124–168 (128–162)	10	–	92.0
Gag	MLKET**INEEAAEWDRVHP**VHAGPIA	200–224 (205–217)	4	5	99.0
Gag	ILG**LNKIVRMYSPTSILDIRQGPKEPFRDYV**DRFY	267–301 (270–297)	5	4	98.4
Nef	FPVTPQV**PLRPMTYKAAVDLSHFLK EKGGLEGLIH**SQRRQDI	68–109 (75–102)	5	6	99.9
Nef	RQDILD**LWIYHTQGYF**PDWQNY	106–127 (112–121)	4	–	81.4
Nef	EWRFD**SRLAFHHVARE**LHPEY	182–202 (187–197)	–	4	80.5
Pol	YTAFTIP**SINNETPGIRYQYNVL**PQGW	282–308 (289–304)	5	–	95.6
Pol	IE**ELRQHLLRWGLTTPD KKHQKEPP**FLWMGY	357–387 (359–381)	7	–	96.1
Pol	IQK**QGQGQWTYQIYQE**PFK	484–502 (487–499)	4	–	931
Pol	VQKITT**ESIVIWGKTPKFKLPIQK**ETWETW	527–556 (533–550)	6	–	88.1
Pol	WTEYW**QATWIPEWEFVNTPP**LVK	557–579 (562–576)	5	–	97.1
Pol	QVRDQAE**HLKTAVQMAVFIHNFKRK**GGIGGY	879–909 (886–903)	5	–	94.2
Pol	ITK**IQNFRVYYRDSRN**PLW	932–950 (935–947)	–	4	85.7
Rev	RSGD**SDEELIRTVRLI**KLLY	4–23 (8–19)	4	–	94.0
Rev	RWRE**RQRQIHSISERILGTYLGRSAEP**VPLQLPP	44–77 (48–70)	7	–	95.2
Rev	VPL**QLPPLERLTLDCNEDCGTSGTQ**GV	71–97 (74–95)	–	6	84.6
Rev	T**QGVGSPQILVESPTV**L	94–110 (95–109)	4	–	93.9
Vif	VWQV**DRMRIRTWKSLVKHHMYVSGKARG**WFYRHHY	11–44 (14–37)	5	4	88.5
Vif	SLQY**LALAALITPKKIKPPLPSVTKLTEDRWN**KPQKTK	144–181 (148–175)	9	–	99.5
Vpu	VIIEYR**KILRQRKIDRLID**RLIER	26–49 (32–44)	4	4	90.7

In each cluster sequence the core region of overlapped T-cell epitopes is highlighted in bold and underscored, providing further information on the amino acid position in each protein, the number of putative T-cell epitopes and the predicted population coverage in the target population.

≥ 80% in each target region (hotspots), providing a comprehensive description in **Supplementary Material** for putative clusters specific for the populations of the United Kingdom (**Table S14**), South Africa (**Table S15**), Brazil (**Table S16**) and Japan (**Table S17**). One particular region that rises interest spans positions 150 to 185 (KSWMESEFRVYSSANNCTFEYVSQPFLMDLEGKQ),

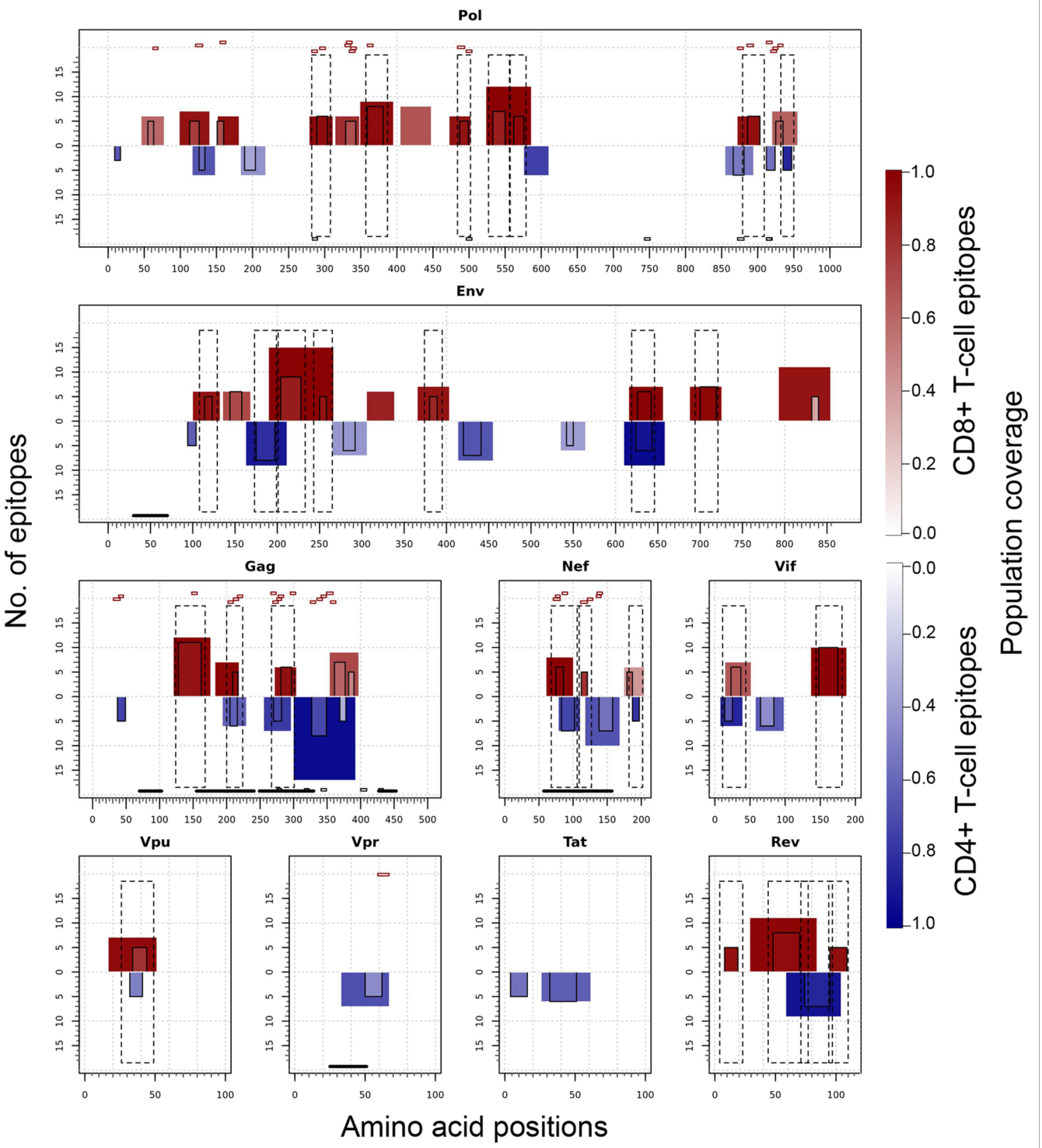

FIGURE 7 | T-cell epitope clusters and hotspots predicted by Predivac-3.0 in the HIV-1 proteome, both for CD8+ T-cell epitopes (magenta bars) and CD4+ T-cell epitopes (blue bars). The left axis indicates the number of T-cell epitopes associated with each cluster and population coverage potentially afforded by the clusters in the Japanese population is represented according to a color scale in the range 0-100%. Potentially the most reactive (immunodominant) regions with population coverages ≥ 80% (hotspots) are highlighted inside dotted-line rectangles, while horizontal bars in the bottom denote ethnicity relevant sequences identified in a previous work (15) (black) and Japanese-specific HIV-1 vaccine-induced CD8+ T-cell epitopes (dashed). Small red rectangles on top of the clusters indicate the location of CD8+ T-cell epitopes from the HIV-dataset that colocalize with these regions.

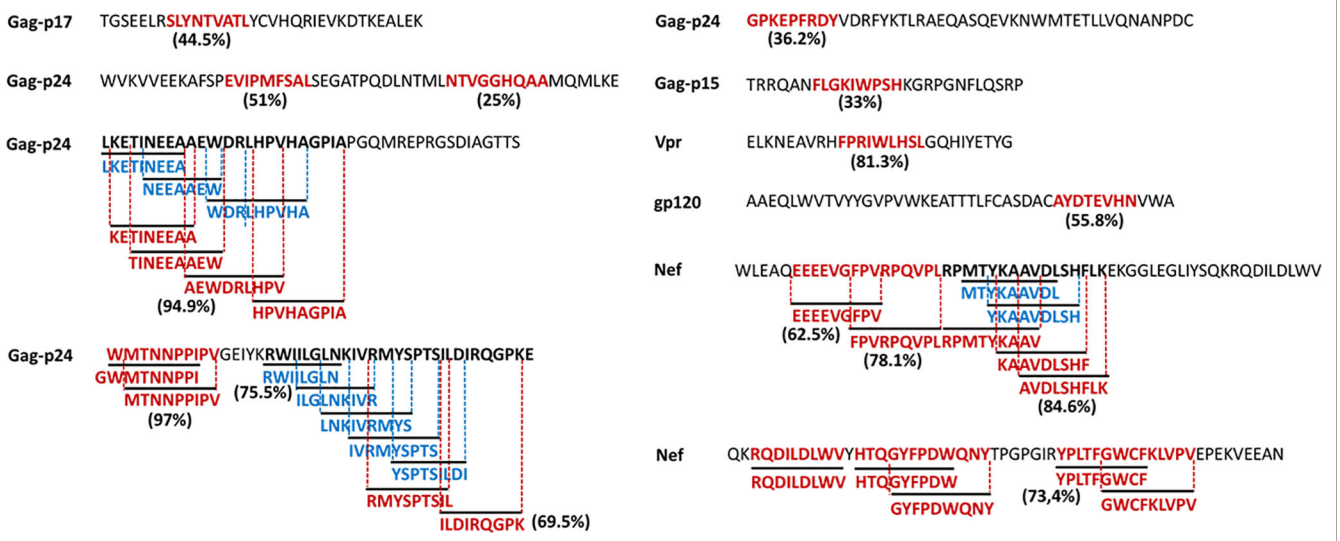

FIGURE 8 | HIV-1 specific T-cell epitopes predicted by Predivac-3.0 for the Japanese population (Epitope Discovery mode), which match immunodominant regions identified across several ethnicities (15). Clusters of overlapped CD8+ (magenta) and CD4+ (blue) T-cell epitopes are highlighted for Gag and Nef, while population coverages associated with the most reactive T-cell epitopes are indicated next to the corresponding sequences.

which comprises both CD8+ and CD4+ T-cell epitope clusters consistently predicted for the populations of the United Kingdom, South Africa, Brazil and Japan.

DISCUSSION

The impact of HLA polymorphism on viral replicative capacity and disease progression has been widely documented in patients infected by HIV-1 (56, 57). For example, HLA-B*57 and HLA-B*27 (protective alleles) are well-known to associate with successful immune control of HIV-1 or slow progression to disease both in Caucasians and African populations, but not in Asians where the frequencies of these alleles are very low (<1%) (58). By contrast, HLA-B*18:01, HLA-B*45:01 and HLA-B*58:02 (disease-susceptible alleles) are strongly associated with high viral load and rapid disease progression in African populations (59). In addition, considerable work has been conducted to determine population-level HLA associations with vaccine-induced immune responses, which are hypothesized as relevant parameters contributing to vaccine failure (17, 60). For example, the lack of response of a recombinant HIV-1 vaccine (ALVAC Env-gp120) designed to induce clade-specific neutralizing antibodies to HIV-1 in the RV144 phase III trial (Thailand) was strongly associated with the presence of certain HLA class II alleles (DRB1*11 and DRB1*16:02) (18), and a recent study assessing the relationship between HLA genotypes and RTS,S vaccine-mediated protection (malaria infections) showed a strong protective association with three allele groups (HLA-A*01, HLA-B*08, and HLA-DRB1*15/*16) (61). In the latter work, the authors discussed the potential impact of these HLA correlations on vaccine immunogenicity and efficacy in risk populations (such as the sub-Saharan African region) where these alleles are present at a lower prevalence than in the UK or USA where these Phase II trials were carried out. This problem is further complicated by the fact that racial and ethnic minority groups generally remain underrepresented in clinical trials (62), limiting the capability to test the efficacy and safety of new clinical interventions across diverse populations and leading to a lack of T-cell data for ethnicities in which viral epidemic currently spreads (15). Therefore, a vaccine that delivers good results for certain groups in Phase I and II trials does not necessarily guarantee strong protective responses in ethnic minority populations that are in more urgent need of new vaccine initiatives (63).

Genetic variability of the HLA system may affect susceptibility and severity of the disease caused by SARS-CoV-2, as recently discussed in a comprehensive *in silico* analysis of viral peptide-HLA class I binding affinity across 145 HLA types (23). Although it is still early within the SARS-CoV-2 pandemic for broad association studies with HLA markers, recent work found that HLA-C*07:29 and HLA-B*15:27 alleles statistically correlated with the occurrence of COVID-19 from a sample of 82 Chinese individuals with COVID-19 that were genotyped for HLA-A, -B, -C, -DRB1, -DRB3/4/5, -DQA1, -DQB1, -DPA1, and -DPB1 loci (20). In addition, several associations between HLA alleles provide susceptibility [e.g., B*46:01 (24), HLA-B*07:03 (64) and HLA-DRB1*12 (65)] or protection [e.g., DRB1*03:01 (66)] to the closely related SARS-CoV-1 have been previously described in Asian populations.

Our immunoinformatics approach addresses this challenge by providing a computational framework to deal with the extent of HLA diversity. We have previously reported the

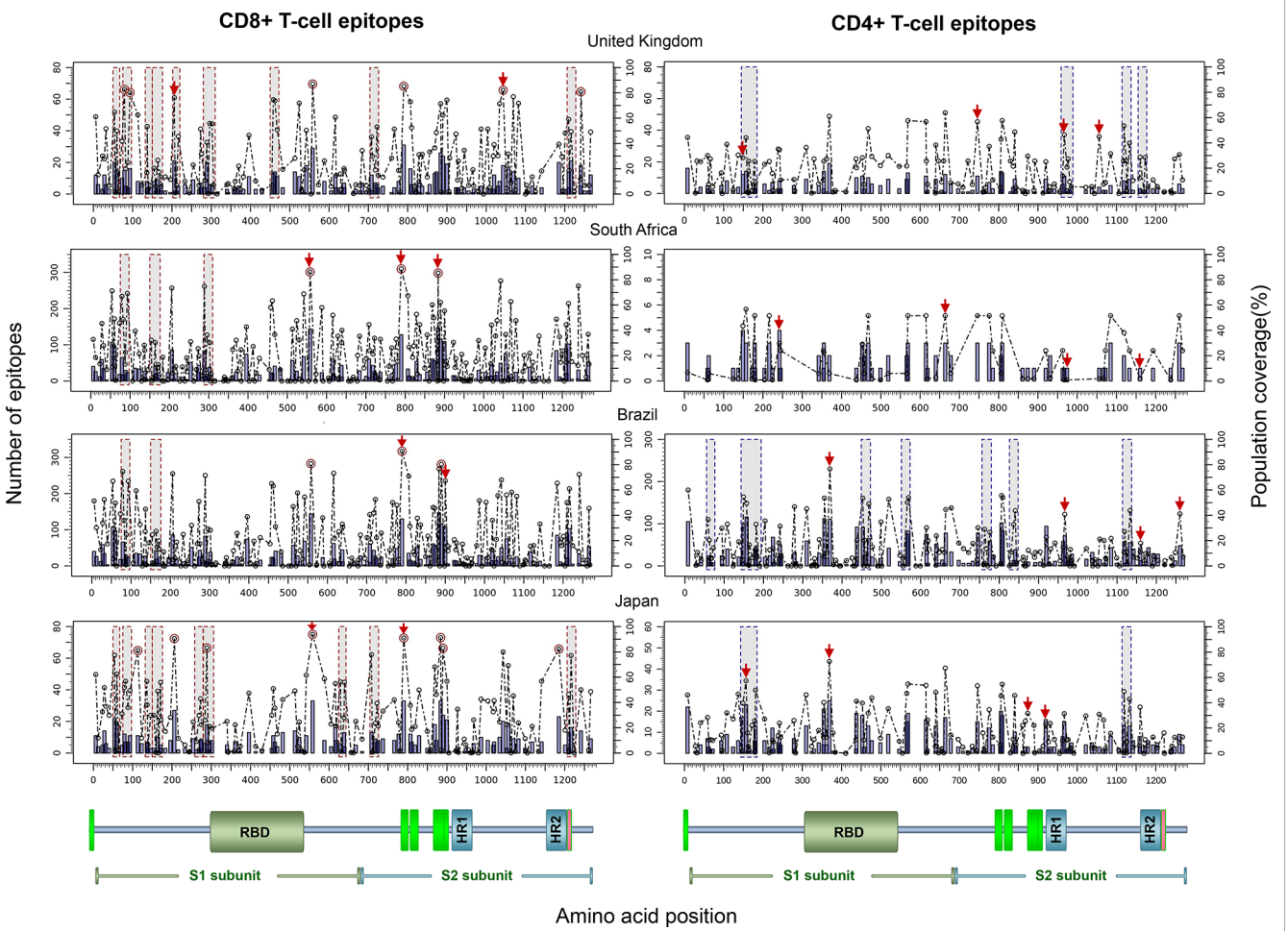

FIGURE 9 | *In silico* T-cell epitope mapping of the SARS-CoV-2 spike glycoprotein (UniProt ID: P0DTC2) targeting the populations of The United Kingdom, South Africa, Brazil and Japan, using the Epitope Discovery and Epitope Optimization modes of Predivac-3.0. Bars in the plots represent the position of putative T-cell epitopes, whose height accounts for the number of CD8+ and CD4+ T-cell epitopes. Circles correspond to the population coverage potentially afforded by the epitopes in the target populations, with epitopes delivering coverages ≥ 60% indicated with red color. Shaded regions mark the position of putative T-cell epitope clusters delivering population coverages ≥ 60% (hotspots). In addition, T-cell epitopes predicted by Predivac-3.0 using the optimization algorithm are indicated over the circles with red arrows. A protein scheme with domain organization is presented on the bottom the figure, highlighting the position of the receptor-binding domain (RBD) and heptad repeat 1 and 2 (HR1 and HR2). Aromatic amino-acid rich pre transmembrane regions (PTM) involved in the mechanism of viral entry are represented in green color.

pan-specific tools Predivac-1.0 (33) and Predivac-2.0 (32) to aid CD4+ T-cell epitope-based vaccine design in the context of genetically heterogeneous human populations. Herein, we describe a significant enhancement in our Predivac approach to contribute to the development of genetics-driven immunization strategies that take into account the ethnic diversity of T-cell recognition in the population to be vaccinated. Predivac-3.0 enables proteome-wide ethnicity-driven CD8+ and CD4+ T-cell epitope prediction to select promiscuous T-cell epitopes priming broad immune responses in target groups, with an additional focus on the identification of immunologically relevant T-cell epitope-rich antigen regions potentially affording high-coverage in the target population (referred as hotspots). As shown in **Figure 3A**, the method was successfully validated in LOOCV experiments for 45 HLA class I alleles (17,425 peptides) represented in PredivacDB (AUC = 0.8), by employing a highly rigorous LOOCV methodology that involves excluding an individual allele from the database and then evaluating performance using the dataset of high-affinity peptides restricted by that particular allele (positive dataset). In addition, the tool proved outstanding performance in the benchmarks (**Figures 3B, C**), delivering comparable accuracies against top-performing state-of-the art methods using the IEDB-dataset (average AUC = 0.915) and DFRMLI-dataset (average AUC = 0.909). These AUC values (~0.9) are indicative of excellent discrimination capability, while the lesser AUC (0.8) obtained in LOOCV is typically expected as a consequence of the more stringent experimental condition of systematically removing 100% of the data for the specific tested allele. To maintain balanced datasets for AUC calculation (LOOCV and

TABLE 4 | List of the top promiscuous CD8+ T-cell epitopes in the SARS-CoV-2 spike glycoprotein predicted by Predivac-3.0 to deliver above 80% coverage in the populations of the United Kingdom, South Africa, Brazil, and Japan (using the Epitope Discovery mode).

Protein	Peptide	Amino acid position	Coverage (%)	No. of HLA class I alleles
The United Kingdom				
Spike	**ESNKKFLPF**	**554–562**	**86.9**	**29**
Spike	**KQIYKTPPI**	**786–794**	**85.2**	**31**
Spike	GTKRFDNPV	75–83	82.8	15
Spike	**RVDFCGKGY**	**1039–1047**	**82.1**	**18**
Spike	MTSCCSCLK	1237–1245	80.9	18
Spike	GVYFASTEK	89–97	80.2	16
South Africa				
Spike	**KQIYKTPPI**	**786–794**	**88.5**	**128**
Spike	**ESNKKFLPF**	**554–562**	**86.1**	**144**
Spike	**GTITSGWTF**	**880–888**	**85.2**	**147**
Brazil				
Spike	**KQIYKTPPI**	**786–794**	**90.6**	**130**
Spike	**ESNKKFLPF**	**554–562**	**80.9**	**144**
Spike	WTFGAGAAL	886–894	80.2	116
Japan				
Spike	**ESNKKFLPF**	**554–562**	**93.8**	**33**
Spike	**GTITSGWTF**	**880–888**	**91.2**	**33**
Spike	**KQIYKTPPI**	**786–794**	**90.9**	**33**
Spike	KIYSKHTPI	202–210	90.5	27
Spike	ITDAVDCAL	285–293	83.0	18
Spike	WTFGAGAAL	886–894	82.9	24
Spike	KEIDRLNEV	1181–1189	82.2	23
Spike	TLDSKTQSL	109–117	81.0	11

In bold, epitope sequences that are potentially immunodominant in most of the countries.

TABLE 5 | Optimal combinations of CD8+ and CD4+ T-cell epitopes selected by Predivac-3.0 in the SARS-CoV-2 spike glycoprotein at the last generation of the genetic (GA) algorithm, which maximize the population coverage in the United Kingdom, South Africa, Brazil, and Japan (Epitope Optimization mode).

T-cell epitopes	Amino acid position	Individual population coverage	Combined population coverage (%)	Putative allele restriction
The United Kingdom				
CD8+ T-cell epitopes				
KIYSKHTPI	202–210	0.762	99.2	A*02:01(0.288),B*07:02(0.152),B*51:01(0.046),A*01:01(0.207),A*03:01(0.137),A*11:01(0.069),A*24:02(0.068),A*25:01(0.021),A*26:01(0.019),B*57:01(0.036)
RVDFCGKGY	1039–1047	0.821		
CD4+ T-cell epitopes				
YHKNNKSWM	145–153	0.283	99.9	DRB1*11:01(0.045),DRB1*13:01(0.043),DRB1*13:02(0.032),DRB1*07:01(0.137),DRB1*01:01(0.088),DRB1*15:01(0.147),DRB1*04:04(0.040),DRB1*04:03(0.024),DRB1*03:01(0.135),DRB1*04:01(0.120),
FPQSAPHGV	1052–1060	0.449		DRB1*04:04(0.040)
VKQLSSNFG	963–971	0.464		
ICGDSTECS	742–750	0.567		
South Africa				
CD8+ T-cell epitopes				
KQIYKTPPI	786–794	0.885	100%	A*23:01(0.085),A*68:02(0.081),A*30:02(0.072),A*30:01(0.056),B*42:01(0.097),B*58:02(0.068),B*08:01(0.059),B*40:06(0.039),B*57:03(0.032),A*23:01(0.085),A*30:02(0.072),A*24:02(0.051),B*42:01(0.097),B*15:03(0.076),B*44:03(0.070),B*08:01(0.059),B*15:10(0.068),B*07:02(0.036),B*14:01(0.032).
ESNKKFLPF	554–562	0.861		
MIAQYTSAL	869–877	0.499		
CD4+ T-cell epitopes				
FQTLLALHR	238–246	0.298	72.4	DRB1*13:01(0.128),DRB1*13:02(0.03),DRB1*10:01(0.004),DRB1*13:05(0),DRB1*03:01(0.137),DRB1*03:02(0.09),DRB1*03:04(0.077),DRB1*09:01(0.008), DRB1*10:01(0.004)
YECDIPIGA	660–668	0.516		

(Continued)

TABLE 5 | Continued

T-cell epitopes	Amino acid position	Individual population coverage	Combined population coverage (%)	Putative allele restriction
FKNHTSPDV	1156–1164	0.0178		
FGAISSVLN	970–978	0.008		
Brazil				
CD8+ T-cell epitopes				
KQIYKTPPI	786–794	0.906	99.2	A*02:01(0.178),A*23:01(0.037),B*48:03(0.045),B*08:01(0.041),B*18:01(0.040),B*51:01(0.038),B*35:06(0.035), B*35:03(0.033),A*68:01(0.079),A*03:01(0.062),B*18:01(0.040),B*35:01(0.033),B*44:03(0.031),
IPFAMQMAY	896–904	0.672		
CD4+ T-cell epitopes				
YSVLYNSAS	365–373	0.766	99.2	DRB1*16:02(0.151),DRB1*08:02(0.136),DRB1*14:02(0.073),DRB1*11:01(0.032),DRB1*04:11(0.066), DRB1*04:04(0.058),DRB1*07:01(0.054),DRB1*09:01(0.034),DRB1*16:02(0.151),DRB1*03:01(0.057)
VKQLSSNFG	963–971	0.408		
FKNHTSPDV	1156–1164	0.179		
FDEDDSEPV	1256–1264	0.411		
Japan				
CD8+ T-cell epitopes				
ESNKKFLPF	554–562	0.938	99.9	A*24:02(0.319),A*26:01(0.072),A*26:02(0.018),B*52:01(0.104),B*15:01(0.083),B*51:01(0.083),B*35:01(0.078), B*44:03(0.070),A*02:01(0.105),A*02:06(0.085),B*52:01(0.104),B*51:01(0.083),B*54:01(0.081),B*40:02(0.078),
KQIYKTPPI	786–794	0.909		B*40:01(0.051),B*40:06(0.047)
CD4+ T-cell epitopes				
YSVLYNSAS	365–373	0.727	99.4	DRB1*04:05(0.117),DRB1*15:02(0.084),DRB1*08:03(0.069),DRB1*13:02(0.052),DRB1*01:01(0.048), DRB1*08:02(0.039),DRB1*14:08(0.118),DRB1*08:03(0.069),DRB1*08:02(0.039),DRB1*14:01(0.032),
MESEFRVYS	153–161	0.574		DRB1*09:01(0.117),DRB1*15:01(0.055)
IAQYTSALL	870–878	0.316		
LYENQKLIA	916–924	0.231		

Putative HLA class I-allele restriction is provided along with their corresponding population frequency in each country (ANFD data).

TABLE 6 | Potentially immunodominant T-cell epitope clusters in the spike glycoprotein of SARS-CoV-2, predicted by Predivac-3.0 to yield coverages ≥ 80% in the populations of the United Kingdom, South Africa, Brazil and Japan.

Cluster			T-cell epitopes	
Sequence	**Amino acid position**	**Population coverage (%)**	**CD8+ T-cell epitopes**	**CD4+ T-cell epitopes**
The United Kingdom				
GTKRFDN**PVLPFNDGV**YFASTEK	82–90 (75–97)	99.9	GTKRFDNPV, RFDNPVLPF, LPFNDGVYF, GVYFASTEK	–
KIY**SKHTPINLVRDLP**QGF	205–217 (202–220)	85.0	KIYSKHTPI, YSKHTPINL, TPINLVRDL, LVRDLPQGF	–
NENGTI**TDAVDCALDPLSETKCTL**KSFTVEK	285–303 (280–310)	93.7	NENGTITDA, ITDAVDCAL, AVDCALDPL, ALDPLSETK, ETKCTLKSF, TLKSFTVEK	–

(Continued)

TABLE 6 | Continued

Cluster			T-cell epitopes	
Sequence	**Amino acid position**	**Population coverage (%)**	**CD8+ T-cell epitopes**	**CD4+ T-cell epitopes**
NYLYRLF**RKSNLKPFE**RDISTEI	457–465 (450–472)	90.6	NYLYRLFRK, RLFRKSNLK, KSNLKPFER, FERDISTEI	–
SVAYSNN**SIAIPTNFT**ISVTTEI	711–719 (704–726)	88.7	SVAYSNNSI, AYSNNSIAI, IPTNFTISV, FTISVTTEI	–
I**ITTDNTFVSGNCDVVI**GIVNNTV	1115–1130 (1114–1137)	89.7	–	IITTDNTFV,ITTDNTFVS, FVSGNCDVV,VSGNCDVVI, VIGIVNNTV
South Africa				
GTKRFDN**PVLPFNDGV**YFASTEK	82–90 (75–97)	99.9	GTKRFDNPV, RFDNPVLPF, VLPFNDGVY, LPFNDGVYF, GVYFASTEK	–
Brazil				
GTKRFDN**PVLPFNDGV**YFASTEK	82–90 (75–97)	99.9	GTKRFDNPV, RFDNPVLPF, VLPFNDGVY, LPFNDGVYF, GVYFASTEK	–
VYYHKNN**KSWMESEFRVYSSANNCTFEYVSQPFLMDLEGKQGNFKNLR**EFVF	150–190 (143–194)	84.2	–	VYYHKNNKS,YHKNNKSW, MESEFRVYS,FRVYSSANN, VYSSANNCT,YSSANNCTF, FEYVSQPFL,FLMDLEGKQ, LMDLEGKQG,MDLEGKQGN, FKNLREFVF
YNYLY**RLFRKSNLKPFERD**ISTEI	454–467 (449–472)	84.3	–	YNYLYRLFR,LYRLFRKSN, YRLFRKSNL,LKPFERDIS, FERDISTEI
Japan				
ST**QDLFLPFFSNVTWF**HA	52–65 (50–67)	86.8	STQDLFLPF, TQDLFLPFF, LPFFSNVTW, FSNVTWFHA	-
GTKRFDN**PVLPFNDGV**YFASTEK	82–90 (75–97)	99.0	GTKRFDNPV, RFDNPVLPF, LPFNDGVYF, GVYFASTEK	-
EFQ**FCNDPFLGVYYHK**NNK	135–147 (132–150)	93.5	EFQFCNDPF, FQFCNDPFL, FCNDPFLGV, GVYYHKNNK	-
KSWME**SEFRVYSSANNCTFEYVSQPF**L	155–175 (150–176)	93.3	KSWMESEFR, SEFRVYSSA, YSSANNCTF, SANNCTFEY, TFEYVSQPF, FEYVSQPFL,	-
NENGT**ITDAVDCALDPLSET**KCTLKSF	285–300 (280–306)	95.6	NENGTITDA, ITDAVDCAL, AVDCALDPL, ALDPLSETK, ETKCTLKSF	-

(Continued)

TABLE 6 | Continued

Cluster			T-cell epitopes	
Sequence	**Amino acid position**	**Population coverage (%)**	**CD8+ T-cell epitopes**	**CD4+ T-cell epitopes**
SVAYSNN**SIAIPTNFT**ISVTTEI	711–719 (704–726)	92.6	SVAYSNNSI, AYSNNSIAI, IPTNFTISV, FTISVTTEI	-
YEQYIKW**PWYIWLGFI**AGLIAIV	1213–1221 (1206–1228)	85.6	YEQYIKWPW, EQYIKWPWY, WPWYIWLGF, FIAGLIAIV	-
VYYHKNN**KSWMESEFRVYSSANNCTFEYVSQPFLMDLEGKQ**GN	150–183 (143–185)	88.8		VYYHKNNKS,YHKNNKSW, MESEFRVYS,FRVYSSANN, VYSSANNCT,YSSANNCTF, FEYVSQPFL,FLMDLEGKQ, LMDLEGKQG,MDLEGKQGN

In each cluster, the core region of overlapped T-cell epitopes is highlighted in bold/underscored, showing the amino acid position in each protein, the population coverage in the target populations and the CD8+ or CD4+ T-cell epitopes associated to each cluster.

IEDB benchmark), negative examples were taken from the epitope source protein, by splitting the sequence into overlapping peptides of the same length as the epitope and all peptides except the annotated peptide were assumed as negatives.

We then focused on proteome-wide identification of immunodominant hotspots in order to improve the value of the method for vaccination purposes, in agreement with numerous studies supporting that immunodominant T-cell epitopes are not randomly distributed along the protein sequence, but tend to cluster in limited regions of the antigen undergoing efficient processing (39). The rationale behind this approach is that only a few peptides from complete antigens are7nbsp;capable of inducing significant responses following immunization, which are those peptides presented to T-cells in association with HLA class I and II molecules (67). Accordingly, a significant body of literature underscores the influence of three-dimensional structure of antigens over the likelihood of peptides to be proteolytically released from the source protein, either through the proteasome-mediated endogenous pathway (CD8+ T-cell epitopes) (68) and cathepsin-mediated exogenous pathway (69). However, an alternate reasoning path correlates immunodominant hotspots with promiscuous binding in antigen regions containing a certain density of peptides that bind multiple HLA types (40, 70). Because promiscuous peptides can be presented to T-cells by many individuals (promiscuous T-cell recognition), the identification of regions that are highly enriched in MHC ligands holds potential to define population-based biomarkers as correlates of immunological protection to compare candidate vaccines in efficacy clinical trials (39, 71).

An earlier approach to select promiscuous epitopes is based on the concept of supertypes, which are clusters of HLA molecules sharing overlapping peptide repertories (72). Pepvac is a computational tool based on this approach (30), which depends on pre-calculated population coverages for five HLA class I supertypes (A2, A3, A24, B7, and B15) and accounts for five major American ethnic groups (Black, Caucasian, Hispanic, Native American and Asian). By contrast, allele-based selection methods such as Predivac (32, 33) and OptiTope (31) define promiscuous epitopes as those restricted to as many HLA alleles as possible in a given target population. However, instead of providing population coverage as a function of allele frequency distributions, OptiTope performed "allele coverage" by summing up for each locus the fraction of alleles targeted by predicted T-cell epitopes in a given population. Although we could not compare Predivac-3.0 with OptiTope, since the web-based tool is not currently available, our method offers a more accurate framework by implementing a population coverage algorithm (50) based on HLA genotypic frequencies from the AFND (73), which is the most comprehensive repository of immune gene frequencies of worldwide populations. Therefore, our approach takes into account the fact individuals display different sets of HLA alleles with particular binding specificities and expression frequencies that dramatically differ among different ethnicities (32).

HIV-specific T-cell responses play a pivotal role in the anti-HIV immune response (74). Therefore, the successful identification of HIV-1 specific CD8+ T-cell epitopes in the exploratory analysis for the Japanese population lends support to the utility of the tool in ethnicity-driven T-cell epitope discovery (**Figure 4**). We showed Predivac-3.0 was capable of identifying 46 out of 103 immunodominant T-cell epitopes (44.7% efficiency) from the HIV-dataset with default parameters (PPR = 1; PCT = 0%). Prediction accuracies gradually declined as the population coverage threshold (%) increases for each PPR value (1, 2 and 3), accounting for the growing number of CD8+ T-cell epitopes that are missed as the tool stops selecting epitopes below that threshold limit. However, by filtering T-cell epitopes covering ≥ 20% of the Japanese population (PCT = 20%) the universe of predicted CD8+ T-cell epitopes to be searched

decreased to 46% (from 373 to 201 epitopes), with a slight reduction in the accuracy (from 44.2 % to 31.8 %). A good trade-off must balance the predictive accuracy with a reasonable amount of predicted T-cell epitopes to be experimentally tested in the laboratory, because this number must be kept low enough to make the tool useful in accelerating epitope discovery. Inset plots in **Figure 4** show that for PCT = 0% the number of putative CD8+ T-cell epitopes increased from 373 (PPR = 1) to 583 (PPR = 2) and to 719 (PPR = 3), increasingly loosing utility for experimental validation purposes. This is considered a good and sensitive result, especially because the HIV-1 positive dataset (103 CD8+ T-cell epitopes) accounts for a tiny portion (2.4%) of the HIV-1 proteome (4234 amino acids), providing insight into the methods capability to guide epitope discovery in population context. Furthermore, the HIV-dataset only accounts for currently characterized immunodominant T-cell epitopes, leading to the reasonable supposition that accuracy could potentially become higher as new Japanese-specific CD8+ T-cell epitopes are identified in the future. This assumption is underscored by comprehensive HIV-specific epitope mapping studies showing CD4+ and CD8+ T-cell responses across the entire viral proteome (75, 76).

An interesting study case to explore the utility of the tool is the mosaic bivalent T-cell vaccine tHIVconsvX, which comprises 5 Gag-specific and 6 Pol-specific T-cell epitopes (**Table S3**) with the ability to suppress HIV-1 replication *in vivo* and correlate with better clinical outcome (low pVLs and high CD4 counts) in treatment-naïve HIV-1 clade B-infected Japanese individuals (55). The reactivity of this vaccine has been recently characterized in the Japanese population, proving that Gag-specific T cell epitopes (5 epitopes) were found restricted by HLA-B* 52:01, HLA-A*02:06, HLA-A*33:03, and HLA-B*40:02, while the Pol-specific T-cell epitopes (6 epitopes) were restricted by HLA-A*24:02, HLA-A*33:03, HLA-B*40:02, HLA-B*40:06, HLA-B*51:01 and HLA-B*52:01. HLA-B*57, HLA-B*58, and HLA-B*27 are well-known protective alleles for AIDS progression in Caucasians and Africans infected with HIV-1 (65–67). However, the less-characterized HLA*B*52:01 allele is prevalent in the Japanese population (68) and the HLA-B*52:01-C*12:02 haplotype has been suggested to be protective in Japanese individuals, where HLA-B*57, HLA-B*58, or HLA-B*27 are present at very low frequencies in this population (50). The CD8+ T-cell epitopes predicted by Predivac-3.0 to cover 79.3% of the Japanese population (YTAFTIPSI; 282-290) was consistently predicted with HLA*B*52 allele restriction, which in previous studies has been associated with low viral loads in HIV-infected Japanese individuals (69) and also elicited HLA-B*52:01-restricted CD8+ T-cells with strong ability to suppress HIV-1 replication in this population (70).

Predivac-3.0 was effective in identifying immunodominant T-cell epitopes in the HIV dataset, but also guided the detection of Japanese-specific T-cell epitope clusters (hotspots) in the HIV-1 proteome (**Figures 5** and **6**), in agreement with a recent work that provided evidence in favor of the utility of immunoinformatics tools to identify these regions exclusively based on promiscuous HLA peptide binding (40). Indeed, the method was sensitive to capture information on the location of additional CD8+ T-cell epitopes from the HIV-dataset that overlapped putative CD8+ and CD4+ T-cell epitope clusters (**Figure 7**). Putative clusters predicted by Predivac-3.0 are additionally colocalized with 4 immunodominant regions (2 in Gag and 2 in Nef) that are broadly recognized by HIV-infected subjects from several ethnicities, showing the clustering of several CD8+ and CD4+ T-cell epitopes predicted by Predivac 3.0 (**Figure 8; Table S9**). These results lend support to the reactivity of these regions in the Japanese population, but also about their potential for "universal" T-cell-based vaccination against HIV-1 in heterogeneous ethnic populations (15, 77).

An interesting finding of the current study is the detection of strong immunodominant hotspot signals in the regulatory protein Rev, with four T-cell epitope clusters potentially delivering population coverages above 80% in Japan (positions 4–23; 44–77; 71–97; 94–110). These regions hold interest for vaccine development in this particular ethnic population, suggesting crosslinking between CD8+ and CD4+ T-cell epitopes between positions 71 and 110 of the protein. Indeed, the research has largely focused on the assessment of immune responses directed against Nef and late-expressed HIV-1 structural proteins (Gag, Pol and Env), which concentrate the vast majority of well-defined T-cell epitopes (53). By contrast, regulatory (Tat and Rev) and accessory proteins (Vpr, Vpu and Vif) are less frequently targeted by cytotoxic T-cells in study subjects from clinical interventions (75). This result is consistent with previous works suggesting these proteins might be promising targets for vaccine development (78). A substantial amount of evidence points out that cytotoxic T-cell responses directed against non-structural proteins (Tat, Rev, Vpr, Vpu and Vif) contribute importantly to the total magnitude of the HIV-1 specific cellular immune response (79, 80). Because these proteins are expressed earlier in the viral life cycle, their recognition may occur before Nef down-modulates HLA class I molecules on the surface of the infected cells and thus provide a window of opportunity to the immune system to clear the infected cell before the virus is released (81). Another CD8+ T-cell epitope predicted by Predivac-3.0 within a cluster region of Vpr is the HLA-A*02:01-restricted peptide AIIRILQQL (82, 83), which is located in a functionally important region involved with perinuclear localization of the protein (84, 85). This peptide has been shown to correlate inversely with plasma viral load and positively with CD4 count in a study involving a cohort of HIV-1 infected individuals expressing the HLA-A*02:01 allele (80). A study about HLA haplotype frequencies in the Japanese population states that ~80% of this population could be responsive for a vaccine containing T-cell epitopes presented by HLA-A*02:01 (86).

HLA diversity is likewise a crucial host genetic factor in determining variations in the T-cell responses of HIV-infected patients across multiple ethnicities (15). In this regard, the Japanese-specific density maps (T-cell epitope clusters/hotspots) predicted in the HIV-1 proteome are different to those available at Los Alamos HIV Molecular Immunology

Database, accounting for population-level specificities in HLA class I and II frequencies. Interestingly, T-cell epitope clusters allowed a higher efficiency (63.6%) in detecting the position of immunodominant T-cell epitopes from the HIV-dataset (by means of colocalization) than that obtained through direct epitope prediction (12.3%), while both approaches delivered similar accuracies around 40–45% (default parameters PPR = 1; PCT = 0%). Ethnic specificity of HIV immunodominant patterns has been also discussed in a meta-analysis of epitope mapping data from three large vaccination clinical trials carried out in different countries (Merck16, HVTN 054 and HVTN 502/Step), which showed that HIV-1 T-cell responses clustered into distinct hotspot patterns associated with study subjects with different ethnic background (39). Similarly, our analysis carried out on the SARS-CoV-2 spike glycoprotein showed immunodominance patterns accounting for population-specific T-cell epitopes and clusters in the four studied populations (The United Kingdom, South Africa, Brazil and Japan), but also for a few putative immunodominant T-cell epitopes and regions that are of interest for "universal" vaccination purposes as they bind to multiple HLA alleles of high prevalence in all the populations (**Figure 9**). This is also consistent with a recent work showing association of COVID-19 disease with disproportionate mortality among ethnic populations, as specifically observed by the lower mortality rate in the Indian and South Asian subcontinent than in the West (19). To the best of our knowledge these sequences and regions have not been previously reported and can be of interest in the light of the ChAdOx1 vaccine development, which is currently undergoing phase III clinical trials in human volunteers (The United Kingdom, South Africa and Brazil). These results might be also of value for the mRNA-1273 vaccine and for other adenoviral vaccine candidates encoding the S protein antigen.

In short, we support the perspective that this is an immunoinformatics approach that can provide valuable knowledge of T-cell epitopes and immunodominant regions (clusters) to help understand how variation in HLA may affect vaccine-induced immune responses in a population context. Understanding this layer of complexity is also relevant in the context of vaccination trials, since underrepresentation of minorities is an issue that might lead to a resulting body of clinical knowledge that is not generalizable (skewed findings) and a lesser discovery rate of protective T-cell epitopes in certain populations. Predivac-3.0 provides tools to guide the discovery of population-specific epitopes and clusters in the context of SARS-CoV-2 and of other emerging pathogens (EIDs), holding potential to improve vaccine design and clinical trial protocols for evaluation of vaccine candidates in individuals with different genetic or ethnic backgrounds (phase II/III trials).

CONCLUSIONS

Population-level HLA associations are crucial factors determining variations of vaccine-induced immune responses across multiple ethnicities. Predivac-3.0 addresses this problem by implementing a computational framework for rational design of CD8+ and CD4 + T-cell epitope-based vaccination, which allows guiding epitope discovery according to HLA allele frequencies in specific ethnic populations. Our immunoinformatics tool showed a strong performance in the identification of CD8+ T-cell epitopes by leave-one-out cross-validation (AUC ~0.8) and comparable accuracies when benchmarked against state-of-the-art pan-specific methods (AUC ~0.9). We further proved that Predivac-3.0 was accurate and sensitive for *in silico* identification of HIV-1 specific CD8+ T-cell epitopes that are immunodominant in the Japanese population. The method also captured information at proteome-level of epitope-rich areas of HLA promiscuity (hotspots), shedding light onto its capability to identify HIV-1 vaccine-induced and protective T-cell epitopes. We finally showed the utility of Predivac-3.0 in the context of the current COVID-19 pandemics, by applying the Epitope Discovery and Epitope Optimization tools to predict comprehensive lists of population-specific T-cell epitopes and clusters in the SARS-CoV-2 spike glycoprotein for the countries where phase III clinical trials of the ChAdOx1 vaccine are currently being carried out. Putative T-cell epitopes identified for HIV-1 and SARS-CoV-2 are suitable candidates to be experimentally tested for effective vaccine protection, as they hold the potential to induce broad immune responses in the corresponding target populations. In addition, proteome-wide plots (Circos and hotspots) not only allowed for better visualization of the predictions, but also provide the ability to capture knowledge on ligand enrichment areas (based on promiscuous HLA peptide binding) and to detect interactions between the distribution and density of both ethnicity-driven CD8+ and CD4+ T-cell epitopes. Overall, we propose that incorporation of knowledge about HLA prevalence in the target population and immunological hotspots into the predictive algorithm might contribute to the development of novel vaccination strategies that support a more prominent role of T-cell mediated immune responses against emerging viral pathogens, as well as to gain understanding on how variation in HLA may affect vaccine-induced immune responses in a population context. Our immunoinformatics approach is particularly suited to be applied for EIDs associated with well-defined regions or countries, as it accounts for ethnic-level variations of immune responsiveness in the populations in need of vaccination.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

PO: conceived, designed, supervised the experiment, and wrote the manuscript. MK: performed *in silico* experiments and prepared the figures. VF: rewrote the Predivac code in Python and performed

in silico experiments. PO, MK, and VF: analyzed the data. All authors contributed to the article and approved the submitted version.

FUNDING

This research was supported by ANID Chile/FONDECYT Iniciación (Grant No.11170638) and by supercomputing infrastructure from UDEC Southern GPU-cluster (FONDEQUIP EQM150134).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.598778/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.

frontiers in Immunology

ORIGINAL RESEARCH
published: 09 March 2021
doi: 10.3389/fimmu.2021.627688

Ebola-GP DNA Prime rAd5-GP Boost: Influence of Prime Frequency and Prime/Boost Time Interval on the Immune Response in Non-human Primates

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OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: *10 November 2020*
Accepted: *19 February 2021*
Published: *09 March 2021*

Citation:
Marcus H, Thompson E, Zhou Y, Bailey M, Donaldson MM, Stanley DA, Asiedu C, Foulds KE, Roederer M, Moliva JI and Sullivan NJ (2021) Ebola-GP DNA Prime rAd5-GP Boost: Influence of Prime Frequency and Prime/Boost Time Interval on the Immune Response in Non-human Primates. Front. Immunol. 12:627688. doi: 10.3389/fimmu.2021.627688

Heterologous prime-boost immunization regimens are a common strategy for many vaccines. DNA prime rAd5-GP boost immunization has been demonstrated to protect non-human primates against a lethal challenge of Ebola virus, a pathogen that causes fatal hemorrhagic disease in humans. This protection correlates with antibody responses and is also associated with IFNγ^+ TNFα^+ double positive CD8$^+$ T-cells. In this study, we compared single DNA vs. multiple DNA prime immunizations, and short vs. long time intervals between the DNA prime and the rAd5 boost to evaluate the impact of these different prime-boost strategies on vaccine-induced humoral and cellular responses in non-human primates. We demonstrated that DNA/rAd5 prime-boost strategies can be tailored to induce either CD4$^+$ T-cell or CD8$^+$ T-cell dominant responses while maintaining a high magnitude antibody response. Additionally, a single DNA prime immunization generated a stable memory response that could be boosted by rAd5 3 years later. These results suggest DNA/rAd5 prime-boost provides a flexible platform that can be fine-tuned to generate desirable T-cell memory responses.

Keywords: vaccine, Ebola, prime, boost, CD8$^+$, CD4$^+$, T-cell, non-human primates

INTRODUCTION

DNA vaccines have been demonstrated to induce both durable cellular and humoral responses; however, these responses, while broad, were weak in primates when DNA was given alone without an additional vaccine (1–8). Both humoral and cellular immune responses can be significantly enhanced by combining a priming immunization with a heterologous boost vaccination (2, 6, 9–19). Homologous prime-boost immunization regimens, where the initial vaccine agent is re-administered via the same immunization route, have been used since the beginning of vaccine development and are common strategies for many licensed vaccines. The benefit of homologous prime-boost immunization is the efficient boosting of the humoral response and relative simplicity. In the case of DNA vaccines against HIV, three immunizations with DNA generated a superior humoral response than two immunizations, and this humoral response was maintained over time (20–22). However, the memory CD8$^+$ T-cell response was relatively weak (20).

In contrast to homologous prime-boost regimens, heterologous prime-boost vaccinations are more effective in generating a memory $CD8^+$ T-cell response of higher quality and magnitude (2, 9, 11–16, 23–26), and can bypass anti-vector immunity (27, 28). DNA prime followed by a protein or vector-based boost generates strong T-cell responses in addition to a potent antibody response (7, 29, 30). Various strategies consisting of a single or multiple DNA vaccines followed by recombinant adenovirus serotype 5 (rAd5) boost have been tested (31–35). While several of these regimens were reported to efficiently induce multifunctional $CD4^+$ and $CD8^+$ T-cell responses (35, 36), other strategies failed to induce strong $CD8^+$ T-cell responses. For example, three DNA prime immunizations followed by rAd5 boost 1 month later elicited a high frequency of SIV-specific $CD4^+$ T-cells but failed to induce $CD8^+$ T-cells against some subdominant epitopes (37). Immunogenicity data obtained from clinical trials using HIV DNA prime, NYVAC, or rAd boost have demonstrated a high magnitude T-cell response with $CD4^+$ T-cells observed more frequently than $CD8^+$ T-cells (38). It is known that DNA immunization could skew the cellular immune response toward $CD4^+$ T-cell responses (33, 39) but how variation in DNA/Ad5 prime-boost strategies affect memory humoral and cellular responses has not been systematically examined.

In this study, we evaluated the impact of multiple DNA vaccination and the time intervals between DNA prime and rAd5 boost on Ebola vaccine immunogenicity in non-human primates. Ebola virus (EBOV) is a member of the *Filovirus* family that causes hemorrhagic fever with a 32–89% fatality rate in humans (40). We have previously demonstrated that the combination of three immunizations with a DNA plasmid encoding Ebola glycoprotein (GP) followed by a rAd5-GP boost, as well as a single rAd5-GP immunization, protected 100% of non-human primates (NHP) against lethal EBOV challenge (41, 42), and antigen-specific IgG antibody level is a correlate of protection (43). At the same time, $CD8^+$ cellular immunity is required for uniform protection (42). By changing the frequency of DNA vaccination and prime-boost interval, we found that all regimens induced high and durable antibody responses. However, in contrast to the $CD8^+$ T-cell dominated response generated after a single DNA prime-rAd5-GP boost, multiple DNA primes resulted in higher $CD4^+$ T-cell magnitude and reduced $CD8^+$ T-cell responses. Extending the time interval between the multiple DNA primes and the Ad5-GP boost reversed the $CD4^+$ T-cell dominancy. Importantly, $CD8^+$ effector memory cells expressing both IFNγ and TNFα, a phenotypic quality associated with Ebola vaccine protection (44), could be preferentially expanded by modifying vaccine component order, frequency, or time interval. Our data demonstrate the importance of fine-tuning the immunization regimens according to the desired immune responses.

RESULTS

The Number of DNA Primes Impacts Antibody Responses to rAd5 Boost

To assess the impact of DNA immunization frequency on the immunogenicity of DNA prime/rAd5-GP boost regimen, we immunized groups of four macaques with single or multiple doses of DNA vaccine before boosting them with rAd5-GP (**Figure 1**). Anti-GP ELISA IgG specific responses were measured 2 weeks after the last DNA immunization (**Figure 2**). Single DNA prime induced modest plasma antibody titers with an average

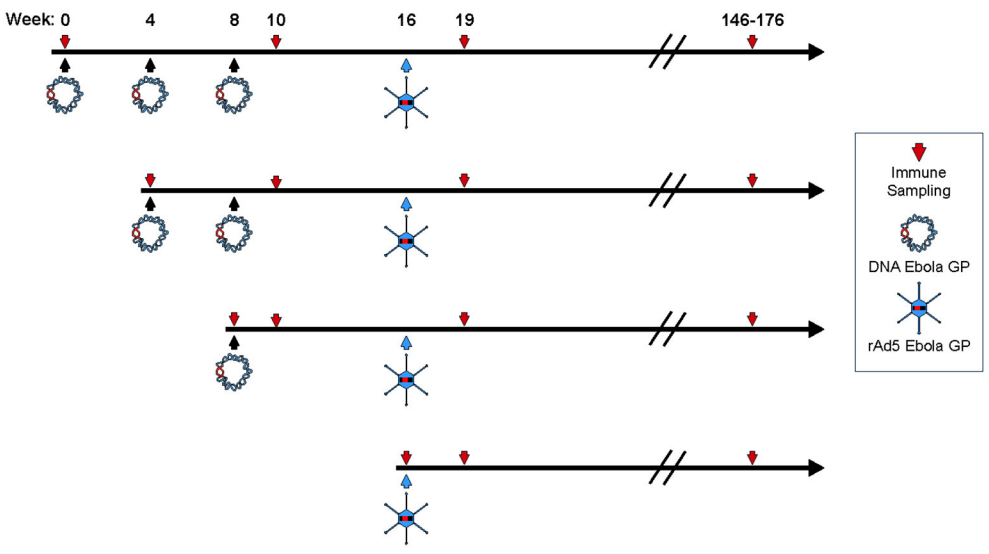

FIGURE 1 | Study design. Four female cynomolgus macaques in each group were primed with plasmid vectors encoding GP(Z) and GP(S/G) 1, 2, or 3 times. Eight weeks after the last DNA immunization some NHP's were boosted with 10^{11} PFU of rAd5-GP. Blood sampling for anti-GP antibody and T-cell analysis were collected as indicated by the downward arrows.

effective concentration (EC_{90}) of 807. Subsequent additional DNA immunization increased GP-specific antibody titer to 1,410 and 4,996 with 2x DNA and 3x DNA, respectively. However, even with 3x DNA primes, the GP-specific IgG titer was significantly lower than that induced by a single rAd5 immunization ($p = 0.026$, **Figure 2**), which is consistent with the hypothesis that DNA is weaker for antibody induction than rAd5.

Following boost immunization with rAd5-GP, GP-specific IgG titers measured at week three post boost were one to two orders of magnitude higher than those induced by DNA immunization (**Figure 2**). A significant increase in GP-specific IgG titers was observed in all DNA immunization groups after the rAd5 boost compared to DNA immunization alone ($p = 0.026$, 0.038, and 0.002 for groups with 1x, 2x, or 3x DNA vaccines, respectively). Furthermore, consistent with higher GP-specific IgG titers induced by multiple DNA immunization, we observed a trend of higher post-boost titers being associated with an increased number of DNA primes. The average post-boost titer of the 3x DNA prime group was significantly higher than the titer of the single DNA prime group ($p = 0.04$, **Figure 2**), which in turn was significantly higher than the titer induced by single rAd5 immunization ($p = 0.039$, **Figure 2**). These results suggest that DNA prime imprints a humoral response even when the titers are moderate after the prime, and allows rAd5 boost to elicit a stronger humoral response than the rAd5 prime alone.

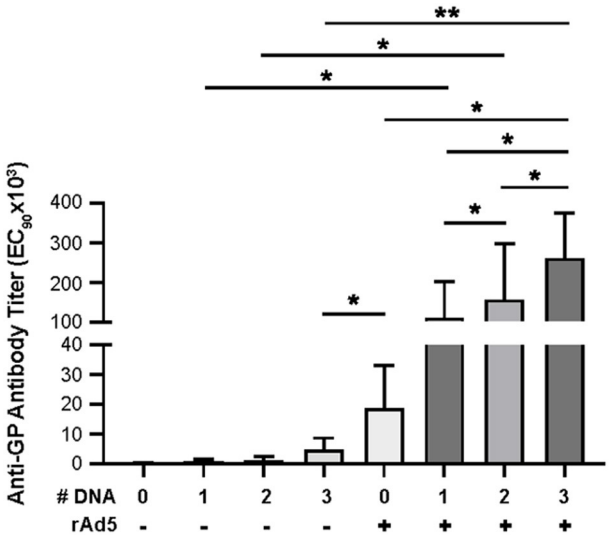

FIGURE 2 | Anti-GP antibody secretion. Anti-GP specific antibodies were detected in immunized NHP sera using ELISA. Sera were diluted from 1:50 to 1:50,000 in half-log increments. Goat anti-human IgG HRP 1:5,000 was used as secondary antibody. ELISA titers are expressed as EC_{90} values, the dilution at which there is a 90% reduction in antigen binding. Statistical analysis between more than two groups was performed using a one-way ANOVA with Tukey's *post hoc* test; $^*p < 0.05$; $^{**}p < 0.01$; mean ± standard deviation shown.

Multiple DNA Primes Change the Dominance of Post Boost T-Cell Response From a $CD8^+$ to a $CD4^+$ T-Cell Response

Having shown that the DNA prime strategy impacts the magnitude of humoral response after the rAd5 boost, we next evaluated the influence of the number of DNA prime doses on the cellular immune response. We used intracellular cytokine staining to measure the antigen-specific T-cell magnitude, defined as the frequency of memory T-cells expressing any one of three cytokines, IFNγ, TNFα, and IL-2 (**Figure 3A**, see **Supplementary Figure 1** for cytokine gating strategy). At 3 weeks post rAd5-GP boost, the 1x DNA prime/rAd5 boost regimen generated $CD4^+$ and $CD8^+$ T-cell responses with magnitudes similar to those induced by a single immunization with rAd5-GP (**Figure 3B**). In both cases, average $CD8^+$ T-cell responses were stronger than $CD4^+$ T-cell responses, accounting for 90% of the total T-cell response (**Figure 3C**). In contrast to a single DNA prime, multiple DNA primes were associated with an increase in the magnitude of $CD4^+$ T-cell response, but not in the magnitude of $CD8^+$ T-cell response. The proportion of the $CD4^+$ T-cell response among total T-cell responses post boost increased from 11% in 1x DNA prime group to 60% in the 2x DNA prime group, and further to 90% in the 3x DNA prime group (**Figures 3B,C**). Thus, multiple DNA primes favor the development of $CD4^+$ T-cell responses, and as a result, a $CD4^+$ dominant memory T-cell response was generated after the rAd5 boost.

In addition to the magnitude of T-cell response, T-cell quality, defined as the frequency of T-cells with certain combinations of effector functions, is important for vaccine-induced protection (45, 46). We tested if the DNA prime frequency also affects post-boost T-cell quality. Quality analysis of the $CD4^+$ T-cell response revealed a significant increase in the frequency of $IFN\gamma^+IL2^+TNF\alpha^+$ $CD4^+$ T-cells with the addition of each DNA prime immunization (**Figure 4A**). In contrast, the magnitude of $IFN\gamma^+TNF\alpha^+$ $CD8^+$ T-cells, a subset that is associated with rAd5 vaccine-induced protection against Ebola virus infection (47), was significantly reduced by 3- and 4.5-fold with the addition of the second and third DNA primes, respectively (**Figure 4B**). Thus, increasing the number of DNA primes may not only affect the magnitude but also the quality of the T-cell response.

Immune Responses Induced by DNA Prime or DNA Prime/rAd5 Boost Are Durable

To assess the durability of the T-cell response following DNA prime and rAd5-GP boost, the frequencies of antigen-specific $CD4^+$ and $CD8^+$ T-cells were analyzed 130–160 weeks after the rAd5-GP boost (**Figure 1**, first and third regimens). Single or multiple DNA primes followed by rAd5-GP boost 8 weeks later resulted in sustained $CD4^+$ T-cell responses that lasted for at least 2.5 years. The magnitude of the $CD4^+$ response 2.5 years after the boost was found to be similar between the 1x DNA/rAd5 and the 3x DNA/rAd5 groups (**Figure 5A**). This response was higher than the $CD4^+$ response that was measured 3 weeks post the rAd boost in the case of single DNA prime immunization but lower in the case of 3x DNA prime immunization (**Figure 3C**). In contrast, post rAd5 boost, specific $CD8^+$ T-cells could be detected mainly in animals that received a

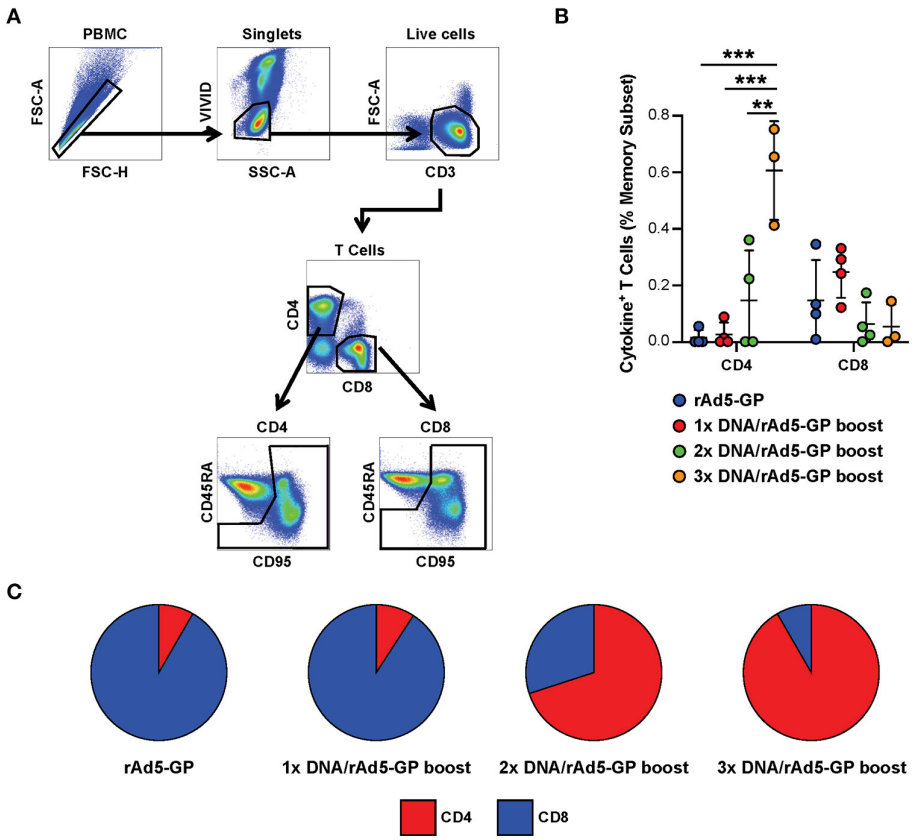

FIGURE 3 | Reduction of DNA priming reverses the CD4/CD8 ratio in heterologous DNA prime rAd5 boost vaccination. Ebola GP reactive $CD4^+$ and $CD8^+$ T-cells secreting any of the three cytokines measured (IFNg, IL-2, or TNFa) were detected in the memory ($CD95^+CD45RA^{hi}$ and $CD95^+CD45RA^{lo}$) subset of the NHP's PBMCs following 6 h *in vitro* stimulation with EBOV GP peptide pool or DMSO control. **(A)** T-cell gating tree. **(B)** Magntitude of cytokine positive T-cells in animals vaccinated with: rAd5 GP (blue), 1x DNA prime-rAd5-GP boost (red), 2x DNA prime-rAd5-GP boost (green), and 3x DNA prime-rAd5-GP boost (orange). **(C)** The relative proportion of $CD4^+$ or $CD8^+$ T-cell responses in each group. Statistical analysis between more than two groups was performed using a one-way ANOVA with Tukey's *post hoc* test; $^{**}p < 0.01$, $^{***}p < 0.001$; mean ± standard deviation shown.

single DNA prime immunization and were barely detected in the multiple DNA primed animals (**Figure 5C**). Quality analysis of the $CD4^+$ T-cells 2.5 years after boost revealed the dominancy of $TNF\alpha^+$ single positive cells (**Figure 5B**). The $CD8^+$ T-cell dominance in the single DNA prime rAd5-GP consisted mainly of $IFN\gamma^+IL2^+TNF\alpha^+$ and $IFN\gamma^+IL2^-TNF\alpha^+$ secreting cells, indicating a memory phenotype (**Figure 5D**) (48). Anti-Ebola GP IgG could also be detected 130–160 weeks after the rAd5 boost of either single or multiple DNA primed groups. Anti-GP IgG titers were significantly higher in NHPs that received a single DNA prime/rAd5 boost than NHPs that were boosted after multiple DNA primes (**Figure 5E**).

Interestingly, 3 years after a single DNA immunization with no additional boost, low yet measurable $CD4^+$ T-cells (data not shown), as well as anti-Ebola GP antibody could be detected (**Figure 5E**). To assess the immunological memory established by a single DNA prime long-term, this group was boosted with rAd5-GP 3 years later. High titers of anti-GP antibody (200,373 ± 127,700) were detected (data not shown). We found that previously undetected $CD8^+$ T-cell responses could be boosted after 3 years, and shifted the memory phenotype to one that slightly favored $CD8^+$ T-cells (**Figure 5F**). These data indicate that durable and stable immunological memory can be established after a single DNA prime and leaves a large window of time for a subsequent boost with rAd5.

Increasing the Time Interval Between Multiple DNA Primes and rAd5 Boost Can Reverse the T-Cell Dominancy From $CD4^+$ to $CD8^+$ T-Cells

To assess whether the long time interval following prime could improve $CD8^+$ T-cell responses in the multiple DNA prime/rAd5 strategy, we boosted four animals with rAd5 1 year after multiple DNA immunizations (**Figure 6A**). In contrast to $CD4^+$ T-cell dominant responses observed when the boost immunization was given 8 weeks post-prime (**Figure 3B**), a long time interval between the DNA prime and rAd5-GP boost resulted in high magnitudes of both $CD4^+$ and $CD8^+$ T-cell responses, with $CD8^+$ T-cell responses dominating (**Figure 6B**). Furthermore,

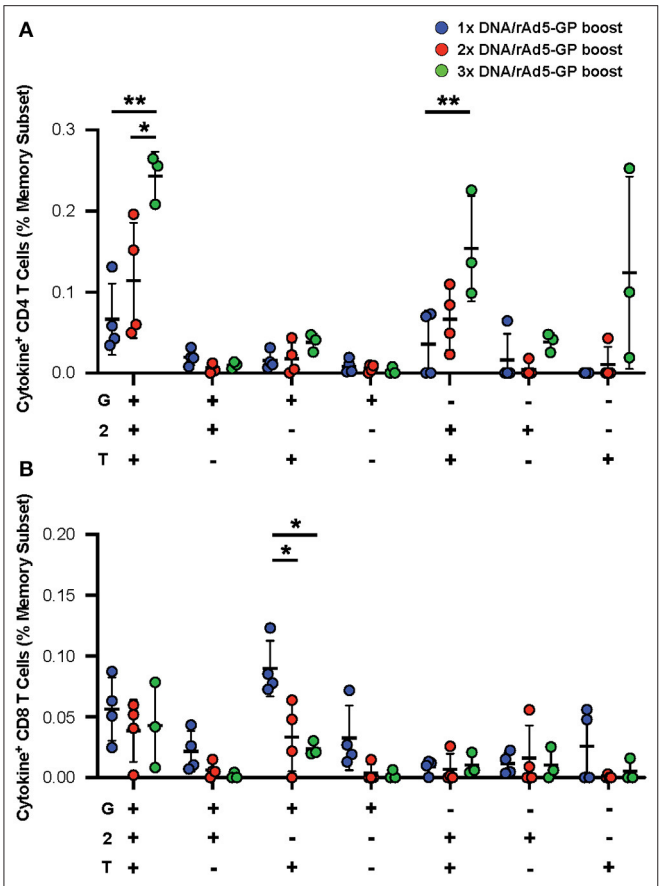

FIGURE 4 | GP specific polyfunctional T-cell. PBMCs were stimulated with EBOV GP peptides. The functionality of antigen-specific $CD4^+$ **(A)** and $CD8^+$ **(B)** T-cell responses were assessed in DNA prime rAd5 boost NHP's by analyzing the individual cytokine (G:IFNγ, T:TNFa, and 2:IL-2) response pattern. Statistical analysis between more than two groups was performed using a one-way ANOVA with Tukey's *post hoc* test; $^*p < 0.05$, $^{**}p < 0.01$; mean ± standard deviation shown.

boosting with rAd5 1 year after multiple DNA immunizations yielded the same high levels of GP-specific IgG titers as boosting with rAd5 8 weeks after multiple DNA immunizations (240,000 ± 186,000 and 262,000 ± 111,000, respectively) (**Figure 6C**).

DISCUSSION

Following vaccination with rAd5 Ebola GP, high quality Ebola-specific memory $CD8^+$ T-cells secreting multiple cytokines are needed to confer protection against lethal challenge, and moreover, the protection in this vaccination regimen was found to be correlated specifically with $IFN\gamma^+TNF\alpha^+$ $CD8^+$ T-cells (42). In addition, a high antibody titer was found to be a quantitative predictor for rAd5 Ebola GP vaccine efficacy (43). Therefore, generating a large population of this specific high-quality memory $CD8^+$ T-cell population is an essential goal. Based upon experience with licensed vaccines, multiple vaccinations as a prime-boost regimen is a feasible approach to rapidly generate a large population of memory $CD8^+$ T-cells.

The initial immunization has a major influence on the nature of the immune response that follows the boost. One important variable that impacts how strongly the $CD8^+$ T-cell population can be boosted is the length of time separating the primary and secondary antigen administrations. A time period of at least 40–60 days is required before optimal boosting of the $CD8^+$ T-cell population is possible (49). A single DNA prime immunization before the rAd5 boost 8 weeks later resulted in the expansion of the $CD8^+$ T-cell population (mainly $IFN\gamma^+TNF\alpha^+$ cells that are known to correlate with protection against Ebola infection), while the $CD4^+$ T-cell population was very small. In contrast, after three prime immunizations with DNA, we could not detect expansion of the $CD8^+$ T-cells after the rAd5 GP boost, while the expansion of the $CD4^+$ T-cell population could be observed. The reduction in the $CD8^+$ T-cells observed after multiple DNA prime immunizations were mainly in the $IFN\gamma^+TNF\alpha^+$ and $IFN\gamma^+$ secreting cells. The increase in the $CD4^+$ T-cell population after a single DNA prime rAd boost was mainly in the polyfunctional memory T-cells. The induction of high magnitude, high quality $CD8^+$ T-cells is likely beneficial in the control of viral replication at its initiation and therefore might confer protection against lethal challenge. Thus, a single DNA prime followed by a rAd5-GP boost 8 weeks later is beneficial for the generation of $CD8^+$ T-cells in a shorter time period than multiple DNA prime immunizations.

As shown in our work, DNA prime immunization resulted in low anti-GP antibody titers and tracked with the number of DNA primes. As a high antibody titer was found to be a surrogate marker for vaccine efficacy, this regimen by itself may not be sufficient for protection against Ebola. However, this prime generates humoral immunological memory that lasts for at least 3 years, even after a single DNA prime, and that memory could be activated by the rAd5-GP boost at any time. Thus, prime immunization with a single DNA vaccine followed by a rAd5-GP boost given when needed might be a useful approach for generating a polyfunctional effector $CD8^+$ T-cell population for the rapid development of protective immunity that is mediated by $CD8^+$ T-cells and antibodies.

The impact of the prime frequencies on the durable antibody response is interesting and is likely associated with memory B-cells. It was not surprising to observe a drop in Ag-specific IgG levels 130–160 weeks after the boost given that there was no additional exposure to antigen. It was, however, interesting that we observed a higher Ag-specific antibody response in animals vaccinated with a single DNA plus rAd5-GP vs. 3x DNA plus rAd5-GP 130–160 weeks post boost. The low Ag-specific IgG titers in the 3x DNA group could be explained by our observation that increasing time after multiple DNA primes favors $CD8^+$ T-cell responses. In contrast, a single DNA plus rAd5-GP over time may favor $CD4^+$ T-cells. Thus, since $CD4^+$ T-cells support B-cell function, this may be the obvious explanation. Furthermore, the presence of specific anti-GP antibodies years after immunization is probably due to long lived plasma cells in survival niches. It might be that multiple DNA primes followed by rAd5-GP boost leads to a very high Ag-specific antibody response that results

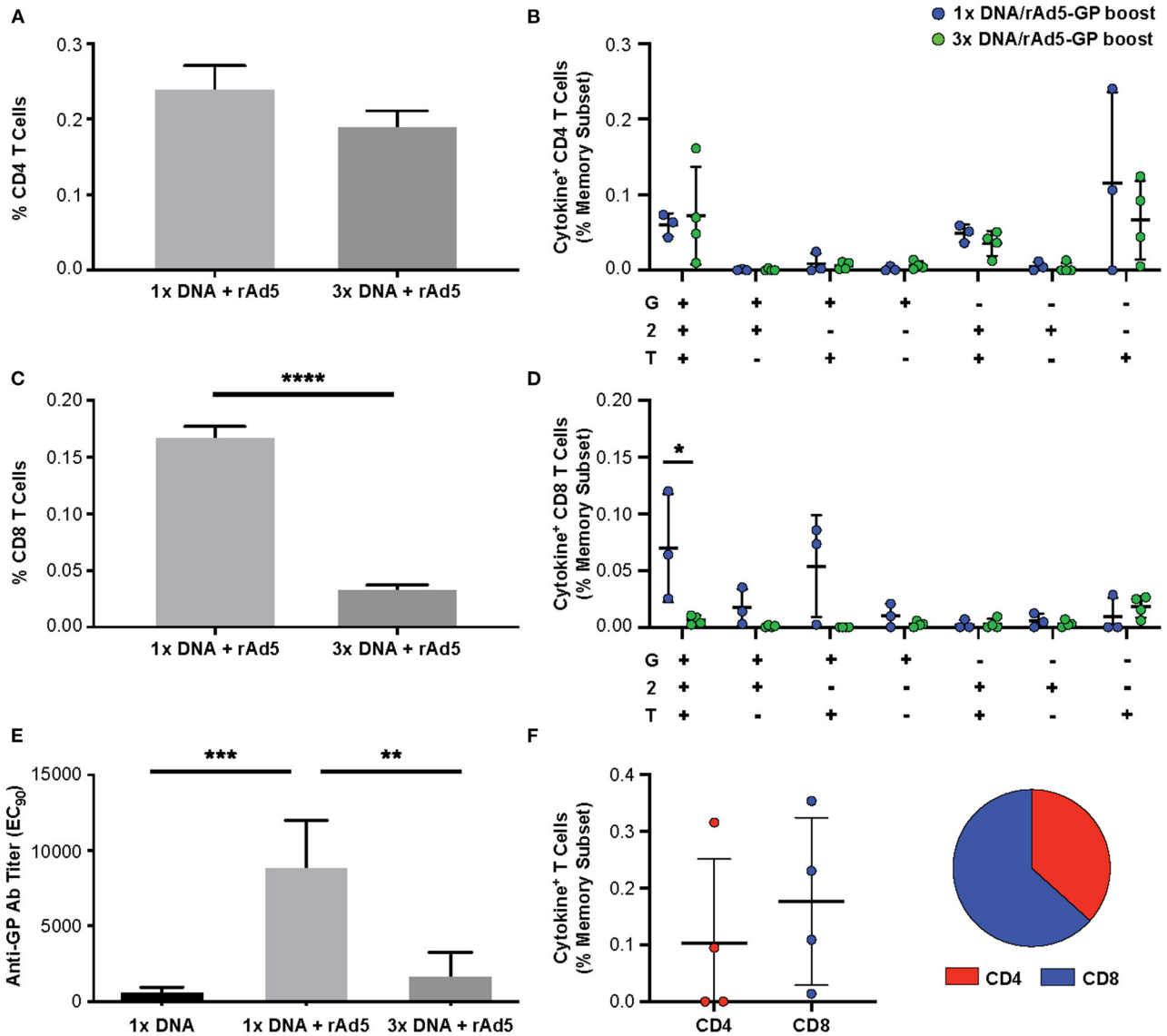

FIGURE 5 | Durability of the immune response following DNA prime rAd5-GP boost. Cynomolgus macaques were primed with plasmid vectors encoding GP(Z) and GP(S/G) one or three times. Eight weeks after the last DNA immunization 4 NHP's from each group were boosted with 10^{11} PFU of rAd5-GP vaccine. Blood sampling for anti-GP antibody and T-cell analysis were collected 130–160 weeks after the rAd5-GP boost, and at 159 weeks from four animals which received only a single DNA prime immunization without a boost. PBMCs were stimulated with EBOV GP peptides. The magnitude of antigen-specific cytokine response and pattern of $CD4^+$ **(A,B)** and $CD8^+$ **(C,D)** T-cells (G:IFNγ, T:TNFa, and 2:IL-2). **(E)** Specific anti-GP antibody titers were measured using ELISA. **(F)** Ebola GP specific $CD4^+$ and $CD8^+$ T-cells after rAd5 GP boost that was given 159 weeks after a single DNA prime. Statistical analysis between two groups was performed using an unpaired student's *t*-test, comparison between more than two groups were performed using a one-way ANOVA with Tukey's *post hoc* test; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$,$^{****}p < 0.0001$; mean ± standard deviation shown.

in B-cell exhaustion and elimination. The outcome might be a decreased number of long lived plasma cells leading to low levels of anti-GP antibodies. A single DNA immunization is probably insufficient for the generation of a substantial antibody response and long-lived plasma cells.

The $CD8^+$ T-cells from all treatment groups showed dominance of $IFN\gamma^+TNF\alpha^+$ and $IFN\gamma^+$ $IL2^+TNF\alpha^+$, with similar polyfunctional profiles and the major difference being found in the magnitude of the specific T-cell subpopulations. Thus, DNA prime regimens may not influence the maturation of the T-cells, but instead affect the expansion of the T-cell population, with the $CD4^+$ T-cells expanded following multiple DNA primes at the expense of the $CD8^+$ T-cells. The effect of the number of DNA primes on cellular and humoral immune responses suggests a unique and complex immune mechanism rather than simply impairing the expression of the GP protein by the rAd5-GP boost (**Figure 7A**). Our results revealed that compared to multiple DNA immunizations, a single DNA prime

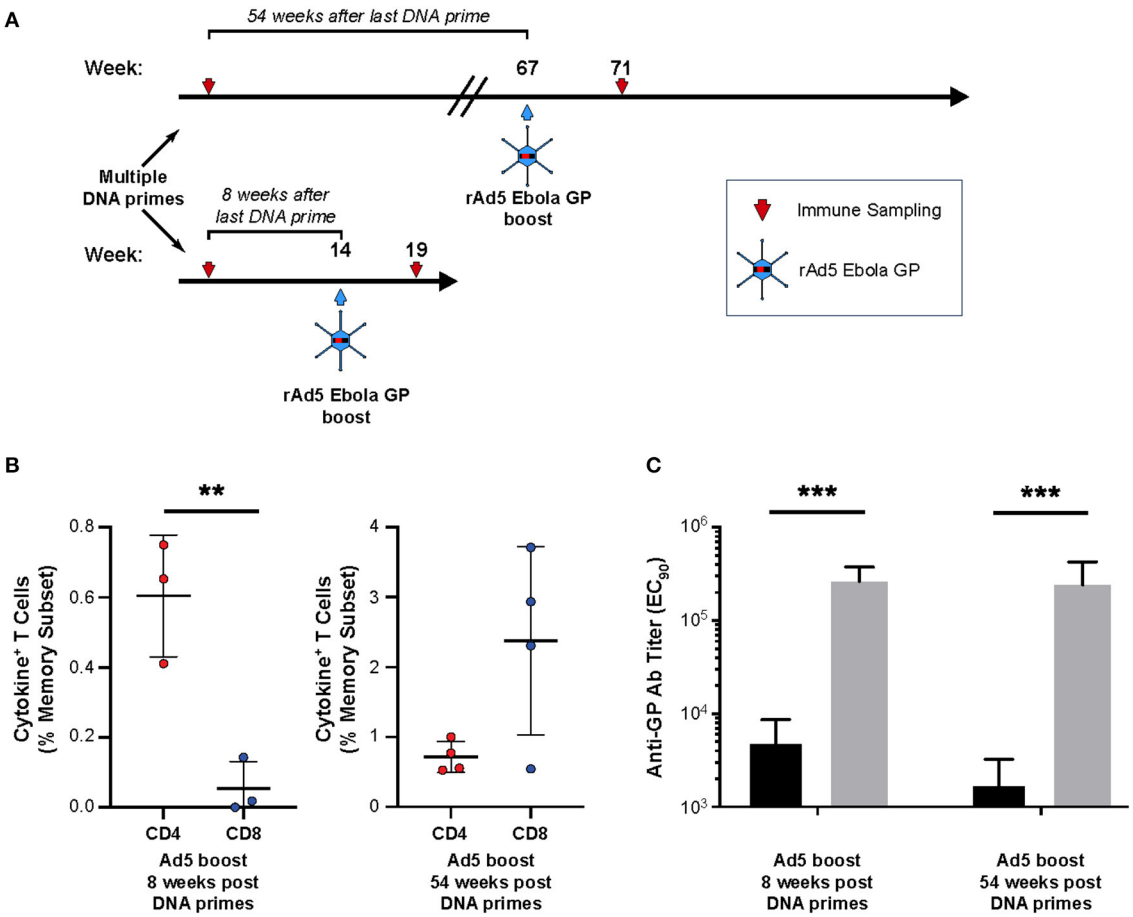

FIGURE 6 | Effect of time interval between prime and boost on GP responses. **(A)** Study design: Four cynomolgus macaques were primed with multiple plasmids encoding Ebola GP(Z) and GP(S/G). Eight or 54 weeks after the last DNA immunization NHP's were boosted with 10^{11} PFU of rAd5-GP vaccine. Blood sampling for anti-GP antibody and T-cell analysis was taken before and after the rAd5 boost. **(B)** Antigen-specific $CD4^+$ and $CD8^+$ T-cells analyed 4 weeks after the boost in the short interval or 4 weeks after the boost in the long interval. **(C)** Anti-GP antibody titers, pre boost (black), post boost (gray). Statistical analysis between two groups was performed using an unpaired student's *t*-test; $^{**}p < 0.01$, $^{***}p < 0.001$; mean ± standard deviation.

skewed toward $CD8^+$ T-cell responses after the rAd boost. This observation is likely explained by the fact that adenoviral vectors are strong inducers of $CD8^+$ T-cell responses, while a single DNA vaccination alone generates relatively weaker responses. Therefore, it is not surprising that a single DNA plus rAd5-GP boost is very similar in T-cell phenotype to that of a single rAd5-GP prime without a boost. Similarly, a long-time interval (1 year) between multiple DNA primes and the rAd5-GP boost allowed the robust rAd-induced $CD8^+$ T-cells to dominate the DNA-induced memory $CD4^+$ T-cells, and thus reversed the $CD4^+/CD8^+$ T-cell ratio observed following multiple DNA primes, with a short time interval before rAd5-GP boost (8 weeks), from a $CD4^+$ T-cell dominance to that of a $CD8^+$ T-cell dominance (**Figure 7B**). These observations could be explained by the immune response generated following the prime. If the immune response after the prime was not fully contracted, the response generated by the boost will be influenced by the prime. On the other hand, if the response generated by the prime was fully contracted and low, the boost may be efficiently boosting the primed (memory) response, and instead could more closely resemble a primary response from the "boost." In essence, the strength and nature of the primary immunization could imprint particular qualities on the memory T-cells irrespective of the boosting agent. Additionally, the magnitude of T-cell reactivation could also have an impact on the quantity and quality of the memory T-cell response after the boost. There are several studies in which a prolonged time interval between the prime and the boost was found to be critical for the establishment of a memory response (50–53). Since $CD8^+$ cells are important in the context of rAd5-GP-induced immune protection against Ebola virus infection, an immunization regimen that induces robust $CD8^+$ cells may be beneficial.

The data presented herein suggest it is possible to tailor immune responses and provide a framework for testing the relative contribution of skewed or balanced immune responses to immune protection against Ebola or other pathogens using infectious challenge models. Our study indicates that the DNA prime regimen, when reduced to a smaller number of injections,

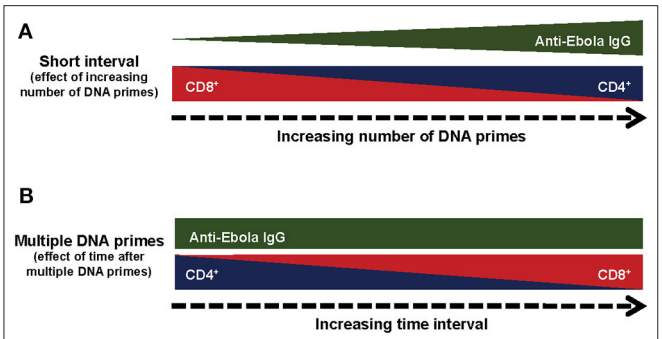

FIGURE 7 | Summary of the immunological responses to different regiments of DNA prime rAd5-GP Boost. **(A)** Effect of the number of prime immunizations. **(B)** Effect of time after multiple DNA prime immunizations.

can contribute to the expansion of the $CD8^+$ T-cells without changing their polyfunctional properties. The benefits of such a prime-boost regimen are the induction of a high magnitude antibody response, without sacrificing the $CD8^+$ T-cell advantage induced after the rAd5-GP immunization, and in a much shorter time than the three DNA prime-rAd5 boost regimen. Thus, when considering the DNA prime rAd5 boost regimen, a single DNA prime will allow for the establishment of a robust and durable immunological response in a shorter period of time with the benefit of high magnitude antibody responses and $CD8^+$ T-cell responses. The same approach could be applied for a condition in which the rapid generation of both antibodies and polyfunctional $CD8^+$ T-cell responses are desired, either for vaccine design or treatment of human diseases.

While these studies were focused on Ebola vaccines, the results show that vaccine platforms can drive the relative balance of immune responses. Therefore, similar $CD4^+$ to $CD8^+$ phenotypes would be expected with a similar DNA/rAd5 vaccination strategy expressing a different protein, although this would have to be systematically evaluated. A vaccination strategy requiring four administrations to drive specific immune response ratios has pragmatic limitations, but the data provide proof of principle that vaccination strategies can be tailored to induce $CD4^+$ or $CD8^+$ T-cell responses depending on the desired outcome.

MATERIALS AND METHODS

Vaccines

The vaccine vectors used in this study have been described previously (41). DNA and replication-defective rAd5 GP vectors were cloned and purified as described previously (54).

Animal Study and Safety

Animal experiments were conducted in full compliance with all relevant federal guidelines and NIH policies. All animal experiments were conducted under protocols approved by NIH Animal Care and Use committees. Female cynomolgus macaques (*Macaca fascicularis*) 3–5 years of age and weighing between 2–3 kg were obtained from Covance for all studies. Monkeys were housed individually and given enrichment regularly as recommended by the Guide for the Care and Use of Laboratory Animals (DHEW number NIH 86-23). Animals were anesthetized with ketamine prior to blood sampling or vaccination. Each vaccination group in this study contained three or four cynomolgus macaques.

Macaque Immunization

DNA immunizations were administered to cynomolgus macaques to both deltoids by Biojector intramuscular injection with a mixture of two milligrams each of two plasmid vectors encoding the glycoprotein from *Zaire ebolavirus* Mayinga strain, GP(Z), and the glycoprotein from *Sudan ebolavirus* Gulu strain, GP(S/G). One to four DNA immunizations were administered as indicated in each experiment. Following the final DNA priming immunization, at a time indicated for each group subjects received a boost immunization with 10^{11} particle units (PU) of rAd5 encoding the glycoprotein from *Zaire ebolavirus* Mayinga strain, GP(Z). The boost was performed by intramuscular injection in the deltoids by needle and syringe containing the vectors.

Anti-EBOV GP IgG ELISA

Anti-EBOV GP IgG ELISA titers were measured as described previously (55). Transmembrane-deleted EBOV GP (EBOV GPΔTM) was generated by calcium phosphate-mediated transient transfection of 293T cells using the Promega ProFection® Mammalian Transfection System and the plasmid VRC6008 [pVR1012-GP(Z) delta Tm]. 293T cells were plated in complete media [high glucose DMEM (ThermoFisher Scientific, Waltham, MA) containing 10% fetal bovine serum (Gemini Bio, Sacramento, CA)] 14–18 h prior to transfection. Two to three hours before transfection the media was removed and replaced with fresh complete media. The cells were transfected at 30–60% confluency. Eight hours post transfection, the media was once again replaced with complete media. Twenty-four hours post transfection, the media was replaced with serum-free media (DMEM without FBS). The cell culture supernatant was harvested 36 h later, centrifuged, and filtered using 0.22 mm filters. This cell culture supernatant served as the antigen. Polyvinyl chloride enzyme-linked immunosorbent assay (ELISA) plates (Nunc, Rochester, NY) were coated with 100 μl of antigen per well and incubated at 4°C overnight. Subsequent incubations were performed at room temperature. Plates were washed with PBS containing Tween 20 after antigen coating. Test sera were serially diluted and added to the antigen-coated wells for 60 min. The plates were washed followed by incubation with the detection antibody, goat anti-human IgG (H+L; SouthernBiotech/Millipore, Billerica, MA) conjugated to horseradish peroxide. SigmaFast o-phenylenediamine dihydrochloride (Sigma, St. Louis, MO) substrate was added to the wells, and the optical density was determined (450 nm). A positive control serum sample from a single animal with a known EBOV GP IgG response was run every time the assay was performed. Background-subtracted ELISA titers are expressed as EC_{90}, reciprocal optical density values, which represent the dilution at which there is a 90% decrease in antigen binding.

Intracellular Cytokine Staining

Intracellular cytokine staining was performed as described previously (44). After the 6 h stimulation with EBOV GP peptides or DMSO control, PBMC were stained with a mixture of antibodies against lineage markers [CD3-Cy7-APC clone SP34-2 (BD Biosciences), CD4-QD605 clone M-T477 (BD Biosciences), CD8-PerCP Cy5.5 clone RPA-T8, CD95 Cy5-PE clone DX2 (BD Biosciences), CD28 Alexa 488 clone 28.2 (Biolegend), CD45RA QD655 clone 5H3] at room temperature for 20 min. After two washes the cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) followed by staining with antibodies against cytokines: tumor necrosis factor (TNF)α-APC clone MAb11 (BD Biosciences), interleukin (IL)-2 PE clone MQ17H12 (BD Biosciences), and IFNγ PE-Cy7 clone B27. The viability dye ViViD (Invitrogen) was included to allow discrimination between live and dead cells (56). EBOV-specific cytokine positive cells were defined as a percentage within $CD4^+$ and $CD8^+$ T-cell memory subsets ($CD95^+CD45RA^{hi}$ and $CD95^+CD45RA^{lo}$) secreting any of the three cytokines measured (IFNγ IL-2 or TNFα) above DMSO background. Cells were acquired on BD-LSR II cytometer collecting up to 1,000,000 total events, resulting in >100,000 events in the CD4/CD8 T-cell gates. Typically, this yields >10 total events in the cytokine gates of the antigen-stimualted samples. Samples were analyzed using FACSDiva and analyzed with FlowJo 9.4.9 (Tree Star, Inc.) and SPICE software (57).

Statistical Analysis

Comparison of anti-GP ELISA IgG titers and intracellular cytokine production by T-cell memory subsets was performed using an unpaired two-tailed Student's *t*-test for comparisons between two groups or one-way ANOVA with Tukey's *post hoc* test for comparisons between more than two groups in GraphPad Prism version 9 software.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by VRC ACUC.

AUTHOR CONTRIBUTIONS

HM and NS designed these studies. HM, ET, CA, MD, KF, and DS executed experiments. NS and CA wrote animal study protocols. MB and CA executed vaccination. HM, YZ, JM, and NS wrote the manuscript. MR provided scientific support. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Intramural Research Program of the Vaccine Research Center, the National Institute of Allergy and Infectious Diseases, and the National Institutes of Health.

ACKNOWLEDGMENTS

We thank the Vaccine Research Center's Non-human Primate Immunogenicity Core and the Flow Cytometry Core for NHP sample processing. We thank Ruth Hunegnaw, Ph.D. for her careful review of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.627688/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.

frontiers in Immunology

REVIEW
published: 18 March 2021
doi: 10.3389/fimmu.2021.629636

The Integration of Human and Veterinary Studies for Better Understanding and Management of Crimean-Congo Haemorrhagic Fever

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Outbreaks that occur as a result of zoonotic spillover from an animal reservoir continue to highlight the importance of studying the disease interface between species. One Health approaches recognise the interdependence of human and animal health and the environmental interplay. Improving the understanding and prevention of zoonotic diseases may be achieved through greater consideration of these relationships, potentially leading to better health outcomes across species. In this review, special emphasis is given on the emerging and outbreak pathogen Crimean-Congo Haemorrhagic Fever virus (CCHFV) that can cause severe disease in humans. We discuss the efforts undertaken to better understand CCHF and the importance of integrating veterinary and human research for this pathogen. Furthermore, we consider the use of closely related nairoviruses to model human disease caused by CCHFV. We discuss intervention approaches with potential application for managing CCHFV spread, and how this concept may benefit both animal and human health.

Keywords: CCHF, NSDV, One Health, Hazara, vaccines, veterinary vaccines, mouse model, NHP model

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: *15 November 2020*
Accepted: *01 March 2021*
Published: *18 March 2021*

Citation:
Gilbride C, Saunders J, Sharpe H, Maze EA, Limon G, Ludi AB, Lambe T and Belij-Rammerstorfer S (2021) The Integration of Human and Veterinary Studies for Better Understanding and Management of Crimean-Congo Haemorrhagic Fever. Front. Immunol. 12:629636. doi: 10.3389/fimmu.2021.629636

INTRODUCTION

Zoonotic diseases are caused by pathogens which circulate in vertebrate hosts and periodically spillover into human populations (1). It has been estimated that over 60% of pathogenic species in humans originally arose from animal populations (2). In the 21st century alone, several zoonotic pathogens have caused epidemics such as SARS (3) and MERS coronaviruses (4), avian influenza (5), and Ebolavirus (6, 7), as well as pandemics of swine flu (8) and the newly emerged SARS-CoV-2 (9), which alone has caused an estimated 2.25 million deaths up to February 2021 (10).

While it is difficult to estimate the continuous economic burden of zoonotic disease, localised epidemics and worldwide pandemics can induce instant and deep economic shock which has important secondary impacts on global health outcomes. The 2015 MERS outbreak in South Korea infected 186 confirmed individuals and caused a total of 36 deaths, with the overall economic damage to South Korea approximated at USD$8.5 billion (0.6% GDP) (11, 12). Estimates for the

larger 2002-2003 SARS epidemic place the economic costs at $3.7 billion (2.6% GDP) in the Hong Kong epicentre (13, 14), while the recent COVID-19 pandemic economic impact may be greater with estimates for the second quarter alone of 2020 showing a 2% fall in GDP worldwide (15).

Economic damage can directly cause human mortality, particularly in low- or middle-income countries where the healthcare systems may be less robust. The 2014-2016 Ebolavirus epidemic resulted in approximately 11,000 direct deaths (7), but the subsequent overwhelming of the healthcare systems in Guinea, Sierra Leone, and Liberia caused an estimated 10,000 further deaths (16). Avian influenza virus strains can cause fatal disease in humans and introduction into Indonesia in 2009 caused devastation to livelihoods as a result of culling 11 million poultry. The subsequent closure of 30% of the country's farms created severe disparities in education and nutrition in the worst affected communities (17).

Human activities, such as accelerated deforestation, encroachment into natural animal habitats, or climate change (18), exponentially increase the likelihood of exposure and spillover of novel zoonotic pathogens (19). Due to their potential impact to human health, the WHO and other healthcare agencies have identified pathogens of concern with epidemic or pandemic potential (**Table 1**). The list represents zoonotic diseases that have caused or may lead to outbreaks, and many lack effective and timely control measures that are crucial for preserving human and animal health.

The One Health approach acknowledges the interdependent relationship between human and animal health, together with our shared environment. The need to increasingly consider all parts of the ecosystem arose as a result of global concerns including antimicrobial resistance and the prevalence of emerging infectious diseases transmitted between animals and humans (36). One Health approaches aim to address such issues using control measures that are ultimately rooted in achieving better veterinary and human health outcomes. Control measures can include detailed epidemiology facilitating identification of transmission routes, disease surveillance, or more overt strategies such as the development of therapeutic and preventative healthcare measures (37). A conceptual framework encompassing a One Health strategy requires concerted inter-disciplinary efforts from many professions including social science, healthcare and epidemiology. This level of collaboration is necessary to scale solutions from local to national to global levels to help manage the spread of zoonotic diseases (38).

An identified zoonotic disease of concern, Crimean-Congo Haemorrhagic Fever (CCHF), is a tick-borne disease caused by CCHFV, which affects humans and can cause severe haemorrhagic symptoms with fatal outcomes (39). As noted in **Table 1**, numerous vertebrates can act as reservoirs of CCHFV to help maintain the virus. Reservoir animals can experience transient viremia and may develop antibody responses towards CCHFV, but no clinical disease is observed (40). As such, further work is needed to identify the main drivers of disease underpinning pathogen transmission.

In this review we discuss the value of integrating veterinary and human research in the field of CCHF by discussing findings from recently developed animal models and underlining the benefit of exploring closely related nairoviruses such as Nairobi Sheep Disease Virus (NSDV) and Hazara Virus (HAZV). In addition, we address current vaccine candidates for preventing CCHF and a range of approaches that could be key aspects to One Health approaches to combat CCHF.

CRIMEAN-CONGO HAEMORRHAGIC FEVER

The control and management of zoonotic diseases, such as CCHF, relies heavily on knowledge of the disease. Frequently the causes and impacts of zoonoses are complex and poorly understood (41). Animal reservoirs of CCHFV encompass domestic ungulates, while small mammal and bird populations are thought to play a role in immature tick maintenance and CCHFV transmission (42, 43). For a successful One Health approach, it is crucial that sufficient understanding is acquired on animal reservoirs, amplifying hosts and transmission patterns to adequately understand both human and animal disease, while considering CCHFV maintenance and circulation in ticks (44). Attainment of greater knowledge in these areas will support the implementation of control measures, such as prophylactic vaccines for human or animal use (45).

TABLE 1 | WHO Blueprint Diseases and their principal mammalian reservoirs.

WHO Blueprint Disease (20)	Mammalian reservoir(s)
COVID-19 (recently added)	Fruit bats (speculative) (21), pangolin (speculative) (22)
Crimean-Congo Haemorrhagic Fever (CCHF)	Cattle (23), goats (24), sheep (23), camels (25), horses (26), donkeys (27)
Ebola virus disease	Fruit bats (28)
Marburg virus disease	Fruit bats (29)
Lassa fever	Multimammate mouse (*Mastomys natalensis*) (30)
MERS coronavirus disease	Bats, alpacas, camels (31)
SARS coronavirus disease	Horseshoe bats, palm civets (32)
Nipah virus disease	Flying foxes (33), pigs (33)
Rift Valley Fever	Sheep, goats, cattle (34)
Zika	Rhesus monkeys, sheep, goats, cows, horses, bats, carabaos, orangutans (35)
Disease X (future disease outbreak of unknown origin)	Unknown

CCHFV is maintained through a tick-vertebrate-tick transmission cycle (46, 47). Ticks require a blood feed on small vertebrates to progress in their life cycle from larvae to nymph, and then feeding on large mammals, such as cattle and sheep, to progress from nymph to adult tick (47). Though their role is not fully understood, flighted birds can carry ticks and are thought to contribute to the expansion of tick populations to other territories (43, 48, 49). CCHFV is contracted through two major routes. The first is through direct contact with infected ticks, either through the bite of an infected tick (44) or tick pulverisation on open wounds exposing an individual to the virus (50). The second transmission route is contact through an open wound with the blood or bodily fluids of an infected person or animal. High risk occupations for CCHFV infection include veterinarians, farmers, and abattoir workers in endemic areas that are in close proximity to livestock (50–52). Human-to-human transmission can occur following close contact with infected individuals, posing considerable risk for nosocomial outbreaks (53–55).

CCHF is one of the most widespread tick-borne diseases (42). Cases in humans are frequently reported in countries across Asia, Africa and Europe (39) and CCHFV is increasingly being identified in new geographical regions (56, 57). Human infections can range in severity from subclinical or mild disease with non-specific symptoms, to severe disease which can cause fatal haemorrhagic disease (54). The mortality rate during annual outbreaks is estimated to be between 5-30% (39, 46). There are potential differences in CCHF severity between CCHFV endemic geographical regions and it is thought that many human CCHF cases remain subclinical which affect calculated mortality rates (58). A sero-epidemiological survey in Turkey estimated that 88% of CCHFV infections were subclinical but it is unclear if similar asymptomatic rates occur in other endemic countries (59).

Controlling and curbing CCHFV circulation represents a public health priority, especially in countries where the virus is endemic. Better understanding of disease is needed to accelerate therapeutic and interventional treatments. Animal models are critically important in underpinning our knowledge of disease dynamics and progression. As discussed in the following section, understanding of CCHF can be supported through the study of animal models and closely related nairoviruses.

Animal Models of CCHFV Infection

CCHFV is typically studied in BSL-4 conditions due to the high risk of severe or fatal haemorrhagic disease in humans, as at present there are no therapeutics or vaccines available (60). Mouse and non-human primate (NHP) models exist for studying CCHFV pathogenesis in these high containment settings. There are a limited number of CCHFV mouse models due to the need to supress or pre-empt the immune system, as infection of immunocompetent mice with CCHFV does not result in overt disease (61). Historically, the first model involved new-born mice, but it was considered inadequate as pathogenesis in new-born mice involved severe damage to the central nervous system (46), a pathology not representative of human disease. Typical murine models involve knock-out of IFN-I receptor (61, 62) or STAT1 (63) targeting the type-I interferon (IFN-I) response. The same phenotype can be induced in immunocompetent mice by treatment with antibodies against IFN-1 that block IFN-1 signalling prior to CCHFV infection (64). Such knock-out models have been useful, as some of the main clinical symptoms and disease pathogenesis of CCHF manifest, particularly infection of the liver and spleen, within 4-5 days of inoculation (61–63). These animal models have been informative in determining viral tropism and have been used to assess vaccine candidates (65, 66).

Development of NHP models of infection are often considered a pinnacle of replicating human infections in an animal model. However, disease pathology can differ across NHP species for many emerging pathogens, requiring infection studies on a breadth of species to determine a suitable model. For CCHFV, cynomolgus macaques are a suitable model of infection mimicking human disease (67). In cynomolgus macaques, disease pathology appears to be similar to that of humans, with all infected animals developing mild to severe disease, and a proportion of infections displaying a lethal outcome (67). A larger immunocompetent animal model of infection, such as a NHP model, is generally deemed crucial to assess vaccines and therapeutics against CCHFV infection, however, they are more expensive than small animal knock-out models.

CCHFV Analogues for Understanding *Orthonairovirus* Pathogenicity

In order to overcome the difficulties of studying pathogens that cause severe disease in humans, researchers frequently use animal–specific pathogens that have a close phylogenetic relationship and pathology profile to those that cause the disease of interest in humans (68).

NSDV, known as Ganjam virus in India, is an *Orthonairovirus*, closely related to CCHFV (69). NSDV causes Nairobi Sheep Disease (NSD), first described in Kenya during a 1917 outbreak of severe haemorrhagic gastroenteritis in sheep that were relocated from a NSDV-free area to one where NSDV circulated (70). Since then, outbreaks of NSDV have been observed infrequently in sheep and goats.

Like CCHFV, NSDV is also transmitted by ticks. The main symptoms of small ruminant NSDV infection are a febrile illness with diarrhoea, followed by haemorrhages with mortality rates up to 90% (71–73). However, NSDV has a low level of zoonotic potential with evidence of human infection rarely documented (74, 75).

There are a number of analogues between NSDV infection of sheep and CCHFV infection of humans, represented in **Table 2**. Individuals and animals generally undergo a febrile illness, followed by a haemorrhagic phase in the same organs, predominantly the gastrointestinal tract; both produce leukopenia and injury of the liver and spleen (85). Infection of susceptible hosts with NSDV or CCHFV induces a similar pro-inflammatory immune reaction (71, 86) as well as a long-lasting antibody response (52, 87). NSDV infects more organs than CCHFV, and death from NSDV typically occurs within 10 days post infection (72, 73), compared to 5-14 days following onset of illness in CCHFV infection (46).

TABLE 2 | Features of Crimean-Congo Haemorrhagic Fever Virus and Nairobi Sheep Disease virus.

Feature	CCHFV	NSDV
Virus structure	Enveloped negative sense ssRNA virus with tripartite genome (76)	Enveloped negative sense ssRNA virus with tripartite genome (76)
S segment amino acid sequence similarity	62-63% (77)	
Vector	Ixoxid ticks, predominantly *Hyalomma* genus (78)	Ixoxid ticks, predominantly *Rhipicephalus* and *Haemophilus* genus (69)
Clinical Disease	Humans (79)	Sheep and goats (69)
Mortality	5-30% (46)	90% (80)
Symptoms in susceptible host	Fever, myalgia, headache, nausea, soft tissue haemorrhage, epistaxis, hematemesis (81)	Fever, diarrhoea, gastro-intestinal haemorrhage, soft tissue haemorrhage (71)
Tissue pathology	Isolated in lung, liver, and spleen (82, 83)	Isolated in lung, liver, spleen, and intestines (84)

The major advantage of using NSDV infection in small ruminants as a model for CCHFV infection over the current mouse models is the ability to study lethal *Orthonairovirus* infection in an immuno-competent organism. The limitation of NSDV is that the virus is of a different species to CCHFV, with a different host tropism. However, the close phylogenetic relationship between NSDV and CCHFV increases the chance that results may be translatable, and the similarities in haemorrhagic pathogenesis suggest that NSDV infection can be used to investigate systemic and immunological effects of *Orthonairovirus* infection, at a lower risk to human health compared to using NHP models of CCHFV.

HAZV is another nairovirus that may be used as a model of CCHFV infection. Discovered in Pakistan in *Ixoxid* ticks (88), HAZV can be handled in BSL-2 facilities and is non-pathogenic to humans, unlike CCHFV (88, 89). Significantly, HAZV is considered the virus most closely related to CCHFV; HAZV is in the same sero- (90) and genogroup (76) as CCHFV and there is a high level of structural homology between the nucleoprotein of CCHFV and HAZV (91, 92).

Researchers have used HAZV infection for pre-clinical investigations, such as *in vitro* assays to assess the effects of the therapeutic agent ribavirin in combination with other treatments at preventing HAZV infection (93). Ribavirin is commonly prescribed for treating CCHFV (94) and therapeutics which show efficacy against HAZV have been speculated to be effective against CCHFV (93). Parallels between the pathology of CCHFV and HAZV infection have also been observed in immunocompromised mouse models. Infection of IFN-1 knock-out mice with HAZV resulted in lethal disease in all mice (95). The pathology, mortality, and clinical signs were highly similar to those induced by CCHFV infection in the same immunocompromised mouse strains (96). In contrast, wild-type mice were not susceptible to HAZV or CCHFV infection (95).

The close similarity of HAZV with CCHFV has also led to the suggestion to use HAZV to investigate nairoviral infections in the amplifying hosts of CCHFV, namely sheep, goats, and cattle (97). Experimental challenge of domestic animals, including sheep and cattle, with HAZV does not cause symptomatic disease, and HAZV replication was also not observed in sheep or cattle during a recent challenge study (97). Viremia has been observed upon HAZV challenge in a number of other animals including rhesus monkeys and donkeys (98).

As HAZV does not cause overt disease in human or animals, unlike CCHFV and NSDV respectively, HAZV cannot be used to investigate the haemorrhagic pathogenesis observed in human CCHFV infection. A second limitation of HAZV as a model for CCHFV is the lack of natural infection reported in domestic animals and an unknown host species, which limits corollaries being drawn regarding transmission modes or model studies.

Due to the high levels of homology between HAZV and CCHFV, HAZV could provide a viable model virus to study the molecular biology and pathogenicity of CCHFV. HAZV may also be used for the screening of preventative or therapeutic measures that could be translatable for CCHFV, with the use of HAZV precluding the need for experimentation in BSL-4 containment.

CONTROLLING ZOONOTIC TRANSMISSION OF CCHFV

Despite the presence of CCHFV across a large geographical range and the severity of outbreaks, it is difficult to estimate the disease burden due to the low level of active CCHF surveillance and reliance on passive surveillance with high levels of under-reporting. There is also limited diagnostic capability in many endemic regions, with the uncertain frequency of subclinical infections adding to this issue. As a result, it has been suggested that the burden of CCHF disease is greater than estimated from official case reports (99, 100). Asymptomatic infections may be common and mild disease can present as non-specific febrile symptoms (101). The awareness of CCHF disease and symptoms is low among physicians and veterinarians even in endemic countries (102), and increasing the awareness of CCHF clinical symptoms among physicians and veterinarians is the first step towards improving access to diagnosis for mild cases, and to prevent nosocomial outbreaks (103).

Implementation of better diagnostic frameworks to improve surveillance strategies for CCHFV infections is an important aspect for a comprehensive One Health approach for disease management (**Table 3**). A key part of this strategy is the use of rapid laboratory diagnosis for humans by reverse transcription polymerase chain reaction (RT-PCR), a highly sensitive tool to detect CCHFV RNA in individuals (104). Crucially, there are not always laboratory facilities equipped to perform PCR and

TABLE 3 | The different control measures that may facilitate a CCHF One Health approach and the current status or challenges for their implementation.

Control measure strategy	Current status
Immunisation of humans	• Limited licensure of one inactivated vaccine in Eastern Europe • Multiple vaccine candidates show promise in pre-clinical studies • No published assessment of candidates in human trials
Immunisation of animals	• Multiple vaccine candidates show promise in pre-clinical studies • Lack of disease burden means a lack of economic incentive to vaccinate
Tick control	• Acaricidal agents known to be effective at reducing vector-borne disease rates • Environmental implications and logistical issues of widespread usage
Diagnostic, education, and surveillance	• PCR available but often limited in capacity or inaccessible • Sero-surveillance rates increasing (44)

diagnose CCHF in less developed rural areas, or they may lack the capacity needed during outbreaks (105). This can result in distant reference laboratories being relied upon, delaying diagnosis (100). The lack of specific clinical presentation also adds difficulty to seeking diagnosis. Improved diagnostic capacity and accessibility in both clinical and field-based settings by the establishment or improvement of regional laboratories in endemic areas would allow earlier detection of positive cases and pre-emptive interventions to be undertaken (106). Increased clinical diagnosis would also identify more asymptomatic and mild cases and improve the estimation of CCHF disease burden.

Active CCHF surveillance is also vital, as has been displayed by the increasing frequency of serological studies to detect anti-CCHFV antibodies in human populations (99, 107). The data from these sero-surveillance investigations have provided a key insight into the seroprevalence of populations and can be used to evaluate potential risk of exposure in endemic areas (44). Interpretation of such studies should be considered with caution as is that the serological methodologies are often inconsistent and accuracy is not always quantified (58). Additionally, seroprevalence measurement does not resolve the missing CCHF knowledge regarding incidence of subclinical infections versus symptomatic disease. Serological studies in both domestic and wild animal species, as well as surveying distribution and viral presence in tick species, should also be integrated into CCHF surveillance strategies (47, 108). Assessing CCHFV in the amplifying and natural hosts can increase the understanding of the geographical spread of CCHFV and provide estimates of circulating viral quantities, though there is additional complexity to animal and tick surveillance (99, 109). Establishment of CCHF surveillance programmes could determine potential levels of risk to human populations in endemic areas by monitoring the prevalence of virus in humans, animals, or tick vectors and this may identify where disease management interventions could be most useful.

CCHF impacts human health, with hundreds of cases officially reported each year and an obvious need for improved control measures including preventative vaccinations or therapeutic treatments (**Table 3**). There are currently no CCHFV vaccines licenced for widespread usage (110), although one has been licenced in Eastern Europe which uses an inactivated virus platform (111). Studies investigating the immune response to this formalin inactivated vaccine have demonstrated that after four doses, low levels of neutralising antibodies are induced (112). Multiple other vaccine candidates for CCHFV have been developed using a wide range of platforms to deliver vaccine antigens (113–116). For example, two doses of a protein-based subunit CCHFV vaccine that targeted Gn and Gc of the glycoprotein precursor achieved neutralising antibody responses, but did not confer protection in mouse challenge models (117). A DNA vaccine expressing the CCHFV full-length glycoprotein induced humoral and cellular immunity and was protective after two doses in mouse challenge models (118). Viral vectored vaccines that used the MVA vector to deliver full length glycoproteins as immunogens against CCHFV also induced humoral and cellular responses; the vaccine was 100% protective against CCHFV in immunocompromised mouse models (119). Efforts are now needed to test and then translate putative vaccine candidates into veterinary and human vaccines which can protect against CCHFV (120). It is likely that these medicinal interventions would be stockpiled in endemic countries and used in outbreak situations as has been suggested for other outbreak pathogens such as Nipah or MERS coronavirus (121).

While immunising livestock and poultry against infectious pathogens is an economically important practise to avoid disruption to the food and textiles industry (122), vaccination of farmed animals against diseases can also reduce the likelihood that zoonotic pathogens will be transmitted to humans (123, 124). There are, however, barriers to the development of effective CCHFV vaccines for use in veterinary settings (**Table 3**). There are no economic incentives for farmers, livestock producers or agri-industry to vaccinate against CCHFV. Without an appreciable level of disease in animal hosts, and the accompanying loss of income to those working with animals, the incentive to vaccinate animal reservoirs purely for the benefit of human health may be limited. As such, outside investment may be needed to incentivise this approach. Alternatively combining vaccines that combat impactful veterinary pathogens with a vaccine against CCHFV may persuade key stakeholders to implement vaccination regimens.

Further methods may be considered to manage CCHFV in animal reservoirs. Targeting the disease vector is common in reducing arbovirus transmission (125) and therefore reservoir livestock can be treated with acaricidal agents to remove ticks (**Table 3**) (126). Unlike with vaccination against CCHFV, treatment of ticks offers direct benefits to animal health (127).

However, the acaricidal agents can contaminate animal products (128), and annual deaths from organophosphates greatly outnumber those from CCHFV (126). Due to the environmental implications of acaricide usage (129), it would not be feasible to apply acaricides across the large areas needed to supress wild tick populations. Furthermore, the eradication of ticks across large regions of lands would negatively affect the ecosystem, causing unspecified environmental damage (130). While likely to be insufficient alone, careful control of ticks in livestock would be a valuable tool alongside vaccination to reduce CCHFV infections in humans.

CONCLUSION

To mitigate the risk and impact posed by CCHF it is vital that sufficient knowledge on human infection and the interplay with the animal reservoir is delineated. Though limited in the past, the increasing availability of animal models has supported the study of CCHF as a disease and the causative agent CCHFV. These models are already playing a significant role assessing the preclinical efficacy of CCHF vaccines. Similarly, the study of closely related nairoviruses such as NSDV and HAZV that are non-pathogenic to humans can further advance our understanding of CCHFV, due to the similarities of the virus infection and subsequent disease. These closely related pathogens can represent valuable models for CCHFV infection, though all research must be viewed with the caveat that there are differences between CCHFV and the model pathogens which must be taken into account.

CCHF has historically been overlooked as a disease of impact due to being largely under reported in humans and the asymptomatic nature of CCHFV in animal reservoirs that enables the virus to circulate undetected. There is little incentive for treatment of animals or surveillance until zoonotic transmission occurs. Without co-ordinated rapid diagnostic testing in tandem with sero-surveillance mechanisms, the first evidence of circulation is frequently after zoonotic transmission, with those working closely with animals put at considerable risk with no foreknowledge (50).

Given the high mortality rates seen during sporadic (but now almost annual) outbreaks of CCHF, better management approaches are essential in countries where CCHFV is endemic and significant health risks exist. This is urgently needed due to the potential for increased incidence of CCHF cases and growing geographical distribution of *Hyalomma* ticks resulting from environmental changes. There are obvious challenges to alleviating the threat of CCHFV, but ultimately, implementation of a One Health approach to CCHFV management and control while focusing on integrating human and veterinary studies would be of huge benefit to human health.

AUTHOR CONTRIBUTIONS

CG, JS and EA contributed to writing of the original draft. CG, JS, HS, EA, GL, AL, TL and SB-R contributed to review and editing to produce the final manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This research was funded by the Biotechnology and Biological Sciences Research Council (BBSRC) [BB/R019991/1] and the National Institute of Health Research (NIHR) [16/107/06].

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