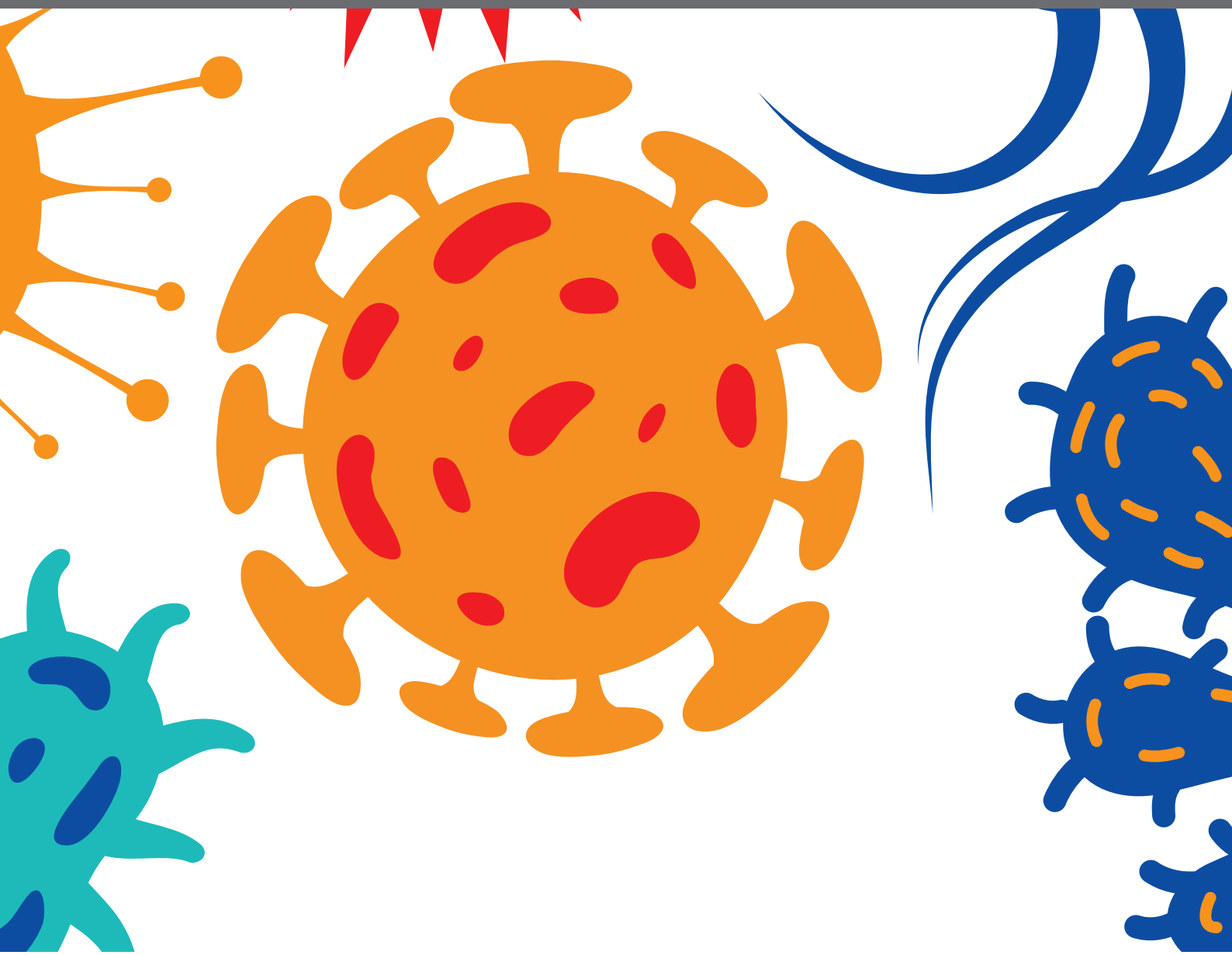
MOLECULAR VACCINES AGAINST PATHOGENS IN THE POST-GENOMIC ERA

EDITED BY: Angel Alejandro Oñate, Alberto Moreno and Yanmin Wan
PUBLISHED IN: Frontiers in Cellular and Infection Microbiology



frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88963-513-9
DOI 10.3389/978-2-88963-513-9

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

MOLECULAR VACCINES AGAINST PATHOGENS IN THE POST-GENOMIC ERA

Topic Editors:
Angel Alejandro Oñate, University of Concepcion, Chile
Alberto Moreno, Emory University School of Medicine, United States
Yanmin Wan, Fudan University, China

Citation: Oñate, A. A., Moreno, A., Wan, Y., eds. (2020). Molecular Vaccines against Pathogens in the Post-Genomic Era. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88963-513-9

Table of Contents

05 ***Editorial: Molecular Vaccines Against Pathogens in the Post-genomic Era***
Angel Alejandro Oñate, Yanmin Wan and Alberto Moreno

08 ***Structure-Based Design of Porcine Circovirus Type 2 Chimeric VLPs (cVLPs) Displays Foreign Peptides on the Capsid Surface***
Dongliang Wang, Sujiao Zhang, Yawen Zou, Wanting Yu, Yifan Jiang, Yang Zhan, Naidong Wang, Yanpeng Dong and Yi Yang

18 ***Back to the Future: Lessons Learned From the 1918 Influenza Pandemic***
Kirsty R. Short, Katherine Kedzierska and Carolien E. van de Sandt

37 ***The Combination Vaccine Adjuvant System Alum/c-di-AMP Results in Quantitative and Qualitative Enhanced Immune Responses Post Immunization***
Thomas Ebensen, Simon Delandre, Blair Prochnow, Carlos A. Guzmán and Kai Schulze

46 ***Oral Vaccination With a Formulation Combining*** **Rhipicephalus microplus** ***Subolesin With Heat Inactivated*** **Mycobacterium bovis** ***Reduces Tick Infestations in Cattle***
Marinela Contreras, Paul D. Kasaija, Octavio Merino, Ned I. de la Cruz-Hernandez, Christian Gortazar and José de la Fuente

55 ***Toward DNA-Based T-Cell Mediated Vaccines to Target HIV-1 and Hepatitis C Virus: Approaches to Elicit Localized Immunity for Protection***
Zelalem A. Mekonnen, Branka Grubor-Bauk, Makutiro G. Masavuli, Ashish C. Shrestha, Charani Ranasinghe, Rowena A. Bull, Andrew R. Lloyd, Eric J. Gowans and Danushka K. Wijesundara

63 ***The Future of Flu: A Review of the Human Challenge Model and Systems Biology for Advancement of Influenza Vaccinology***
Amy Caryn Sherman, Aneesh Mehta, Neal W. Dickert, Evan J. Anderson and Nadine Rouphael

72 ***A Pertussis Outer Membrane Vesicle-Based Vaccine Induces Lung-Resident Memory CD4 T Cells and Protection Against*** **Bordetella pertussis*****, Including Pertactin Deficient Strains***
María Eugenia Zurita, Mieszko M. Wilk, Francisco Carriquiriborde, Erika Bartel, Griselda Moreno, Alicja Misiak, Kingston H. G. Mills and Daniela Hozbor

83 ***A Multi-Stage*** **Plasmodium vivax** ***Malaria Vaccine Candidate Able to Induce Long-Lived Antibody Responses Against Blood Stage Parasites and Robust Transmission-Blocking Activity***
Jessica N. McCaffery, Jairo A. Fonseca, Balwan Singh, Monica Cabrera-Mora, Caitlin Bohannon, Joshy Jacob, Myriam Arévalo-Herrera and Alberto Moreno

99 ***Chimeric Murine Polyomavirus Virus-Like Particles Induce*** **Plasmodium** ***Antigen-Specific CD8⁺ T Cell and Antibody Responses***
David J. Pattinson, Simon H. Apte, Nani Wibowo, Yap P. Chuan, Tania Rivera-Hernandez, Penny L. Groves, Linda H. Lua, Anton P. J. Middelberg and Denise L. Doolan

111 ***Delineating the Plausible Molecular Vaccine Candidates and Drug Targets of Multidrug-Resistant* Acinetobacter baumannii**
Shama Mujawar, Rohit Mishra, Shrikant Pawar, Derek Gatherer and Chandrajit Lahiri

133 ***The Quest for a Truly Universal Influenza Vaccine***
Yo Han Jang and Baik Lin Seong

frontiers in Cellular and Infection Microbiology

EDITORIAL
published: 22 January 2020
doi: 10.3389/fcimb.2019.00443

Editorial: Molecular Vaccines Against Pathogens in the Post-genomic Era

Angel Alejandro Oñate[1]*, *Yanmin Wan*[2] and *Alberto Moreno*[3,4]

[1] Laboratory of Molecular Immunology, Department of Microbiology, Universidad de Concepción, Concepción, Chile, [2] Department of Infectious Disease, Huashan Hospital, Fudan University, Shanghai, China, [3] Emory Vaccine Center, Yerkes National Primate Research Center, Emory University, Atlanta, GA, United States, [4] Division of Infectious Diseases, Department of Medicine, Emory University, Atlanta, GA, United States

Keywords: vaccines, systems biology, multi-omics, immunoinformatics, modern vaccinology

Editorial on the Research Topic

Molecular Vaccines Against Pathogens in the Post-genomic Era

The development and implementation of vaccines are one of the greatest Public Health achievements in human history (Centers-for-Disease-Control-and-Prevention, 2011a,b). In the first decade of the twenty-first century, the Expanded Program on Immunizations averts more than 2.5 million deaths every year (WHO, 2009). Vaccines not only prevent deaths, disease, and disability but also provide community protection by reducing the spread of the disease within a population (Orenstein and Ahmed, 2017). In the US alone, it has been estimated that the prevention of clinical cases and deaths by vaccination for a single birth cohort represents a net savings of $68.8 billion in total societal costs (Zhou et al., 2014). Although there are vaccines internationally available against 26 infectious diseases, nearly half of all deaths from infectious diseases are caused by pathogens for which no vaccine is available (Piot et al., 2019), including emerging and re-emerging pathogens (Williamson and Westlake, 2019). Interestingly, the majority of these vaccines have been developed empirically with limited information available on the mechanisms involved in protection (Pulendran and Ahmed, 2011). The development of high-throughput technologies and the advances in bioinformatics allow the massive generation and integration of datasets from multiple components of a biological system to understand in-depth physiological or pathological events (Pezeshki et al., 2019). This holistic approach of systems biology, when applied to studies of vaccine-induced immune responses, is known as system vaccinology (Pulendran et al., 2010). This research field will provide tools not only for the rational vaccine design but also for the development of novel adjuvants and vaccine delivery systems (Raeven et al., 2019). In this *Frontiers* Research Topic, some concepts of modern vaccinology are explored.

INFLUENZA PANDEMIC PREPAREDNESS

In light of the new technological advances in systems biology, Short et al. reviewed host, pathogen, and environmental factors that determined the high mobility and mortality rates reported for the 1918 influenza pandemic. The progress in understanding the immune responses induced by influenza vaccines using system biology tools is reviewed by Sherman et al. The authors summarized the molecular signatures of B cell responses that have been reported to correlate with the response to vaccination. Signatures of long-lasting antibody responses and immunosenescence defined after influenza vaccination are also discussed. The availability of influenza challenge models combined with system biology tools has started to reveal differences in gene expression signatures in volunteers exposed either to H1N1 or H3N2. Furthermore, access to human challenge models has also shown the dynamics of the viral evolution within the host. Jang and Seong review

OPEN ACCESS

Edited and reviewed by:
Nahed Ismail,
University of Illinois at Chicago, United States

***Correspondence:**
Angel Alejandro Oñate
aonate@udec.cl

Specialty section:
This article was submitted to Clinical Microbiology, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *12 November 2019*
Accepted: *10 December 2019*
Published: *22 January 2020*

Citation:
Oñate AA, Wan Y and Moreno A (2020) Editorial: Molecular Vaccines Against Pathogens in the Post-genomic Era. Front. Cell. Infect. Microbiol. 9:443. doi: 10.3389/fcimb.2019.00443

recent data on the advances and challenges on the development of universal influenza vaccines (UIVs). The search for a broadly protective vaccine capable of redirecting the immune responses from the variable immunodominant regions to conserved subdominant has identified potential candidates that can be delivered using novel vaccine platforms. Among the strategies, novel immunoinformatics tools to define consensus or ancestral sequences by phylogenetic analyses are discussed. Systems vaccinology approaches are needed to understand the mechanism of protections induced by UIVs.

INTERACTOME MINING TO REVEAL PATHOGEN TARGETS

Characterizing the patterns of molecular interaction of proteins, using protein interaction networks or interactomes, is essential for understanding the cellular function and pathogen–host interactions. Mujawar et al. report the development of protein interactomes for *Acinetobacter baumannii*, a causative agent of nosocomial infections, to identify potential vaccine candidates and virulence factors for immunological or pharmacological targeting. The identified proteins are then mapped onto the whole genome protein interactome for *in silico* verification to generate a short list of proteins for future *in vivo* validation.

ADJUVANT FORMULATIONS

The development of vaccines based on subunits is a promising strategy given its excellent safety profile. However, candidates are usually poorly immunogenic and require the formulation with potent adjuvants. In their article, Contreras et al. present new data on tick vaccination, using a combination of the recombinant subolesin from *Rhipicephalus microplus* described as protective for this arthropod in combination with heat-inactivated *Mycobacterium bovis* and administered orally. The results of this study confirmed the efficacy of subolesin-based vaccines for the control of cattle tick infestations and expanded to oral vaccination using an immunostimulant. Ebensen et al. report the use of a combination of c-di-AMP, a STING agonist, and a promising adjuvant capable of stimulating an effective Th1/Th2 and cytotoxic immune response, with the well-known adjuvant alum. This adjuvant system was tested with a model antigen showing the induction of a balanced humoral and cellular immune responses.

DELIVERY SYSTEMS

In the search for novel vaccine delivery systems, several strategies have been proposed. Zurita et al. address the need to develop more effective acellular pertussis vaccines. In eliciting tissue-resident memory CD4+ T cells, a critical effector subset involved in protection, the authors show that an outer membrane vesicle (OMV)-based vaccine is more effective than a commercial acellular vaccine, having a better ability to induce protection and immunological memory. Chimeric virus-like particles (VLPs) using the murine polyomavirus are reported to be an effective platform to deliver subunit vaccines by Pattinson et al. In proof-of-concept studies, the authors produced chimeric VLPs genetically modified for surface expression of CD8+, CD4+, or B cell epitopes derived from the *Plasmodium yoelii* circumsporozoite protein. The vaccine platform was efficient to induce CD8+ T cell and antibody responses, but limited CD4+ T cell responses. The potential of using Porcine Circovirus Type 2 (PCV2) Chimeric VLPs for surface expression of exogenous peptides is presented by Wang et al.. The availability of three-dimensional structural data of the PCV2 capsid protein allows the authors to use homology modeling to characterize surface displaying. The platform allows the insertion of foreign peptides without altering the virus assembly and its entrance to the host cell.

DNA vaccines consist of plasmid vectors that, after immunization, allows intracellular expression of the encoded antigens. Protective efficacy is achieved by the induction of a strong humoral and cellular immune response dependent on B and T cells. In a mini review by Mekonnen et al., the limitations and strategies for using DNA vaccines against human immunodeficiency virus (HIV)-1 and hepatitis C virus (HCV) in humans are discussed. The authors review the potential of DNA vectors for elicit protective compartmentalized CD8 + T cells in the liver for HCV and the genito-rectal mucosa for HIV.

Conjugation of poorly immunogenic antigens to carrier protein is the strategy used for glycoconjugate vaccines. McCaffery et al. reported the use of genetic conjugation to deliver a *Plasmodium vivax* sexual-stage vaccine candidate. The authors take advantage of a highly immunogenic chimeric protein that they designed targeting a blood-stage antigen to create a bifunctional vaccine by genetic linkage to the transmission-blocking vaccine candidate Pvs25. This approach addresses the need for the development of effective multi-stage malaria vaccines.

CLOSING PERSPECTIVES

The wealth of information provided by systems biology approaches can be integrated into product design for the development of novel vaccines. This Research Topic offered a glimpse into some strategies in modern vaccinology. Although these tools are essential for the development of effective vaccines against agents with complex host–pathogen interactions such as HIV, tuberculosis, and malaria, from the global health perspective, it is also critical to identify and deal with factors associated with persistent social disparities. It has been estimated that only 5% of all children born worldwide receive all 11 vaccines recommended by the WHO (Mantovani and Santoni, 2018). It is therefore critical that in conjunction with research efforts to develop novel vaccines, global health initiatives such as the Global Alliance for Vaccines and Immunizations (GAVI) that promotes equal access to vaccines are strengthened (Ikilezi et al., 2019; Rappuoli et al., 2019; Zerhouni, 2019).

AUTHOR CONTRIBUTIONS

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

FUNDING

AO received funded from the Fondo Nacional de Desarrollo Científico y Tecnológico, FONDECYT grants 1180122. AM received funded from the National Institutes of Health, NIAID grants R21 AI094402-01A1 and R21AI135711-01. YW received founded by project 81671636 and 81971559 from NSFC.

REFERENCES

Centers-for-Disease-Control-and-Prevention (2011a). Ten great public health achievements–worldwide, 2001–2010. *MMWR Morb. Mortal. Wkly. Rep.* 60, 814–818.

Centers-for-Disease-Control-and-Prevention (2011b). Ten great public health achievements–United States, 2001–2010. *MMWR Morb. Mortal. Wkly. Rep.* 60, 619–623.

Ikilezi, G., Augusto, O. J., Dieleman, J. L., Sherr, K., and Lim, S. S. (2019). Effect of donor funding for immunization from Gavi and other development assistance channels on vaccine coverage: evidence from 120 low and middle income recipient countries. *Vaccine*. doi: 10.1016/j.vaccine.2019.10.057. [Epub ahead of print].

Mantovani, A., and Santoni, A. (2018). Mandatory vaccination in Italy: time for engagement of immunologists. *Eur. J. Immunol.* 48, 12–14. doi: 10.1002/eji.201870016

Orenstein, W. A., and Ahmed, R. (2017). Simply put: vaccination saves lives. *Proc. Natl. Acad. Sci. U.S.A.* 114, 4031–4033. doi: 10.1073/pnas.1704507114

Pezeshki, A., Ovsyannikova, I. G., McKinney, B. A., Poland, G. A., and Kennedy, R. B. (2019). The role of systems biology approaches in determining molecular signatures for the development of more effective vaccines. *Expert. Rev. Vaccines* 18, 253–267. doi: 10.1080/14760584.2019.1575208

Piot, P., Larson, H. J., O'Brien, K. L., N'kengasong, J., Ng, E., Sow, S., et al. (2019). Immunization: vital progress, unfinished agenda. *Nature* 575, 119–129. doi: 10.1038/s41586-019-1656-7

Pulendran, B., and Ahmed, R. (2011). Immunological mechanisms of vaccination. *Nat. Immunol.* 12, 509–517. doi: 10.1038/ni.2039

Pulendran, B., Li, S., and Nakaya, H. I. (2010). Systems vaccinology. *Immunity* 33, 516–529. doi: 10.1016/j.immuni.2010.10.006

Raeven, R. H. M., van Riet, E., Meiring, H. D., Metz, B., and Kersten, G. F. A. (2019). Systems vaccinology and big data in the vaccine development chain. *Immunology* 156, 33–46. doi: 10.1111/imm.13012

Rappuoli, R., Santoni, A., and Mantovani, A. (2019). Vaccines: an achievement of civilization, a human right, our health insurance for the future. *J. Exp. Med.* 216, 7–9. doi: 10.1084/jem.20182160

WHO, UNICEF, and World-Bank (2009). *State of the World's Vaccines and Immunization, 3rd Edn.* Geneva: World Health Organization. Available online at: https://www.who.int/immunization/sowvi/en/

Williamson, E. D., and Westlake, G. E. (2019). Vaccines for emerging pathogens: prospects for licensure. *Clin. Exp. Immunol.* 198, 170–183. doi: 10.1111/cei.13284

Zerhouni, E. (2019). GAVI, the vaccine alliance. *Cell* 179, 13–17. doi: 10.1016/j.cell.2019.08.026

Zhou, F., Shefer, A., Wenger, J., Messonnier, M., Wang, L. Y., Lopez, A., et al. (2014). Economic evaluation of the routine childhood immunization program in the United States, 2009. *Pediatrics* 133, 577–585. doi: 10.1542/peds.2013-0698

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 Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 09 July 2018
doi: 10.3389/fcimb.2018.00232

Structure-Based Design of Porcine Circovirus Type 2 Chimeric VLPs (cVLPs) Displays Foreign Peptides on the Capsid Surface

Dongliang Wang[1], Sujiao Zhang[1], Yawen Zou[1], Wanting Yu[1], Yifan Jiang[1], Yang Zhan[1], Naidong Wang[1], Yanpeng Dong[2] and Yi Yang[1]**

[1] *Hunan Provincial Key Laboratory of Protein Engineering in Animal Vaccines, Laboratory of Functional Proteomics, Research Center of Reverse Vaccinology, College of Veterinary Medicine, Hunan Agricultural University, Changsha, China,* [2] *Jiangsu Nannong Hi-Tech Co., Ltd, Jiangyin, China*

Although porcine circovirus-like particles can function as a vector to carry foreign peptides into host cells, displaying foreign peptides on the surface of virus-like particles (VLPs) remains challenging. In this study, a plateau, consisting of the middle portion of Loop CD (MP-Lcd) from two neighboring subunits of PCV2 capsid protein (Cap), was identified as an ideal site to insert various foreign peptides or epitopes and display them on the surface of PCV2 VLPs. One of the goals of this work is to determine if the surface pattern of this plateau can be altered without compromising the neutralizing activity against PCV2 infections. Therefore, biological roles of MP-Lcd regarding VLPs assembly, cell entry, and antigenicity were investigated to determine whether this was a universal site for insertion of foreign functional peptides. Three-dimensional (3D) structure simulations and mutation assays revealed MP-Lcd was dispensable for PCV2 Cap assembly into VLPs and their entry into host cells. Notably, substitution of MP-Lcd with a foreign peptide, caused surface pattern changes around two-fold axes of PCV2 VLPs based on 3D structure simulation, but was not detrimental to VLPs assembly and cell entry. Moreover, this substitution had no adverse effect on eliciting neutralizing antibodies (NAbs) against PCV2 infection in pigs. In conclusion, MP-Lcd of the PCV2 Cap was a promising site to accommodate and display foreign epitopes or functional peptides on the surface of PCV2 VLPs. Furthermore, chimeric VLPs (cVLPs) would have potential as bivalent or multivalent vaccines and carriers to deliver functional peptides to target cells.

Keywords: porcine circovirus type 2 (PCV2), chimeric virus-like particles (cVLPs), bivalent or multivalent vaccines, capsid protein, 3D structure prediction

OPEN ACCESS

Edited by:
Alberto Moreno, Emory University School of Medicine, United States

Reviewed by:
Christian Nelson, SUNY Cortland, United States
Karl William Boehme, University of Arkansas for Medical Sciences, United States

****Correspondence:***
Yi Yang
yiyang@hunau.edu.cn
Naidong Wang
naidongwang@hunau.edu.cn

Received: *23 April 2018*
Accepted: *19 June 2018*
Published: *09 July 2018*

Citation:
Wang D, Zhang S, Zou Y, Yu W, Jiang Y, Zhan Y, Wang N, Dong Y and Yang Y (2018) Structure-Based Design of Porcine Circovirus Type 2 Chimeric VLPs (cVLPs) Displays Foreign Peptides on the Capsid Surface. Front. Cell. Infect. Microbiol. 8:232. doi: 10.3389/fcimb.2018.00232

INTRODUCTION

Porcine circovirus (PCV), a member of the *Circovirus* genus in the *Circoviridae* family, has a small, single-stranded and circular DNA genome. There are two main genotypes, namely porcine circovirus type 1 (PCV1) and PCV2, both of which share high levels of nucleotide homology and a common genomic organization (Segales et al., 2013). PCV2, the key causative agent of porcine circovirus-associated diseases (PCVADs), causes severe economic losses worldwide. Based on genome or *cap* gene (it encodes the sole structural protein of PCV2 capsid) analysis of PCV2

isolates, PCV2 is divided into four main subtypes (PCV2a, PCV2b, PCV2c, and PCV2d) (Franzo et al., 2015). Furthermore, PCV2a is subdivided into five clusters (2A, 2B, 2C, 2D, and 2E), PCV2b into three clusters (1A, 1 B, and 1C), whereas PCV2c has only been reported in Denmark, with only three isolates in GenBank (An et al., 2007; Olvera et al., 2007). Recently, a novel PCV genotype was identified (Phan et al., 2016; Palinski et al., 2017; Zhang et al., 2017) and temporarily designated PCV3, as its genomic DNA only shared ∼30% nucleotide identities with PCV1 and PCV2 (Palinski et al., 2017). However, pathogenicity and potential lesions caused by PCV3 remain to be determined.

The circular genome of PCV2 contains 1766–1768 nucleotides, encoding two main viral proteins: the capsid protein (Cap) and virus replication associated proteins (Rep and its isoform of Rep', formed via alternative splicing) (Cheung, 2003). The PCV2 Cap, the sole structural protein of this virus, is composed of ∼233 residues, with 60 of the Caps capable of self-assembly into a virus-like particle (VLP) *in vitro* (Khayat et al., 2011). Furthermore, the VLP has been successfully used as the main antigen in commercial vaccines against PCV2 infection (Fachinger et al., 2008). In addition, PCV2 VLPs instead of virus, were exploited to study interaction of virus-host cells (Misinzo et al., 2006) and mechanism of virus entry into various swine host cell lines (i.e., PK15 and 3D/4 cell lines) (Misinzo et al., 2005, 2009).

The three-dimensional (3D) structure of the PCV2 Cap was elucidated by two research groups (Khayat et al., 2011; Liu et al., 2016). The Cap contains a typical jelly-roll fold composed of eight β-strands, connected via seven loops (Khayat et al., 2011). There are indications that these loops are responsible for assembly of VLPs and that they determine surface patterns of the PCV2 capsid (Khayat et al., 2011; Wang et al., 2016). Further, based on 3D structure analyses, loops exposed on the outer surface of the capsid have substantially diverged during PCV2 evolution (Wang et al., 2016). In addition, both the NH_2- and carboxyl-terminus (NT & CT) of the Cap contain a non-structured stretch. The NT, rich in basic amino acids, has a typical nuclear localization signal (NLS), responsible for distribution of the PCV2 Cap in nucleus (Liu et al., 2001) and viral genome packaging (Khayat et al., 2011). Therefore, in a stable PCV2 capsid, the NT is located inside the capsid and interacts with viral genomic DNA, whereas the CT is exposed on the surface of the capsid, as illustrated in 3D structures (Khayat et al., 2011; Liu et al., 2016; Wang et al., 2016). Although both termini of the Cap have been engineered as targeted sites for insertions of or substitutions with foreign epitopes or functional peptides to generate chimeric VLPs (cVLPs) or chimeric PCV, foreign peptides located at the NT of the Cap may hide within PCV2 VLPs and consequently may not be displayed on the exterior surface of the capsid if the NT is not externalized. Chimeric PCV were constructed by insertion of various epitopes or tags into the 3'-terminal end of the *cap* gene (the CT of the Cap), and specific antibodies against these tags or foreign peptides were produced in response to these chimeric PCVs (Beach et al., 2011; Piñeyro et al., 2015a,b). Furthermore, maximal insertion size of foreign amino acid residues at the 3'-terminal end of the *cap* gene has also been studied. Chimeric porcine circovirus (PCV) containing amino acid epitope tags in the C terminus of the capsid gene are infectious and elicit both anti-epitope tag antibodies and anti-PCV type 2 neutralizing antibodies in pigs (Beach et al., 2011).

Our laboratory previously designed and prepared PCV2 cVLPs to display a foreign epitope on the exterior surface of the cVLPs, based on 3D structural analyses of the PCV2 Cap and its assembly of the capsid (Hu et al., 2016). Several loops were considered for insertions of the GP5 epitope B of porcine reproductive and respiratory syndrome virus (PRRSV), although only Loop CD was an ideal site for insertion of the GP5 epitope B, since this insertion had no negative effects on either assembly or cell entry of cVLPs *in vitro*.

In this study, a plateau consisting of MP-Lcd around two-fold axes of PCV2 VLPs was used to determine, based on 3D structure analyses and simulations, its potential as a universal platform for insertions of various foreign peptides without compromising assembly and cell entry of PCV2 cVLPs. Notably, roles of MP-Lcd in PCV2 VLPs assembly, cell entry and antigenicity were evaluated.

MATERIALS AND METHODS

Reagents, Genes, Plasmids, and Cell Lines

Unless otherwise stated, all chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA). PCV2 *cap* gene (GenBank accession number: JF504708) was optimized and synthesized, as we have described (Hu et al., 2016). Mutated PCV2 cap genes were constructed by overlapping polymerase chain reaction (PCR), as described (Hu et al., 2016). Resultant DNA fragments were subcloned into a pET100 vector (Invitrogen, Carlsbad, CA, USA; **Figure 2A**). After confirmation by DNA sequencing, recombinant plasmids were transformed into BL21 (DE3) competent cells (TransGen, Beijing, China) for protein expression.

PCV2 Cap Expression, Purification, and Self-Assembly of VLPs *in Vitro*

Protein expression, purification, and VLPs assembly *in vitro* of PCV2 Cap wild type and its mutants were done as described (Zhang et al., 2016). For western blots, proteins from cell lysates were first separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad, Hercules, CA, USA). Thereafter, proteins were transferred to a polyvinylidene difluoride membrane (PVDF; Life Technologies, Carlsbad, CA, USA) in a protein transfer device (Bio-Rad) at 80 V for 1 h. The membrane was then blocked with 3% bovine serum albumin (BSA; Roche, Basel, Switzerland) in phosphate-buffered saline (1×PBS, pH 7.4) for 1 h, and then primary antibody (rabbit anti-PCV2 Cap, 1:500) was incubated with the membrane overnight. A horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:5,000, Promega, Madison, WI, USA) was the secondary antibody and signals were visualized with High-sig ECL western blotting substrate (Tanon, Shanghai, China). Formation of VLPs was confirmed by transmission electron microscopy (TEM). The PCV2 VLPs were adsorbed on to carbon-coated copper grids for 10 min and stained with 1% phosphotungstic acid for 10 min. Subsequently, VLPs were

examined with TEM (CM100, Philips Electron Optics, Zurich, Switzerland).

Cap Sequences Alignment and 3D Structure Simulations of PCV2 Cap and Capsid

Typical Cap sequences from PCV1, 2, and 3 were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/). Subtype and cluster of each PCV2 isolate were confirmed via previous phylogenetic analyses (Zhan et al., 2016). Amino acid sequences were aligned using a web server (http://multalin.toulouse.inra.fr/multalin/). For each Cap mutant, a 3D structural model was generated via homology modeling using a crystal structure of PCV2 Cap (PDB accession number: 3R0R) as a template, with protein modelings done using Modeller (http://salilab.org/modeller/). Then, icosahedral structures (3D) of all capsid mutants were generated, based on the monomeric structure of the PCV2 Cap mutant, by applying a VMD 1.9 matrix transformation using Tcl script (Humphrey et al., 1996) and displayed with PyMol (Version 1.8.4.0, http://www.pymol.org/).

Preparation of Anti-PCV2 Cap Sera

Female New Zealand rabbits ($n = 2$), 2 mo old, were used to prepare anti-PCV2 Cap serum. Rabbits were initially immunized with 2 ml of PCV2 VLPs (wild type; 0.2 mg/ml) mixed with Alhydrogel adjuvant (InvivoGen, San Diego, CA, USA), and then similarly boosted twice more at 2-week intervals (Weeks 2 and 4). Blood samples were collected 6 wk after final immunization and serum was isolated and stored at −20°C.

Indirect Immunofluorescence Assays (IFAs)

Cell entry of PCV2 VLPs were detected with IFAs, as described (Misinzo et al., 2005). PK15 cells cultured in 24-well plates were incubated with 2 μg of PCV2 VLPs in each well for 1 h. After incubation, PK15 cells were washed three times with PBS to remove unbound VLPs. As a control, PBS without VLPs was added to PK15 cells. For immunofluorescent staining, PK15 cells were fixed in 4% (wt/vol) paraformaldehyde in PBS at12 h post-inoculation of PCV2 VLPs. Cells were subsequently washed and permeabilized with 0.1 % Triton X-100 for 5 min at room temperature. Cells were washed three times with PBS, incubated with rabbit anti-PCV2 serum (1:1,000) for 1 h, and washed three times with PBS. Then, cells were incubated with fluorescein isothiocyanate (FITC) conjugated donkey anti-rabbit IgG (1:2,000, Life Technologies) for 1 h in the dark, and subsequently washed three times. Finally, cells were mounted with Prolong® Gold Anti-fade Reagent with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen) for nucleic acid staining and imaged with confocal microscopy (LSM 710 NLO & DuoScan System, Carl Zeiss, Germany).

Immunization and Serum Collection

Fifteen commercial 3-week-old pigs, serologically negative for PCV2 and porcine reproductive and respiratory syndrome virus (PRRSV), were used. These pigs were randomly allocated into three groups (five per group). Groups 1 and 2 were immunized with intramuscular injections of 200 μg of PCV2 wild type VLPs or cVLPs-M8 (**Figure 2A**) mixed with MontanideTM Gel 01 ST adjuvant (SEPPIC), respectively, whereas Group 3 was immunized with PBS only (negative control). Each pig was injected only once. Sera were collected 21 and 28 d post-primary immunization (dpi) and tested by indirect enzyme-linked immunosorbent assay (ELISA) and PCV2 neutralization assays. All animal care and use protocols in this study were conducted in accordance with the guidelines of the Animal Care and Use Committee of Hunan Agricultural University and were approved by the Institutional Ethics Committee (IEC) of Hunan Agricultural University.

Indirect ELISA and PCV2 Neutralization Assay

PCV2 VLPs assembled from wild type PCV2 Cap (Cap WT) were coated as an antigen to detect specific anti-PCV2 antibodies by ELISA, as described (Zhang et al., 2016). The PCV2 neutralization assay was also performed as described (Fort et al., 2007). All serum samples from each group were heat-inactivated (56°C for 30 min) and 50 μl of inactivated sera was serially two-fold diluted with PBS, and then mixed with 50 μl of PCV2 virus (200 TCID50; GenBank accession number: KP112484) for 1 h at 37°C. Then, serum-virus mixtures were inoculated to confluent PK-15 cells cultured in 96-well plates and incubated at 37°C for 72 h. Cells were fixed with absolute ethyl alcohol for 30 min, 0.1% triton for 10 min and then blocked with 3% BSA for 1 h at room temperature. Thereafter, cells were incubated with rabbit anti-PCV2 serum (1:1,000) as the primary antibody and FITC-conjugated donkey anti-rabbit IgG (1:2,000 dilution, Life Technologies) as second antibody, each for 1 h. Cells were observed using a fluorescent microscope (Olympus IX73, Tokyo, Japan). The reciprocal of the highest dilution at >50% fluorescent focus reduction was the neutralizing antibody titer.

RESULTS

MP-Lcd From Two Neighboring Cap Subunits Formed a Plateau on the Two-Fold Axis of PCV2 Capsid

Loop CD, connecting β-strands C and D in PCV Cap, was a highly divergent loop among distinct genotypes of porcine circoviruses (PCV1, PCV2, and PCV3), based on amino acid sequence alignment (**Figure 1A**). There was no strictly conserved residue present in this loop among the three genotypes (**Figure 1A**). In contrast, 6 of 18 residues (81PP82, 84GG85, 87N, and 92P) between Caps of PCV1 and 2 were invariant (solid red dots at top of **Figure 1A**). In addition, 5 of the 6 conserved residues were located within the middle portion (eight residues from positions 80–87; red rectangle in **Figure 1A**) of Loop CD. Therefore, the portion composed of these eight residues was designated MP-Lcd (Middle Portion of Loop CD). In the 3D structure, Loop CD of the PCV2 Cap formed the second elevation on the exterior surface of the PCV2 capsid, with most residues in Loop CD located on surfaces of both the PCV2 Cap and the capsid (**Figures 1B–D**) (Khayat et al., 2011; Wang et al., 2016), whereas MP-Lcd formed a plateau on the surface of the two-fold

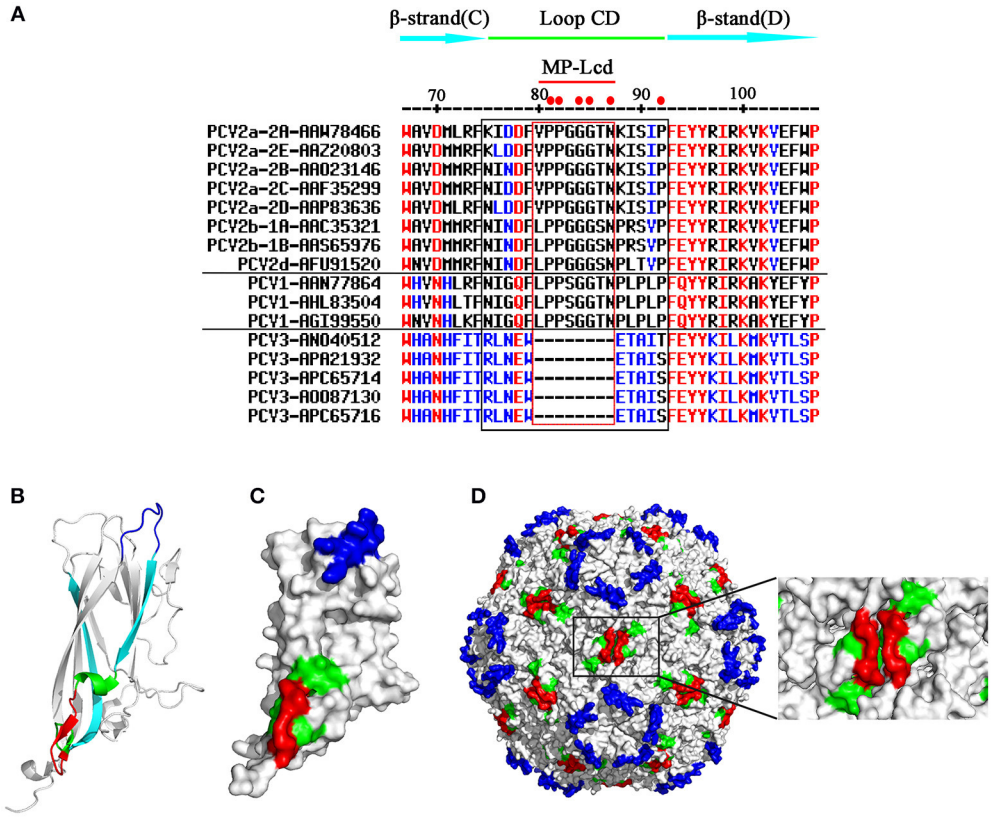


FIGURE 1 | Primary and 3D structures of Loops CD among PCV Caps. **(A)** Comparative sequence alignments of β-stand C, Loop CD, and β-stand D among various PCV Caps (top). Numbers on the top indicate positions of residues in PCV2 Caps. Genotype, subtype, and GenBank accession number of each sequence are shown in the left column. A *cap* gene derived from subtype of PCV2b-1B was used for this study. Residues within the black rectangle represent Loops CD, whereas residues within the red rectangle indicate MP-Lcd. Red dots on the top indicate residues conserved between PCV1 and PCV2 Caps. Hyphen (–) means residue is missing at the indicated position. **(B,D)** 3D localizations of MP-Lcd on PCV2 Cap backbone **(B)**, PCV2 Cap surface **(C)**, and PCV2 capsid **(D)**. Data of 3D structure of PCV2 Cap and capsid were retrieved from Protein Data Bank (http://www.rcsb.org/; PDB accession number: 3R0R). MP-Lcd was labeled in red; β-strands C and D were labeled in cyan and Loop CD (except MP-Lcd) was labeled in green. Loop BC colored in blue demonstrates surfaces of icosahedral five-fold axes of PCV2 capsid.

axes of the PCV2 capsid (red portions in **Figure 1D**). Of note, MP-Lcd was absent among PCV3 Caps (**Figure 1A**). Based on structural features and absence of MP-Lcd in PCV3 Cap, together with previous study that an epitope derived from PPRSV GP5 was successfully inserted Loop CD (between 85G and 86S of PCV2 Cap) (Hu et al., 2016). we inferred that MP-Lcd was an optimal target site for inserting foreign peptides and displaying them on the surface of the PCV2 capsid. To confirm this hypothesis, a series of PCV2 Cap mutants (Cap-M1-6 in **Figure 2A**) were designed to test tolerances of MP-Lcd to foreign peptides.

MP-Lcd Had High Tolerances to Insertions of Various Foreign Peptides

Four peptides, including two epitopes derived from structural proteins of porcine epidemic diarrhea virus (PEDV) (Sun et al., 2008) and porcine parvovirus (PPV) (Sun et al., 2015), respectively (**Figure 2A**, Cap-M1 and -M2), and two artificial peptides (**Figure 2A**, Cap-M3 and -M4), were designed. Based on simulated 3D structures, these four peptides projected from a plateau formed by the MP-Lcd formed (orange in **Figure 3A**) after they were inserted into the PCV2 Cap. To test effects of insertions of various peptides in MP-Lcd on VLPs assembly and cell entry, Cap mutants (Cap-M1 to -M4, **Figure 2A**) were successfully expressed and purified (**Figures 2B,C**). Of note, these Cap mutants were capable of self-assembling into VLPs after *in vitro* dialysis (**Figure 3B**). Moreover, insertion of various peptides into MP-Lcd had no adverse effect on entry of these VLPs into PK15 cells (**Figure 3C**). Therefore, MP-Lcd tolerated insertions with various foreign peptides (Cap-M1 to 4), including those with highly charged residues (Cap-M3 and 4).

Capacity for MP-Lcd to Accommodate Foreign Peptides

Having established that MP-Lcd allowed insertions of various foreign peptides without compromising *in vitro* assembly of VLPs and entry into PK15 cells, maximal insertion size was studied. Two new Cap mutants were designed. One contained an insertion of two distinct epitopes (derived from structural proteins of PEDV and PPV) spaced by a "GS" linker (Cap-M5, **Figure 2A**), whereas another had an insertion of three

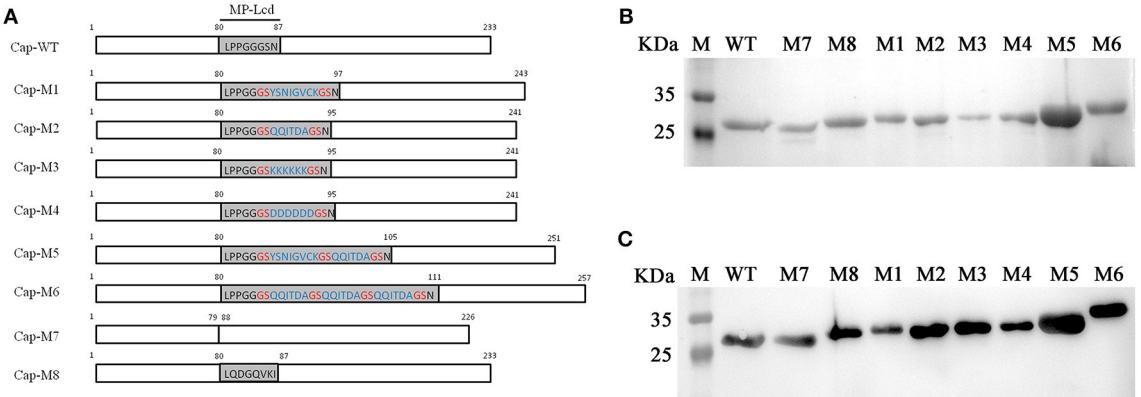


FIGURE 2 | PCV2 Cap WT and mutants. **(A)** Residues within MP-Lcd of PCV2 Cap WT and mutants. Numbers indicate residue positions in PCV2 Cap and mutants. Sequence of MP-Lcd is shown in Cap-WT. Residues in blue represent foreign peptides inserted into MP-Lcd of PCV2 Cap, whereas residues in red indicate "GS" linkers between foreign peptides or foreign peptides and PCV2 Cap. Note: MP-Lcd is substituted by an epitope derived from PEDV spike protein in Cap-M8. **(B)** SDS-PAGE of purified Cap WT and Cap mutants via Ni-NTA affinity chromatography. Lane M, protein marker; expected recombinant Cap protein is indicated in each lane. **(C)** Western blots of supernatants from bacterial lysates containing expressed Cap-WT and various mutants. Lane M, protein marker; the expected specific band is present in each lane.

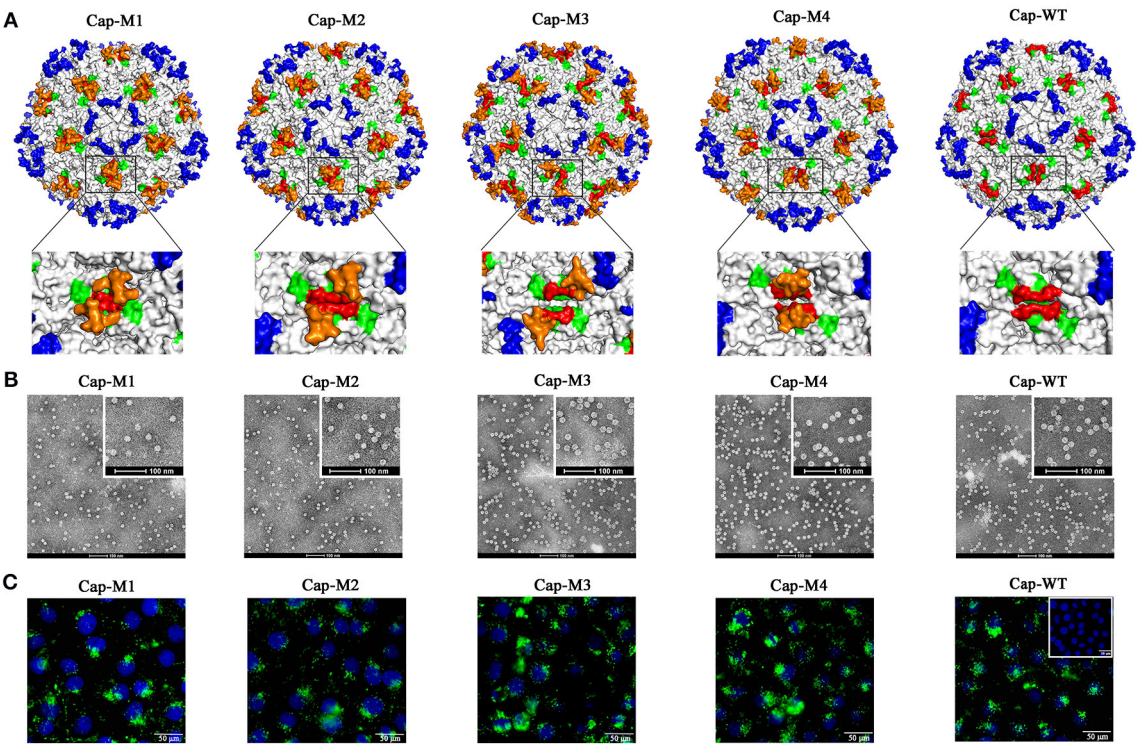


FIGURE 3 | PCV2 VLPs assembly and cell entry into PK15 cells. **(A)** Localizations of foreign peptides or epitopes on the surface of PCV2 capsid in simulated 3D models after assembly of PCV2 Cap-WT and mutants into VLPs. Images on bottom of **(A)** demonstrate (high magnification) 3D structures or orientations of foreign epitopes and artificial peptides on a plateau formed by two neighboring MP-Lcd. Foreign epitopes or artificial peptides were labeled in orange; MP-Lcd was labeled in red and Loop CD, except MP-Lcd was labeled in green and Loop BC-decorated five-fold axes of the icosahedra were labeled in blue on PCV2 capsid. **(B)** PCV2 VLPs formations observed by TEM. Negative staining of VLPs assembled from PCV2 Cap WT or mutants (bars = 100 nm). **(C)** Entry of PCV2 VLPs into PK-15 cells. Internalizations of PCV2 VLPs assembled from PCV2 Cap WT or Cap mutants (M1-M4) were confirmed by confocal microscopy. Inset in PCV2 Cap-WT represents a negative control in which PBS instead of VLPs was added into PK15 cell culture. Green fluorescence represents PCV2 Cap in PK15 cells. Nuclei (blue) of PK-15 cells were stained by DAPI.

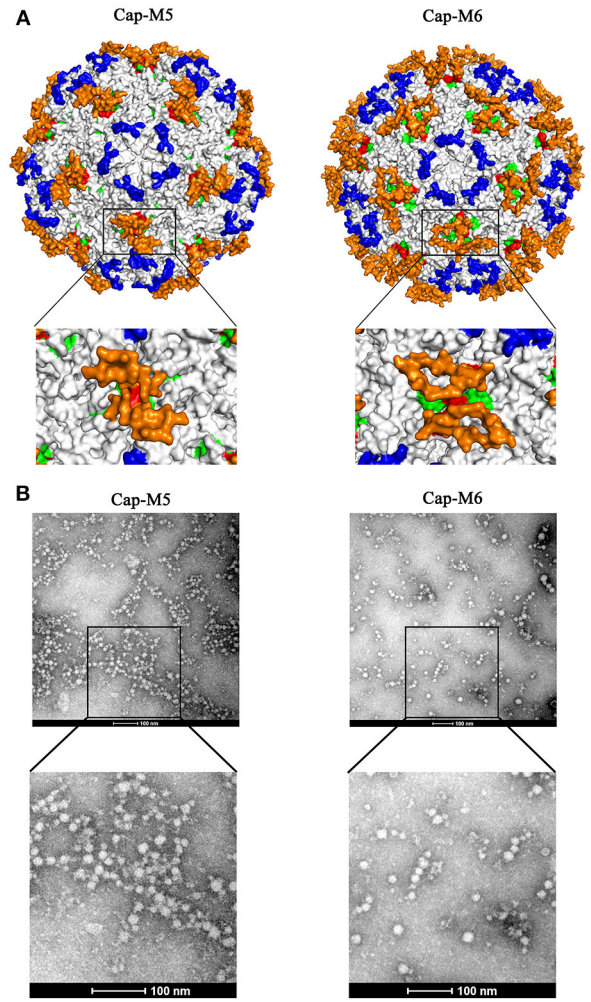


FIGURE 4 | Insertions of large fragments had an adverse effect on PCV2 VLPs assembly. **(A)** Simulated 3D structures of foreign epitopes (Cap-M5 and M6) on the surface of the PCV2 capsid. Images on bottom of **(A)** show top views (high magnification) of foreign epitopes on a plateau formed by two neighboring MP-Lcd. Foreign epitopes were labeled in orange; MP-Lcd was labeled in red and Loop CD, except MP-Lcd was labeled in green and Loop BC-decorated five-fold axes of the icosahedra were labeled in blue on PCV2 capsid. **(B)** Morphologies of PCV2 Cap-M5 and -M6 after dialysis against assembly buffer. Morphologies were observed by TEM after samples were negatively stained.

repeats of the epitope derived from PPV, also spaced by two "GS" linkers (Cap-M6, **Figure 2A**). The simulated 3D structures demonstrated that both foreign peptides might be displayed on the outer surface of PCV2 VLPs (**Figure 4A**). In addition, both Cap mutants were also successfully purified after being expressed in bacteria (**Figures 2B,C**). After the two mutants were dialyzed against assembly buffer *in vitro*, morphologies were distinct from those of VLPs assembled from either the Cap WT or other Cap mutants (**Figure 4B**). Based on TEM results, we inferred that PCV2 VLPs assembly may have been adversely affected by 18 residues, in the absence of further optimization of linker or assembly conditions.

MP-Lcd Was Dispensable for PCV2 VLPs Assembly and Cell Entry

To test functions of MP-Lcd on PCV2 VLPs assembly *in vitro* and cellular uptake, we designed two PCV2 Cap mutants (Cap-M7 and -M8 in **Figure 2A**), of which MP-Lcd was either deleted (M7) or replaced (M8) by a foreign epitope derived from a structural protein of PEDV spike protein (Sun et al., 2008). The 3D structure simulations revealed both PCV2 Cap mutants had a similar jelly-roll fold as wild type **(Figure 5A)**. However, when Cap-M7 was assembled into a capsid, the plateaus composed of MP-Lcd around the two-fold axes had disappeared (**Figure 5A**). In contrast, a new surface pattern around the two-fold axes was formed in a capsid assembled by Cap-M8 (**Figure 5A**), due to replacement of MP-Lcd with the foreign epitope. Further, both mutants were expressed and purified via NTA-Ni2+ chromatography (**Figures 2B,C**). After dialysis *in vitro*, both were capable of assembling into VLPs with a diameter of ∼17 nm, as confirmed by TEM (**Figure 5B**), with very similar morphology as VLPs assembled from the WT of the Cap (**Figure 5B**). Based on IFA, VLPs assembled from both Cap mutants entered PK15 cells similar to wild type (**Figure 5C**). Therefore, MP-Lcd appeared dispensable for VLPs assembly *in vitro* and subsequent entry into PK15 cells.

Replacement of MP-Lcd With a Foreign Epitope Had Minimal Effect on PCV2 Antigenicity and Production of PCV2-Specific NAbs in Pigs

Although replacement of MP-Lcd with the foreign peptide was not detrimental to PCV2 VLPs assembly and cell entry *in vitro*, effects of foreign peptide on immunogenicity of PCV2 VLPs had not been determined. Therefore, pigs were immunized with VLPs assembled either from the Cap-WT or the Cap-M8. Anti-PCV2 antibody was determined by indirect ELISA at 21 and 28 d post immunization (dpi). Antibody titers increased rapidly ($P < 0.01$) from 21 to 28 dpi, compared to the control group (**Figure 6**). Based on ELISA, PCV2 Cap-specific antibodies were successfully induced by PCV2 VLPs assembled from Cap-WT or from the Cap-M8, with no statistical difference in titers (**Figure 6A**). Further, PCV2 NAbs were detected at 21 and 28 dpi, and both anti-PCV2 Cap sera had neutralizing activity against PCV2 infections *in vitro*, whereas no NAbs were detected in the PBS group (**Figure 6B**). Notably, both anti-PCV2 Cap sera had consistent virus-neutralizing titers, which strongly indicated replacing MP-Lcd with the foreign peptide had no adverse effect on eliciting PCV2 Cap-specific NAbs in pigs.

DISCUSSION

Inserting a foreign peptide into the middle of a protein often poses a huge challenge, as this may adversely affect protein folding, and thereafter affect protein functions. Although foreign peptides or tags are usually fused to the NT or CT of proteins to minimize effects on protein backbones, there is no assurance

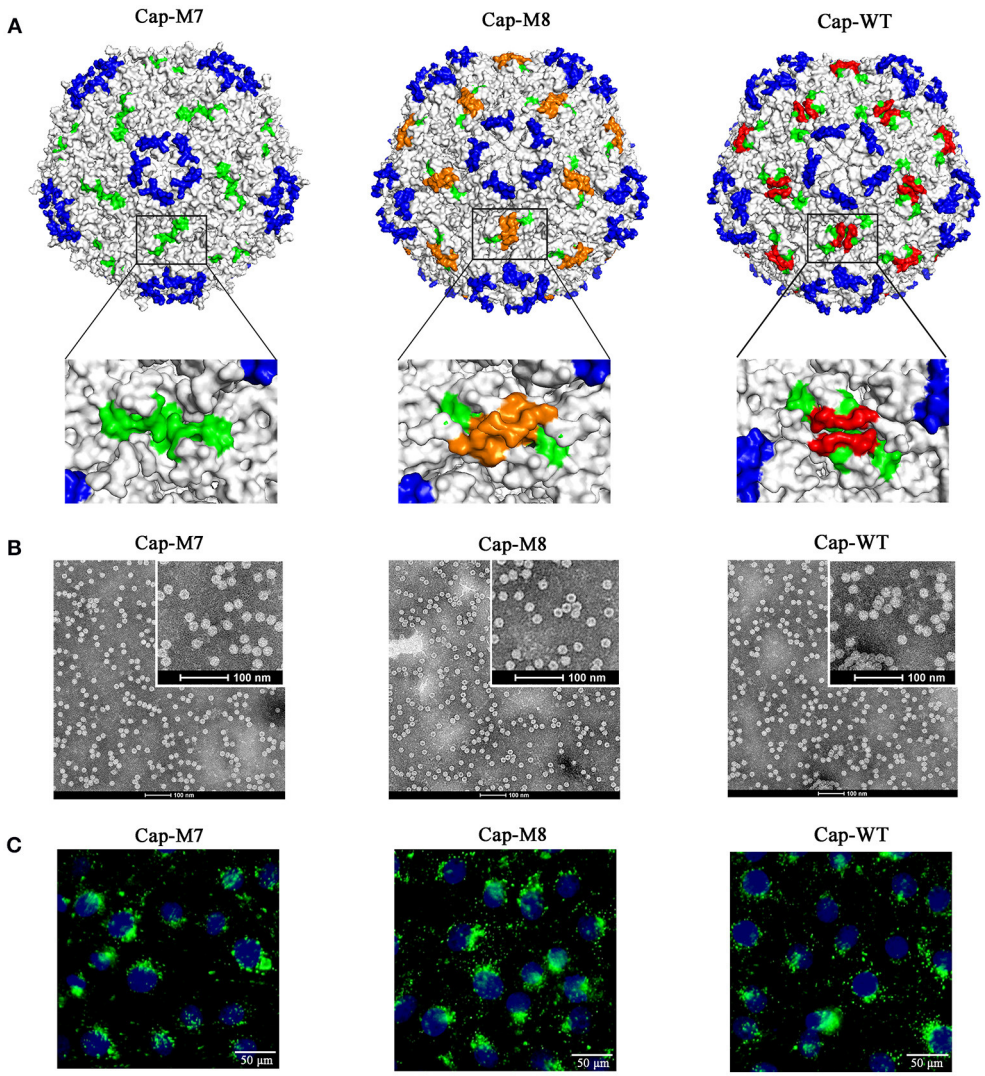


FIGURE 5 | MP-Lcd was not required for PCV2 VLPs assembly and entry into PK15 cells. **(A)** Surface alterations around two-fold axes of PCV2 icosahedra due to ML-Lcd deletion (Cap-M7) or replacement by a foreign epitope (Cap-M8) in simulated 3D models. Magnified images (top view) around a two-fold axis of PCV2 icosahedra were shown on bottom of **(A)**. Loop CD, except MP-Lcd was colored in green; the foreign epitope was colored in orange; MP-Lcd was colored in red, whereas blue indicated Loop BC-decorated five-fold axes of the PCV2 icosahedra. **(B)** Removal of MP-Lcd or replacing it with a foreign epitope had minimal effect on assembly of VLPs. Morphologies of PCV2 VLPs assembled from the Cap-M7, -M8, or WT, respectively were confirmed by TEM (bars = 100 nm). **(C)** Entry of PCV2 VLPs into PK-15 cells. Internalizations of PCV2 VLPs assembled from PCV2 Cap-M7, -M8, or WT were confirmed by confocal microscopy. Green fluorescence represented PCV2 Cap in PK15 cells. Nuclei (blue) of PK-15 cells were stained by DAPI.

that inserted foreign peptides or tags will be displayed on the surface of proteins or protein complexes (i.e., VLPs). However, PCV2 VLPs have been developed to carry foreign peptides, with fusion usually done on either the NT or the CT of the Cap (Li et al., 2013; Zhang et al., 2014). Since the NLS at the NT of the PCV2 Cap is not required for VLPs assembly (Khayat et al., 2011), several PCV2 cVLPs were successfully prepared by replacing the NLS with a T-cell epitope, B-cell epitope, or a T-cell epitope conjugated with a B-cell epitope of classical swine fever virus (CSFV). However, no neutralizing antibodies against CSFV were detected (Zhang et al., 2014). Perhaps these foreign epitopes fused at the NT of the PCV2 Cap were buried inside the PCV2 VLPs after assembly. However, an epitope located on the exterior surface of VLPs may be presented to B lymphocytes by binding to B cells receptor (BCR) (Bachmann and Jennings, 2010). Conversely, the CT of the PCV2 Cap is exposed on the surface of PCV2 VLPs (Khayat et al., 2011; Liu et al., 2016; Wang et al., 2016), and was used to fuse with foreign peptides, although no typical VLPs were observed by TEM (Hu et al., 2016). We also failed several times with fusion of distinct tags to the CT (data not shown). In our previous study, Loop CD exploited a target site to insert a B-cell epitope derived from PRRSV GP5 protein, and the recombinant Cap (rCap) was capable of self-assembling into VLPs *in vitro* (Hu

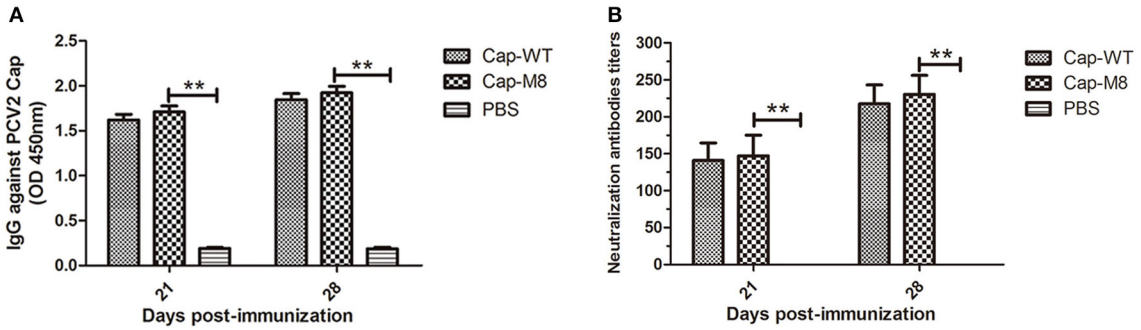


FIGURE 6 | Humoral immune responses to VLPs assembled from PCV2 Cap and Cap-M8. **(A)** Cap-specific antibody in pigs detected by indirect ELISA. Sera were tested for presence of IgG antibodies by indirect ELISA at 21 and 28 d post immunization. PCV2 VLPs were used as an antigen for the ELISA. Optical densities were read at 450 nm. **(B)** Neutralization activity was evaluated by NAbs titers in pig sera. Bars represent arithmetic means ± *SD* of antibody titers. $^{**}P < 0.01$.

et al., 2016). Furthermore, cVLPs entered PK15 cells and induced NAbs against PCV2 and PRRSV infections. Loop CD of PCV2 Cap has several advantages over other loops of this protein for insertion of a foreign epitope (Hu et al., 2016). Recently, a novel genotype of PCV, named PCV3, was reported, although its pathogenesis and lesions remain to be determined (Phan et al., 2016; Palinski et al., 2017). Despite amino acid sequence identity between the Caps of PCV2 and PCV3 being only ∼30% (Palinski et al., 2017), the PCV3 Cap had a similar jelly-roll as the PCV2 Cap, present in many icosahedral viruses (Khayat and Johnson, 2011; Zhan et al., 2017). Surprisingly, MP-Lcd was missing in the PCV3 Cap (**Figure 1A**), which indicated this portion may not be necessary for Cap folding into a jelly-roll structure and VLPs assembly during PCV evolution. Loop CD of the PCV2 Cap is the third largest loop of the Caps with 18 residues and forms the second highest elevation on the capsid surface (Khayat et al., 2011). Furthermore, MP-Lcd in Loop CD contributes the entire plateau portion to the two-fold axis surface of the PCV2 capsid (**Figure 1D**). Simulated 3D structures of the PCV2 cVLPs assembled from the Cap mutants (M1-6) clearly demonstrated that MP-Lcd functioned as a platform to display foreign peptides outward (**Figures 3**, **4**, red). Of note, Loops CD of two neighboring Cap subunits were tightly aligned through their anti-parallel arrangement on the PCV2 capsid surface (Wang et al., 2016). Thus, two MP-Lcd were exactly side by side (**Figure 1D**, red), providing potential bivalent binding sites between foreign peptides (ligands) and their binding partners (receptors). Moreover, foreign peptides caused minimal alteration to the surface of PCV2 VLPs, since they projected from the plateau and were independently displayed. Thus, these foreign peptides might have no negative effect on assembly and antigenicity of PCV2 VLPs. Consequently, conformations of foreign peptides on the plateau also increased the likelihood of interactions with binding partners, e.g. BCR. By virtue of the MP-Lcd orientation within the PCV2 capsid, this is a promising target site for display of foreign peptides. In addition, retaining the plateau of MP-Lcd, instead of deleting it, projects foreign peptides further from the surface of the PCV2 cVLPs, based on the simulated 3D structures.

Further, we tested whether MP-Lcd was biased regarding amino acid composition of foreign peptides. Four PCV2 Cap mutants (Cap-M1 to M4) containing insertions with distinct foreign peptides were capable of self-assembly into VLPs *in vitro* (**Figure 3B**), and these VLPs retained their capacity to enter PK15 cells (**Figure 3C**). In particular, insertion of either poly-lysine or poly-glutamic acid residues into MP-Lcd (Cap-M3 and M4) had no effect on VLP assembly and cell entry (**Figures 3B,C**). Therefore, foreign peptides with highly charged residues may adopt very flexible conformations on MP-Lcd to minimize effects of charge repulsion on VLPs assembly. It also proves MP-Lcd exhibits high tolerance to insertions of various peptides. Indeed, 3D structure simulation also suggested both insertions independently protruded from the surface of MP-Lcd on PCV2 VLPs (**Figure 3**). Finally, maximal insertion size of MP-Lcd was tested. Two PCV2 rCap containing two distinct epitopes or three repeats of the same epitope in tandem, respectively, were successfully expressed and purified (**Figures 2B,C**). Morphologies of both assemblies had different sizes after rCap were dialyzed in assembly buffers (**Figure 4B**). Therefore, we concluded that morphology of PCV2 VLPs may be changed when the insertion size exceeded 18 residues in MP-Lcd. However, morphologies of PCV2 VLPs may also be changed *in vitro* through alterations of assembly conditions (e.g., temperatures and buffer), as reported for other VLPs (Tsukamoto et al., 2007).

Roles of MP-Lcd in PCV2 VLPs assembly, cell entry, and antigenicity were also investigated in the present study. Apparently, removal of MP-Lcd caused the plateau to disappear from the surface, but had no effects on VLPs assembly and cell entry. Therefore, we inferred that MP-Lcd may not be involved in recognition of virus-host cells for PCV2 internalization. Further, deleting MP-Lcd and replacing it with a foreign epitope (Cap-M8) introduced a new surface pattern around the two-fold axes of the PCV2 VLPs, since part of residues of the foreign epitope were present on the surface (orange in **Figure 5A**). Results from EM and IFA suggested that this Cap mutant was still capable of self-assembling into VLPs and entering PK15 cells. We also used this mutant to test effects of surface

alteration around the two-fold axes on antigenicity of PCV2 VLPs in a swine model. Based on ELISA and neutralization experiments, we inferred that removal of MP-Lcd and alteration of the plateau around the two-fold axes of PCV2 VLPs had minimal effects on antigenicity and eliciting NAbs against PCV2 infection.

In conclusion, the MP-Lcd of Loop CD was identified as a very promising site within the PCV2 Cap to develop a functional carrier capable of displaying a variety of foreign peptides or epitopes on the surface of PCV2 VLPs. Of note, it was dispensable for assembly and cellular uptake of PCV2 VLPs. In addition, MP-Lcd was not required for eliciting NAbs against PCV2 infection. Therefore, development of PCV2 VLPs-based bivalent or multivalent vaccines against concurrent infections of PCV2 and other pathogens should be very feasible. Furthermore, based on structure simulation, MP-Lcd functioned as a plateau to project foreign peptides from the surface of PCV2 VLPs. Finally, although insertion size was tested, maximal size for MP-Lcd insertion still needs to be determined, as conditions may be further optimized for VLPs assembly *in vitro*.

AUTHOR CONTRIBUTIONS

DW, NW, and YY are responsible for experimental design. DW, SZ, YZo, WY, YJ, YZh, and YD did experiments, collected and analyzed data. DW and YY wrote the manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

This project was supported by General Program of National Natural Science Foundation of China (Grants No. 31571432); Research Foundation of Hunan Provincial Education Department, China (Grant No. 15A086); Hunan provincial Natural Science foundation of China (Grant No.2018JJ2177); Lateral project funding (Grant No. 17430102000049 and 17430102001846); the Talent Input Project of Hunan Agricultural University (Grant No. 540490317006); and the Youth Science Foundation of Hunan Agricultural University (Grant No. 17QN10).

REFERENCES

An, D. J., Roh, I. S., Song, D. S., Park, C. K., and Park, B. K. (2007). Phylogenetic characterization of *Porcine circovirus* type 2 in PMWS and PDNS Korean pigs between 1999 and 2006. *Virus Res.* 129, 115–122. doi: 10.1016/j.virusres.2007.06.024

Bachmann, M.F., and Jennings, G. T. (2010). Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns. *Nat. Rev. Immunol.* 10, 787–796. doi: 10.1038/nri2868

Beach, N. M., Smith, S. M., Ramamoorthy, S., and Meng, X. J. (2011). Chimeric porcine circoviruses (PCV) containing amino acid epitope tags in the C terminus of the capsid gene are infectious and elicit both anti-epitope tag antibodies and anti-PCV type 2 neutralizing antibodies in pigs. *J. Virol.* 85, 4591–4595. doi: 10.1128/JVI.02294-10

Cheung, A. K. (2003). The essential and nonessential transcription units for viral protein synthesis and DNA replication of *Porcine circovirus* type 2. *Virology* 313, 452–459. doi: 10.1016/S0042-6822(03)00373-8

Fachinger, V., Bischoff, R., Jedidia, S. B., Saalmüller, A., and Elbers, K. (2008). The effect of vaccination against *Porcine circovirus* type 2 in pigs suffering from porcine respiratory disease complex. *Vaccine* 26, 1488–1499. doi: 10.1016/j.vaccine.2007.11.053

Fort, M., Olvera, A., Sibila, M., Segalés, J., and Mateu, E. (2007). Detection of neutralizing antibodies in postweaning multisystemic wasting syndrome (PMWS)-affected and non-PMWS-affected pigs. *Vet. Microbiol.* 125, 244–255. doi: 10.1016/j.vetmic.2007.06.004

Franzo, G., Cortey, M., Olvera, A., Novosel, D., Castro, A. M., Biagini, P., et al. (2015). Revisiting the taxonomical classification of *Porcine circovirus* type 2 (PCV2): still a real challenge. *Virol. J.* 12:131. doi: 10.1186/s12985-015-0361-x

Hu, G., Wang, N., Yu, W., Wang, Z., Zou, Y., Zhang, Y., et al. (2016). Generation and immunogenicity of porcine circovirus type 2 chimeric virus-like particles displaying porcine reproductive and respiratory syndrome virus GP5 epitope B. *Vaccine* 34, 1896–1903. doi: 10.1016/j.vaccine.2016.02.047

Humphrey, W., Dalke, A., and Schulten, K. (1996). VMD: visual molecular dynamics. *J. Mol. Graph.* 14, 33–38. doi: 10.1016/0263-7855(96)00018-5

Khayat, R., and Johnson, J. E. (2011). Pass the jelly rolls. *Structure* 19, 904–906. doi: 10.1016/j.str.2011.06.004

Khayat, R., Brunn, N., Speir J. A., Hardham, J. M., Ankenbauer, R. G., Schneemann, A., et al. (2011). The 2.3-angstrom structure of *Porcine circovirus* 2. *J. Virol.* 85, 7856–7862. doi: 10.1128/JVI.00737-11

Li, W., Wang, X., Bai, J., Ma, T., Li, Z., Li, Y., et al. (2013). Construction and immunogenicity of recombinant *Porcine circovirus*-like particles displaying somatostatin. *Vet. Microbiol.* 163, 23–32. doi: 10.1016/j.vetmic.2012.11.045

Liu, Q., Tikoo, S. K., and Babiuk, L. A. (2001). Nuclear localization of the ORF2 protein encoded by *Porcine circovirus* type 2. *Virology* 285, 91–99. doi: 10.1006/viro.2001.0922

Liu, Z., Guo, F., Wang, F., Li, T-H., and Jiang, W. (2016). 2.9 Å resolution cryo-em 3d reconstruction of close-packed virus particles. *Structure* 24, 319–328. doi: 10.1016/j.str.2015.12.006

Misinzo, G., Delputte, P. L., Lefebvre, D. J., and Nauwynck, H. J. (2009). *Porcine circovirus* 2 infection of epithelial cells is clathrin-, caveolae- and dynamin-independent, actin and Rho-GTPase-mediated, and enhanced by cholesterol depletion. *Virus Res.* 139, 1–9. doi: 10.1016/j.virusres.2008.09.005

Misinzo, G., Delputte, P. L., Meerts, P., Lefebvre, D. J., and Nauwynck, H. J. (2006). *Porcine circovirus* 2 uses heparan sulfate and chondroitin sulfate B glycosaminoglycans as receptors for its attachment to host cells. *J. Virol.* 80, 3487–3494. doi: 10.1128/JVI.80.7.3487-3494.2006

Misinzo, G., Meerts, P., Bublot, M., Mast, J., Weingartl, H. M., and Nauwynck, H. J. (2005). Binding and entry characteristics of porcine circovirus 2 in cells of the porcine monocytic line 3D4/31. *J. Gen. Virol.* 86, 2057–2068. doi: 10.1099/vir.0.80652-0

Olvera, A., Cortey, M., and Segalés, J. (2007). Molecular evolution of *Porcine circovirus* type 2 genomes: phylogeny and clonality. *Virology* 357, 175–185. doi: 10.1016/j.virol.2006.07.047

Palinski, R., Piñeyro, P., Shang, P., Yuan, F., Guo, R., Fang, Y., et al. (2017). A novel *Porcine circovirus* distantly related to known circoviruses is associated with Porcine Dermatitis and Nephropathy Syndrome and reproductive failure. *J. Virol.* 91:e01879–e01816. doi: 10.1128/JVI.01879-16

Phan, T. G., Giannitti, F., Rossow S., Marthaler D., Knutson T. P., Li, L., et al. (2016). Detection of a novel circovirus PCV3 in pigs with cardiac and multi-systemic inflammation. *Virol. J.* 13:184. doi: 10.1186/s12985-016-0642-z

Piñeyro, P. E., Kenney, S. P., Giménez-Lirola, L. G., Heffron, C. L., Matzinger, S. R., Opriessnig, T. et al. (2015a). Expression of antigenic epitopes of porcine reproductive and respiratory syndrome virus (PRRSV) in a modified live-attenuated porcine circovirus type 2 (PCV2) vaccine virus (PCV1-2a) as a potential bivalent vaccine against both PCV2 and PRRS. *Virus Res.* 210, 154–164. doi: 10.1016/j.virusres.2015.07.027

Piñeyro, P. E., Kenney, S. P., Giménez-Lirola, L. G., Opriessnig, T., Tian, D., Heffron, C. L., et al. (2015b). Evaluation of the use of non-*pathogenic Porcine circovirus* type 1 as a vaccine delivery virus vector to express antigenic epitopes

of porcine reproductive and respiratory syndrome virus. *Virus Res.* 213, 100–108. doi: 10.1016/j.virusres.2015.11.005

Segales, J., Kekarainen, T., and Cortey, M. (2013). The natural history of *Porcine circovirus* type 2: From an inoffensive virus to a devastating swine disease? *Vet. Microbiol.* 165, 13–20. doi: 10.1016/j.vetmic.2012.12.033

Sun, D., Feng, L., Shi, H., Chen, J., Cui, X., Chen, H., et al. (2008). Identification of two novel B cell epitopes on porcine epidemic diarrhea virus spike protein. *Vet. Microbiol.* 131, 73–81. doi: 10.1016/j.vetmic.2008.02.022

Sun, J., Huang, L., Wei, Y., Wang, Y., Chen, D., Du, W., et al. (2015). Identification of three PPV1 VP2 protein-specific B cell linear epitopes using monoclonal antibodies against *Baculovirus*-expressed recombinant VP2 protein. *Appl. Microbiol. Biotechnol.* 99, 9025–9036. doi: 10.1007/s00253-015-6790-z

Tsukamoto, H., Kawano, M. A., Inoue, T., Enomoto, T., Takahashi, R. U., Yokoyama, N., et al. (2007). Evidence that SV40 VP1-DNA interactions contribute to the assembly of 40-nm spherical viral particles. *Genes Cells* 12, 1267–1279. doi: 10.1111/j.1365-2443.2007.01134.x

Wang, N., Zhan, Y., Wang, A., Zhang, L., Khayat, R., and Yang, Y. (2016). *In silico* analysis of surface structure variation of PCV2 capsid resulted from loops mutation of its capsid protein (cap). *J. Gen. Virol.* 97, 3331–3344. doi: 10.1099/jgv.0.000634

Zhan, Y., Dong-liang, W., Nai-dong, W., Yi-fan, J., Kun H., Shang-jin, C., et al. (2017). Survey on detection and analyses of cap antigenicity prediction of *Porcine Circovirus* type 3 isolated from partial provinces of Southern China. *Acta Vet. Zootechnol. Sin.* 48, 1076–1084. doi: 10.11843/j.issn.0366-6964.2017.06.012

Zhan, Y., Wang, N., Zhu, Z., Wang, Z., Wang, A., Deng, Z., et al. (2016). *In silico* analyses of antigenicity and surface structure variation of an emerging porcine circovirus genotype 2b mutant, prevalent in southern China from 2013 to 2015. *J. Gen. Virol.* 97, 922–933. doi: 10.1099/jgv.0.000398

Zhang, H., Qian, P., Liu, L., Qian, S., Chen, H., and Li, X. (2014). Virus-like particles of chimeric recombinant *Porcine circovirus* type 2 as antigen vehicle carrying foreign epitopes. *Viruses* 6, 4839-4855. doi: 10.3390/v6124839

Zhang, J., Liu, Z., Zou, Y., Zhang, N., Wang, D., Tu, D., et al. (2017). First molecular detection of *Porcine circovirus* type 3 in dogs in China. *Virus Genes* 54, 140–144. doi: 10.1007/s11262-017-1509-0

Zhang, Y., Wang, Z., Zhan, Y., Gong, Q., Yu, W., Deng, Z., et al. (2016). Generation of E. coli-derived virus-like particles of porcine circovirus type 2 and their use in an indirect IgG enzyme-linked immunosorbent assay. *Arch. Virol.* 161, 1485–1491. doi: 10.1007/s00705-016-2816-9

Conflict of Interest Statement: 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 Cellular and Infection Microbiology

REVIEW
published: 08 October 2018
doi: 10.3389/fcimb.2018.00343

Back to the Future: Lessons Learned From the 1918 Influenza Pandemic

Kirsty R. Short[1,2], *Katherine Kedzierska*[3]* *and Carolien E. van de Sandt*[3,4]*

[1] School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane, QLD, Australia, [2] Australian Infectious Diseases Research Centre, The University of Queensland, Brisbane, QLD, Australia, [3] Department of Microbiology and Immunology, The Peter Doherty Institute for Infection and Immunity, University of Melbourne, Parkville, VIC, Australia, [4] Department of Hematopoiesis, Sanquin Research and Landsteiner Laboratory, Amsterdam, Netherlands

2018 marks the 100-year anniversary of the 1918 influenza pandemic, which killed ~50 million people worldwide. The severity of this pandemic resulted from a complex interplay between viral, host, and societal factors. Here, we review the viral, genetic and immune factors that contributed to the severity of the 1918 pandemic and discuss the implications for modern pandemic preparedness. We address unresolved questions of why the 1918 influenza H1N1 virus was more virulent than other influenza pandemics and why some people survived the 1918 pandemic and others succumbed to the infection. While current studies suggest that viral factors such as haemagglutinin and polymerase gene segments most likely contributed to a potent, dysregulated pro-inflammatory cytokine storm in victims of the pandemic, a shift in case-fatality for the 1918 pandemic toward young adults was most likely associated with the host's immune status. Lack of pre-existing virus-specific and/or cross-reactive antibodies and cellular immunity in children and young adults likely contributed to the high attack rate and rapid spread of the 1918 H1N1 virus. In contrast, lower mortality rate in in the older (>30 years) adult population points toward the beneficial effects of pre-existing cross-reactive immunity. In addition to the role of humoral and cellular immunity, there is a growing body of evidence to suggest that individual genetic differences, especially involving single-nucleotide polymorphisms (SNPs), contribute to differences in the severity of influenza virus infections. Co-infections with bacterial pathogens, and possibly measles and malaria, co-morbidities, malnutrition or obesity are also known to affect the severity of influenza disease, and likely influenced 1918 H1N1 disease severity and outcomes. Additionally, we also discuss the new challenges, such as changing population demographics, antibiotic resistance and climate change, which we will face in the context of any future influenza virus pandemic. In the last decade there has been a dramatic increase in the number of severe influenza virus strains entering the human population from animal reservoirs (including highly pathogenic H7N9 and H5N1 viruses). An understanding of past influenza virus pandemics and the lessons that we have learnt from them has therefore never been more pertinent.

Keywords: influenza, pandemic, 1918, host factors, viral factors, external factors, societal factors, prevention

OPEN ACCESS

Edited by:
Yanmin Wan,
Fudan University, China

Reviewed by:
Ali Ellebedy,
Washington University in St. Louis, United States
Daniel Angel Ortiz,
Laboratory Corporation of America Holdings (LabCorp), United States

***Correspondence:**
Katherine Kedzierska
kkedz@unimelb.edu.au
Carolien E. van de Sandt
cvandesandt@unimelb.edu.au

Received: *10 August 2018*
Accepted: *10 September 2018*
Published: *08 October 2018*

Citation:
Short KR, Kedzierska K and van de Sandt CE (2018) Back to the Future: Lessons Learned From the 1918 Influenza Pandemic. Front. Cell. Infect. Microbiol. 8:343. doi: 10.3389/fcimb.2018.00343

INTRODUCTION

In 1918 a mysterious and deadly disease spread around the world in three consecutive waves (spring 1918, autumn 1918, and winter 1918–19). This pandemic infected over one third of the world's population and killed an estimated 50 million people (Johnson and Mueller, 2002; Murray et al., 2006), with unusually severe clinical manifestations in previously healthy young adults (Collins, 1931; Hoffman, 2011). In 1918, the etiological agent that caused this disease was unknown (Hildreth, 1991). However, we now know that these events represented the largest influenza virus pandemic on record: the catastrophic 1918 influenza pandemic. Since 1918, the world has experienced three additional influenza pandemics: the 1957 "Asian" influenza pandemic, the 1968 "Hong Kong" influenza pandemic and the 2009 so-called "swine flu" pandemic. These pandemics, although mild in comparison to that of 1918, highlight the constant threat that influenza virus poses to human health. Given that almost 100 years have passed since 1918, it behooves us to ask: are we truly better prepared for the next influenza virus pandemic or are there still lessons to be learned? This review gives an overview of lessons learned from the 1918 influenza pandemic, highlighting new insights into our understanding of viral pathogenesis and their impact on our preparedness for the next outbreak of influenza.

The Origins 1918 Influenza Virus

The 1918 influenza pandemic is often colloquially referred to as the "Spanish" influenza pandemic. However, it is unlikely that the 1918 influenza virus originated in Spain. Instead, influenza cases were widely reported in Spain due to the fact that, as a neutral country in World War I, Spain did not practice censorship in the press. In contrast, other countries involved in the war, such as Germany, Britain and France, most likely limited the news of this deadly pandemic, so as not to lower the moral of the troops and raise questions about their military readiness (Johnson, 2006). Today, the general consensus is that the 1918 influenza virus originated in the Midwest of the United States of America (Barry, 2004). Medical records reported the first cases of "influenza of a severe type" around March 1918 in military camps in Kansas (Barry, 2004). From here, the virus is thought to have spread throughout the United Stated and then transported by American troop ships to the battlefields of France, where it gradually spread throughout Europe and the rest of the world (Patterson and Pyle, 1991). The spread of the virus beyond port cities was further facilitated by local transport networks, predominately railways (Patterson and Pyle, 1991; Johnson, 2006). However, it is possible that the predecessor of this killer virus first entered human population prior to 1918 and became more virulent and/or more transmissible over time. Unusual influenza activity was already reported in the United States and several European countries before the first (spring) wave of the 1918 influenza outbreak (Frost, 1919; Johnson, 2006; Hoffman, 2011). Military camps in France already reported influenza infections accompanied with high mortality in the winter of 1916–17 (Hammond et al., 1917), which was followed 2 months later by a similar outbreak near London at Aldershot, one of Britain's biggest military camps (Oxford et al., 1999, 2002, 2005). Interestingly, no records of civilian influenza cases around that time exist, possibly because influenza cases were not recorded at the time or because they got lost with time. Alternatively, it is tempting to speculate that military camps, with their high population density, close proximity to livestock, high mobility, and large number of people with pre-existing lung conditions (due to exposure to toxic gasses in the trenches) served as the perfect breeding ground for the emergence of this catastrophic pandemic (Oxford et al., 2005).

Just as the geographic origins of the 1918 virus remain unclear, the original animal reservoir of the virus also remains controversial. As a segmented virus, influenza virus is capable of undergoing the process of reassortment. Reassortment occurs when two influenza virus strains co-infect the same cell, facilitating the emergence of a new "reassortant" virus which contains a novel constellation of genes. Reassortment between avian and human influenza viruses gave rise to the 1957 and 1968 influenza pandemics (**Figure 1**; Scholtissek et al., 1978; Kawaoka et al., 1989; Schäfer et al., 1993). In contrast, the 2009 influenza pandemic resulted from a reassortment event between avian, human and swine influenza viruses (**Figure 1**; Garten et al., 2009; Smith et al., 2009b). Unlike these more recent influenza pandemics, the 1918 virus is thought to have been directly introduced in the human population (i.e., in the absence of reassortment) from a single unidentified host (Taubenberger et al., 2005). This notion is supported by the fact that the 8 individual gene segments of the 1918 virus appear to have co-evolved in the same host. However, the exact identity of this host remains unclear, as the nucleotide sequence of the virus is genetically distinct to all other known avian and mammalian influenza viruses (Reid et al., 2004a,b; Taubenberger et al., 2005). In contrast, others argue that the 1918 influenza virus could have indeed originated from a reassortment event between avian and mammalian, possibly swine and/or human, influenza viruses in the years prior to the 1918 pandemic (Smith et al., 2009a; Worobey et al., 2014). Unfortunately, in the absence of influenza virus sequence data in the years preceding the 1918 pandemic, this question may never be definitively answered.

A Broad Spectrum of Clinical Disease

During the 1918 influenza pandemic, a broad spectrum of clinical illness was observed (Brundage and Shanks, 2008). In the first spring wave of the pandemic, disease was typically mild and mortality rates were not unusually high (Johnson and Mueller, 2002). However, there was a surprisingly large number of young adults who were affected by the outbreak (Ahmed et al., 2007). The second or autumn wave of influenza emerged in late August 1918 and by the end of 1918 almost no country was spared (Patterson and Pyle, 1991; Johnson, 2006). The striking feature of the autumn wave was its unprecedented virulence (Taubenberger et al., 2001). Patients typically suffered from a high fever, cyanosis, and fluid accumulation in the lungs (Johnson, 2006). In ∼5% of the fatal cases, death occurred rapidly after the onset of clinical symptoms (i.e., within 3 days), although for the majority of cases the time from clinical symptoms to death was ∼7–10 days (Brundage and Shanks, 2008). The third and final wave of influenza emerged at the start of 1919 (Beveridge, 1977). This

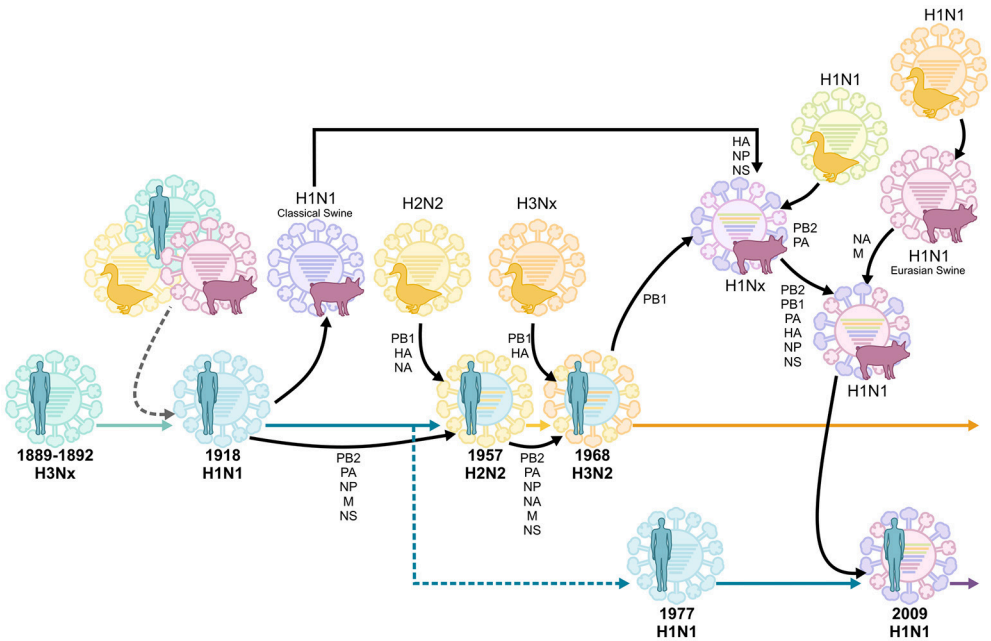


FIGURE 1 | Reassortment events of historic pandemic influenza A viruses, adapted from van de Sandt et al. (2015b). Historic serum analysis suggests that the Russian influenza pandemic of 1889–1892 was of the H3Nx subtype and seasonally circulated up to the 1918 influenza pandemic. It remains undefined whether the 1918 H1N1 pandemic virus originated from multiple reassortment events between avian, swine and human influenza viruses, or if it was introduced by a direct zoonotic transmission event of an avian, swine or other influenza virus. The H1N1 virus continued to circulate, causing seasonal epidemics, until 1957 when it reassorted with an avian H2N2 virus. This virus circulated until 1968, when it reassorted again with the avian H3Nx virus, which has caused seasonal epidemics ever since. In 1977 the H1N1 virus was reintroduced in the human population and co-circulated with H3N2 viruses until the influenza pandemic of 2009 when it was replaced by another H1N1 virus which was the result of multiple reassortment events between avian, swine, and human influenza viruses.

wave was typically not as virulent as the fall wave and it did not affect every country (Beveridge, 1977; Taubenberger and Morens, 2006). During the course of the pandemic, ∼500 million people worldwide were infected, resulting in a case-fatality rate of >2.5% (Johnson and Mueller, 2002; Johnson, 2006; Taubenberger and Morens, 2006). While this fatality rate was certainly higher than other influenza virus pandemics (Taubenberger and Morens, 2006), these data indicate that influenza virus infection was not always fatal and that a large number of people survived the infection. These data raise two intriguing questions: (i) Why was the 1918 influenza pandemic more virulent than other influenza pandemics of the twentieth century; and (ii) Why did some people survive the pandemic and others succumb to the infection?

VIRAL FACTORS ASSOCIATED WITH THE SEVERITY OF THE 1918 INFLUENZA PANDEMIC

It was not until 1933, more than a decade after the devastating pandemic of 1918–19, when the influenza virus was first isolated and demonstrated to be the causative agent of seasonal influenza virus infections (Smith et al., 1933). However, even then, an in-depth understanding of the viral factors that contributed to the severity of the 1918 pandemic was thwarted by the absence of any available biological material from the virus in question. Finally, in the late 1990s the virus' genetic material was successfully isolated from formalin-fixed, paraffin-embedded lung tissue from 1918 influenza victims and from the lungs of a 1918 influenza victim buried in Alaska's permafrost (Taubenberger et al., 1997; Reid et al., 1999). These efforts unraveled a partial viral sequence from four viruses and the complete genomic sequence of one virus (Reid et al., 1999). The fully reconstructed 1918 influenza virus proved to be highly pathogenic in mice (Tumpey et al., 2005), ferrets (Memoli et al., 2009), and macaques (Kobasa et al., 2007). Interestingly, a recent study in ferrets demonstrated that the 1918 influenza virus could spread to, and induce cytokine responses in tissues outside the respiratory tract, which likely contributed to the severity of the infection (de Wit et al., 2018) and could explain the neurological complications observed during the 1918 influenza pandemic (Alexander, 1919; Ravenholt and Foege, 1982). Various reverse genetics experiments suggest that the high pathogenicity exerted by the 1918 influenza virus was most likely an interplay between different virulence factors, in which proteins encoded by the viral haemagglutinin (HA) and polymerase gene segments played a crucial role (Kobasa et al., 2004; Tumpey et al., 2005; Kash et al., 2006; Conenello et al., 2007; Pappas et al., 2008; Watanabe et al., 2009; Jagger et al., 2010).

One of the best-known virulence determinants of influenza virus is the presence of a multibasic cleavage site in the HA

(Horimoto and Kawaoka, 1994; Subbarao et al., 1998). In avian species, influenza viruses without a multibasic cleavage site require the HA to be cleaved by host trypsin-like proteases for infection. Trypsin-like proteases are commonly found in the respiratory tract, thus limiting replication of these viruses to these tissues. However, the presence of the multibasic cleavage site means that these viruses can be cleaved by ubiquitously expressed proteases. The presence of a multibasic cleavage site in modern highly pathogenic avian influenza viruses can be associated with increased virulence in mammalian hosts (Schrauwen et al., 2012; Suguitan et al., 2012). However, none of the available 1918 HA sequences contained a multibasic cleavage site (Kawaoka and Webster, 1988; Taubenberger et al., 1997; Reid et al., 1999). Instead, analysis of the HA sequence of the 1918 viruses revealed that these viruses were adapted to bind to human epithelial cells. The influenza A virus HA protein requires only one amino acid change in order to switch binding to α-2,3-linked sialic acids (typically found on avian cells) to binding to the α-2,6-linked sialic acids (typically found on human epithelial cells in the upper respiratory tract) (Glaser et al., 2005). Compared to victims from the spring wave, an increased incidence of this mutation in the HA was observed in viruses isolated from victims of the more severe autumn wave (Reid et al., 2003; Glaser et al., 2005; Sheng et al., 2011). A second mutation, which strengthens the virus binding to the human receptor, could only be found in some of the 1918 HA sequences (Reid et al., 2003; Sheng et al., 2011). These data suggest that at least two H1N1 influenza viruses circulated in 1918, which differed in their binding affinity for the human receptor. Both viruses displayed a similar cell tropism in the respiratory tract of terminal stage human influenza victims (Sheng et al., 2011). However, this secondary adaptation is essential for effective transmission of the 1918 influenza virus between ferrets (Tumpey et al., 2007). Mutations in other gene segments, including PB2, PA, and PB1-F2 of the 1918 influenza virus can also play a role in host adaptation (Dunham et al., 2009; Jagger et al., 2010; Mehle et al., 2012; Mazel-Sanchez et al., 2018). In the absence of additional influenza virus sequences from 1918, it is hard to establish whether these or other mutations contributed to the dramatic increase in case-fatality seen during the autumn wave of the pandemic (Simonsen et al., 2018).

Gain- and loss-of-function experiments, such as those described above, have provided important insights as to how novel influenza viruses adapt to the human population (Subbarao et al., 1993; Mehle and Doudna, 2009; Herfst et al., 2012; Imai et al., 2012; Belser et al., 2013; Richard et al., 2013; Zhu et al., 2013; Watanabe et al., 2014). Specifically, this information is used to evaluate the pandemic potential of novel influenza viruses, including avian H7N9 and H5N1 viruses, which are frequently crossing the species barrier into the human population. The Global Influenza Surveillance and Response System (GISRS), a surveillance program that monitors which influenza virus strains circulate at a given time (Hay and McCauley, 2018; World Health Organization, 2018a), uses this information for a rapid risk assessment when a potentially pandemic virus is reported to circulate in animals (predominately birds or swine) or has crossed the species barrier into the human population. It is hoped that extensive surveillance activities, in combination with rapid clinical diagnosis, will afford us a "head start" in the case of a future influenza pandemic. However, the success of such surveillance programs is contingent upon their geographical breadth (Krammer et al., 2018). International cooperation and support for influenza surveillance will become even more pertinent in the future as climate changes continues to affect animal reservoirs and avian migration patterns, both of which could lead to the spread of influenza viruses to new locations and across a wider range of avian species (Klaassen et al., 2012; Shaman and Lipsitch, 2013; Audubon, 2018).

HOST FACTORS ASSOCIATED WITH VARIATIONS IN INFLUENZA MORBIDITY AND MORTALITY IN 1918

The 1918 influenza pandemic is notorious for its high morbidity and mortality rates. However, it is important to recognize that there were substantial variations in mortality, both within and between countries (Mills, 1986; Johnson and Mueller, 2002; Johnson, 2006). General estimations assume an overall death rate of 2.5–5 per 1,000 individuals worldwide. Although this might be an accurate estimate for some countries [e.g., Australia (2.8/1000), Austria (3/1000), Demark (4.1/1000)], it represents an overestimation for some countries [e.g., Argentina (1.2/1000), Uruguay (1.4/1000), American Samoa (0/1000)], and a gross underestimation of others [e.g., Nauru (160/1000), Western Samoa (236/1000), Cameroon (445/1000)] (Johnson and Mueller, 2002; Johnson, 2006). These data indicate that in addition to viral factors, host factors had a major impact on the outcome of infection.

Age

An individual's age played a major role in determining one's risk of death during the 1918 influenza pandemic. Typically, when the mortality rates of seasonal influenza are graphed against the age of the population, a "U" shaped curve is produced, as the highest mortality occurs in the very young and old (Johnson, 2006). In contrast, pandemic outbreaks (to various degrees) are characterized by a shift in case-fatality toward younger age groups (Simonsen et al., 1998; Olson et al., 2005; Ahmed et al., 2007; Georgantopoulos et al., 2009). This was particularly pronounced during 1918 pandemic when young adults (15–30 years) displayed such usually high mortality rate that a "W" shaped mortality curve was produced (Olson et al., 2005; Ahmed et al., 2007; Shanks and Brundage, 2012). The underlying mechanisms driving this mortality shift are not fully understood but are likely to be associated with the host's immune status.

Immunopathology

The high mortality rates observed in young adults during the 1918 pandemic has traditionally been attributed to the induction of an aberrant, dysregulated pro-inflammatory response (often referred to as a "cytokine storm"). This hypothesis is based upon experimental studies in various animal models using the reconstructed 1918 influenza virus. These experimental studies showed that the 1918 influenza virus triggered a

potent, dysregulated pro-inflammatory response, which likely contributed to the severe lung lesions observed in victims of the 1918 influenza pandemic (Kobasa et al., 2004, 2007; Kash et al., 2006; Memoli et al., 2009; de Wit et al., 2018). Indeed, this dysregulated immune response has also been observed in natural and experimental infections with both the highly pathogenic avian H5N1 virus and the 2009 pandemic influenza virus (de Jong et al., 2006; To et al., 2010). However, it is important to note that all experimental 1918 influenza virus studies to date have been performed in immunologically-naïve animals. This is not necessarily indicative of the human situation in 1918, as influenza viruses caused epidemics and pandemics prior to 1918 (Dowdle, 1999; Johnson, 2006; Morens and Fauci, 2007; Morens et al., 2009; Valleron et al., 2010). It can therefore be assumed that a large proportion of the human population in 1918, with the possible exception of isolated countries/communities, would have encountered at least one previous influenza virus infection, resulting in pre-existing humoral and cellular immunity. It remains unclear whether such pre-existing immunity would cross-react with the 1918 H1N1 virus, and if so, whether it would enhance or dampen any dysregulated pro-inflammatory response in young adults.

Humoral Immune Response

In contrast to young adults, older adults (aged 30–60 years) fared significantly better during the 1918 pandemic (Luk et al., 2001). This observation is likely to reflect the beneficial effects of pre-existing humoral immunity. It is theorized that an H1 and/or N1 influenza virus circulated in the human population prior to 1889, when it was replaced by a H3 influenza virus that caused the so-called "Russian" influenza pandemic (1889–1892) (Ahmed et al., 2007). Accordingly, individuals born before 1889 (i.e., those 30 years or older during the 1918 pandemic) would have had cross-protective antibodies, while people born after 1889 would have been immunological naïve to the 1918 H1N1 pandemic virus (Dowdle, 1999; Ahmed et al., 2007). The lack of pre-existing 1918 influenza virus-specific or cross-reactive antibodies in children and young adults likely contributed to the high attack rate and rapid spread of the virus (Ahmed et al., 2007). Only people infected during the first "spring" wave of the pandemic acquired a protective immune response against the second, more virulent, "fall" wave of the 1918–19 pandemic (Gibbon, 1919; Shope, 1958; Palmer and Rice, 1992; Barry et al., 2008; Mathews et al., 2010; Shanks et al., 2010, 2011b; Fraser et al., 2011). Interestingly, unlike the majority of elderly populations worldwide, elderly populations in remote settings, including Indigenous Australians, Alaskan Natives, and Latin Americans, experienced high mortality during the 1918 pandemic. This most likely reflects the fact that these remote populations were not exposed to the previously circulating influenza viruses that conferred cross-protection (Ahmed et al., 2007).

Conclusive evidence that protective influenza virus-specific antibody responses are indeed long-lived came from the 2009 influenza pandemic. Here, elderly people who were exposed to the 1918 influenza virus (or its immediate descendant), 60–90 years prior to the pandemic of 2009, were protected from infection and severe disease, as they maintained the antibody response that cross-reacted with the 2009 pandemic strain (Yu et al., 2008; Hancock et al., 2009; Ikonen et al., 2010; Reed and Katz, 2010).

Interesting, a recent study suggested that individuals medically-treated for influenza-like illness in the years prior to the 1918 pandemic (1916–1918) were actually at an increased risk of having clinically significant respiratory illness during the autumn wave of the 1918 pandemic (Shanks et al., 2016a). Similarly, the presence of cross-reactive but non-neutralizing antibodies, was associated with immune complex deposition and increased disease severity during the 2009 influenza pandemic (Monsalvo et al., 2011). These data suggest that protection against disease is dependent not just upon the presence of pre-existing antibodies, but rather their ability to neutralize the influenza virus strain in question.

Cellular Immune Response

The issue of prior influenza virus exposure in the general population prior to 1918 raises the question as to why a pre-existing cellular immune response, in particular cross-reactive $CD8^+$ T cells, offered so little protection to young adults during the 1918 influenza pandemic?

A robust $CD8^+$ T cell response plays an important role in protection against novel influenza virus strains and subtypes. Unlike antibodies, $CD8^+$ T cells can recognize the internal proteins of influenza virus. Since these internal proteins do not undergo rapid antigenic change, $CD8^+$ T cells are able to provide cross-protection against a broad range of different influenza virus strains. Accordingly, pre-existing influenza virus specific $CD8^+$ T cells provided protection against severe disease during the influenza pandemics of both 1957 and 2009 (Slepushkin, 1959; McMichael et al., 1983; Epstein, 2006; Sridhar et al., 2013; Hayward et al., 2015). In addition, seasonally induced influenza virus-specific $CD8^+$ T cells can cross-react with novel potentially pandemic avian influenza viruses (Kreijtz et al., 2008; Lee et al., 2008; van de Sandt et al., 2014) and facilitate more rapid recovery in patients following infection with low pathogenic H7N9 avian influenza virus (Wang et al., 2015). The presence of conserved $CD8^+$ T cell peptides in the viral protein sequences of the 1918 influenza virus (Quiñones-Parra et al., 2014) and the ability the of the 2009 H1N1 pandemic influenza virus to recall influenza virus-specific $CD8^+$ T cells, cross-reacting with the 1918 H1N1 influenza virus (Gras et al., 2010) suggest that pre-existing $CD8^+$ T cells should have been protective against severe infection with the 1918 H1N1 influenza virus, especially in the case of young adults. $CD8^+$ T cells may not have been optimal in very young children (age 0–4 years) due lack of exposure to previous influenza viruses (Bodewes et al., 2011a; Sauerbrei et al., 2014). Similarly, immunosenescence may have impaired $CD8^+$ T cell function in the elderly (>65 years) (Goronzy and Weyand, 2013). This may partially explain the high mortality observed in the youngest and oldest age groups during seasonal epidemics (U-shaped curve). However, individuals between 15 and 65 years of age, who suffered the greatest burden of disease during the 1918 pandemic, are thought to display the "gold-standard" immune response, with optimal cross-reactive $CD8^+$ T cell responses. The absence of protective immunity in this age group is unlikely

to be due to the fact that heterosubtypic immunity is short-lived (Mathews et al., 2010), as the longevity of influenza virus-specific $CD8^+$ T cells in healthy individuals has recently been demonstrated (van de Sandt et al., 2015a). It is possible that the recall of pre-existing influenza virus-specific $CD8^+$ T cell responses was not rapid enough for the extremely virulent 1918 pandemic virus, causing rapid appearance of clinical disease and death within 3 days (Ahmed et al., 2007). Alternatively, pandemic H1N1 influenza viruses (1918 and 2009) may have suppressed immunogenic RIPK3-driven dendritic cell death needed for the induction of an effective $CD8^+$ T cell response (Hartmann et al., 2017). In addition, it is likely that ethnically defined genetic variations in HLA molecules influenced cross-reactive $CD8^+$ T cell responses in influenza virus infected individuals (Quiñones-Parra et al., 2014). This (combined with other socio-economic factors) would leave some ethnicities, like Alaskan Natives and Indigenous Australians, more susceptible to severe influenza virus infections. Indeed, alarmingly high morbidity and mortality rates were observed amongst these populations during the pandemics of 1918 (Ahmed et al., 2007) and 2009 (La Ruche et al., 2009; Flint et al., 2010). Similarly, it is striking to note that the matrix protein of the 1918 virus already contained extra-epitopic amino acid residues that were associated with evasion from the pre-existing influenza virus $CD8^+$ T cells (van de Sandt et al., 2015b), a phenomenon not observed in the comparatively mild 2009 pandemic influenza virus (van de Sandt et al., 2018a,b).

Finally, it is important to note that the highest influenza virus infection rates in 1918 were observed among children of school age (5–15 years). However, this increased infection rate occurred in the absence of high morbidity (Shanks and Brundage, 2012; Mamelund et al., 2016). Thus, school-aged children are thought to be in a "honeymoon period" of superior immunity, whereby they display increased resistance to a variety of different bacterial and viral pathogens (Ahmed et al., 2007). However, the mechanisms underlying such superior immunity are completely unexplored, despite the fact that it holds key information for inducing effective immune responses to influenza viruses.

In this review, we would like to propose an additional hypothesis that might have influenced the effectiveness of the cross-reactive cellular immune response and possibly contributed to the disproportional mortality amongst young adults in 1918; namely immune suppression as a resulting from recent measles infections (Moss et al., 2004; Griffin, 2010; de Vries et al., 2012; Mina et al., 2015). Measles epidemics were frequently reported at the end of the nineteenth and in the early twentieth century (Cliff et al., 1983; Duncan et al., 1997; Shulman et al., 2009; Shanks et al., 2011a, 2014), including a large measles outbreak in the US military camps in the winter of 1917–1918 (Shanks et al., 2014; Morens and Taubenberger, 2015). The elderly population would have experienced measles in their childhood and their immunity would have protected them from contracting measles in the years prior to the 1918 influenza pandemic. However, children and young adults, without prior measles infections, would have been immunologically susceptible to measles in the years preceding the 1918 influenza pandemic. Recent studies have demonstrated that the measles virus infects memory T lymphocytes, resulting in apoptosis and a prolonged state of immune suppression up to 3 years after the initial measles infection (Moss et al., 2004; Griffin, 2010; de Vries et al., 2012; Mina et al., 2015). Influenza virus-specific CTL responses could have been suppressed in young individuals who had to endure a measles infection in the years prior to the 1918 influenza pandemic, which may have increased their susceptibility to a severe influenza infection. The combination of recovering from immunosuppression and an infection with an unexpectedly highly virulent virus might have contributed to severe inflammatory related pathology in a mechanism better known as the immune reconstitution inflammatory syndrome (IRIS) (Hirsch et al., 2004; Morens and Fauci, 2007; Shulman et al., 2009; Barber et al., 2012). Whether recent measles infections indeed led to immunosuppression of the influenza virus-specific T cell responses, resulting in a higher susceptibility for severe influenza virus infections and potential IRIS or alternatively contributed to dampening $CD8^+$ T cell immunopathology remains an important area of future research. Fortunately, measles vaccines are now widely available and have greatly reduced the prevalence of measles worldwide (Moss and Griffin, 2012; Perry et al., 2014; Mina et al., 2015). However, the increased number of measles outbreaks in recent years and declining vaccination rates represent a key point of concern for future influenza virus pandemics.

Together, these data demonstrate that an individual's age (and the associated differences in their immune response) played an important role in determining disease outcome in the context of pandemic influenza virus infections. In 2009, age and pre-existing humoral immunity were taken into account when identifying priority individuals for vaccination. In 2009, the elderly population were less susceptible to severe influenza (Dawood et al., 2012) as they were protected through cross-reactive antibodies and $CD8^+$ T cells acquired during previous seasonal infections, including the antigenically related A/H1N1 virus that circulated prior to 1957 (Yu et al., 2008; Hancock et al., 2009; Ikonen et al., 2010). Based on these findings, the first limited 2009 influenza vaccine stocks were administered to younger individuals, instead of being misdirected to the traditional high risk group: the elderly (National Center for Respiratory Diseases, CDC, and Centers for Disease and Prevention (CDC), 2009). Improving cross-reactive $CD8^+$ T cell responses to influenza vaccinations and natural infections remains a key research priority for the future (Clemens et al., 2018). This includes an understanding of $CD8^+$ T cell functionality in ethnically diverse populations and different age groups (Clemens et al., 2018).

Genetic Differences

In addition to the role of humoral and cellular immunity, there is a growing body of evidence to suggest that individual genetic differences contribute to differences in the severity of influenza virus infections. For example, during the 2009 influenza pandemic several single-nucleotide polymorphisms (SNPs) were strongly associated with severe pneumonia. These included SNPs in the genes for interferon response factor 7 (Ciancanelli et al., 2015), Fc fragment of immunoglobulin G, low-affinity IIA, receptor (Zúñiga et al., 2012), RPA interacting protein (Zúñiga

et al., 2012), complement component 1q subcomponent binding protein (Zúñiga et al., 2012), CD55 (Zhou et al., 2012), IL-1α (Liu et al., 2013), IL-1β (Liu et al., 2013), surfactant protein B gene (To et al., 2014) and interferon induced transmembrane protein 3 (Everitt et al., 2012; Zhang et al., 2013), and IRF9 (Hernandez et al., 2018). Unfortunately, there is insufficient information available to conclude whether mortality variations in 1918 were influenced by any of the aforementioned SNPs. Defining which SNPs confer increased susceptibility to severe influenza remains an important aspect of influenza pandemic preparedness, as it will help to inform which populations are most at risk of severe disease.

Malnutrition

Host nutritional status has long been recognized as an important factor in the outcome of a variety of different infectious diseases (Cohen, 2000). In India in 1918, the effects of malnutrition and famine on influenza severity were particularly pronounced. The 1918 influenza pandemic hit India during a widespread drought, which affected the viability of many important food crops (Mills, 1986). Consequently, many Northern-Western, Western and Central Indian provinces experienced a famine during 1918 (Mills, 1986). It was these provinces which also experienced the highest 1918 influenza mortality rates (Mills, 1986). Due to the unusual age distribution of the pandemic, those who succumbed to the disease were typically young adults who formed the majority of the agricultural labor force (Mills, 1986). The resultant labor shortage only served to exacerbate the severity of the influenza pandemic (Mills, 1986). The exact mechanisms by which malnutrition and famine increase the severity of influenza remain to be defined. However, experimental studies suggest that not only does malnutrition suppress the host's immune response to influenza virus, but that it may also facilitate the emergence of novel viral variants, which display increased pathogenicity relative to the original parental strain (Beck et al., 2004).

Undernutrition (often exacerbated by ongoing civil conflicts) remains a problem for influenza pandemics of the twenty-first century and beyond. Indeed, chronic malnutrition was thought to have contributed to the high morbidity and mortality seen in Guatemalan children during the 2009 influenza pandemic (Reyes et al., 2010). Climate change may result in crop failures and exacerbate any food shortages in the future. However, in any future influenza virus pandemic, we will face a "double burden" of malnutrition whereby a proportion of the world's population will experience severe disease because of undernutrition and a proportion of the world's population will experience severe disease because of overnutrition. Specifically, it is now well accepted that obesity increases one's risk of being hospitalized with, and dying from, an influenza virus infection (Morgan et al., 2010; Louie et al., 2011; Van Kerkhove et al., 2011). Perhaps of even greater concern is the fact that obesity inhibits both virus-specific $CD8^+$ T cell responses and antibody responses to the seasonal influenza vaccine (Sheridan et al., 2012). The challenge for future influenza pandemics is therefore not only to protect those affected by undernutrition (in particularly in light of the growing problem of climate change), but also the growing number of people living with obesity.

UNDERLYING INFECTIONS

Co-infection With Bacterial Pathogens

Historical autopsy reports and examination of lung tissue sections from 1918 to 19 influenza case material indicated that for a significant number of patients, the cause of death was not primary viral pneumonia (Brundage and Shanks, 2008; Morens et al., 2008; Chien et al., 2009). Instead, these individuals succumbed to a secondary bacterial infection, most commonly pneumonia caused by bacteria such as *Streptococcus pneumoniae, Haemophilus influenzae*, *Staphylococcus aureus,* and *Streptococcus pyogenes* (Morens et al., 2008). *H. influenzae* was so frequently observed in influenza patients that it was often cited as the cause of the pandemic (and was thus named accordingly) (Hildreth, 1991). The role of secondary bacterial infections during the 1918 pandemic is consistent with epidemiological observations that while influenza virus attack rates in 1918 were similar among soldiers and civilians, mortality rates were much higher amongst newly arrived soldiers (Shanks et al., 2016b). The unhygienic circumstances in the army camps led to frequent bacterial infections, especially amongst immunologically naïve new army recruits. Thus, following an influenza virus infection, new army recruits were more likely to develop a lethal secondary bacterial pneumonia than civilians or long-serving soldiers (Shanks et al., 2010, 2016b). These observations have been echoed by numerous experimental animal studies, showing that co-infection with influenza virus and bacterial pathogens results in increased disease severity compared to infection with either pathogen alone (Brightman, 1935; Glover, 1941; Francis and de Torregrosa, 1945; Harford et al., 1946; Wilson et al., 1947; Short et al., 2012a, 2013). Different mechanisms have been proposed to explain this viral-bacterial synergism (McCullers, 2006; McAuley et al., 2007; Smith et al., 2013; Hrincius et al., 2015). These include, but are not limited to, reduced mucociliary clearance of inhaled bacteria following influenza virus infection, bacterial adhesion to the basement membrane (Morens et al., 2008; Taubenberger et al., 2012; Chertow and Memoli, 2013) and/or sialic acids exposed by influenza virus (McCullers and Bartmess, 2003; Peltola et al., 2005), viral alterations to the host immune response (Navarini et al., 2006; van der Sluijs et al., 2006; Ballinger and Standiford, 2010; Nakamura et al., 2011; Ellis et al., 2015; Lee et al., 2015) and the bacterial inhibition of epithelial cell repair following initial damage by influenza virus infection (Kash et al., 2011). Importantly, experimental studies suggest that influenza viruses not only increases the severity of secondary bacterial infections, but that it also increases the transmission of *S. pneumoniae* (Diavatopoulos et al., 2010; Short et al., 2012b).

In addition to co-infections with bacterial pathogens such as *S. pneumoniae*, chronic bacterial infections, such as those with *Mycobacterium tuberculosis,* contributed to variations in influenza mortality during the 1918 pandemic. For example, data from a Swiss sanatorium during the 1918 pandemic suggested that the risk of influenza death was higher among tuberculosis (TB) patients than non-TB controls (Oei and Nishiura, 2012).

Similarly, individuals with TB were 2.2 times more likely to contract the 1918 influenza virus than non-TB individuals living in the same household (Noymer and Garenne, 2000; Noymer, 2011). A synergistic relationship between *M. tuberculosis* and influenza viruses has also been supported by experimental studies (Redford et al., 2014). The predominance of TB amongst young adults in 1918 may have contributed to the striking "W shaped" mortality curve associated with the 1918 influenza pandemic (Oei and Nishiura, 2012).

Severe complications and morbidity as a result of bacterial co-infections were not unique to the 1918 influenza pandemic. Rather, bacterial co-infections were also observed in the influenza pandemics of 1957, 1968 and 2009, albeit to a lesser extent than in 1918 (Oswald et al., 1958; Robertson et al., 1958; Louria et al., 1959; Oseasohn et al., 1959; Chertow and Memoli, 2013; Joseph et al., 2013). In the 2009 influenza pandemic, TB was also identified as a risk factor for the development of severe disease (Morales et al., 2017). Thankfully, unlike in 1918, the severity of bacterial infections during these more recent influenza pandemics was likely minimized by the use of antibiotics, advanced medical care and the availability of bacterial vaccines [such as the pneumococcal polysaccharide and *H. influenzae* type b (Hib) vaccine] (Oswald et al., 1958; Robertson et al., 1958; Louria et al., 1959; Madhi et al., 2004; Wahl et al., 2018). However, as the rate of antibiotic resistance continues to rise and as pathogens such as methicillin-resistant *S. aureus* (MRSA) (Memoli et al., 2008) and multidrug-resistant *M. tuberculosis* (Zumla et al., 2013; Millard et al., 2015) become more common, we potentially face a future where antibiotics will be ineffectual in the treatment of bacterial infections. This will have direct and severe implications for any future influenza virus pandemic (Memoli et al., 2008). It must be considered an urgent priority to not only minimize antibiotic resistance, but also to invest in the discovery of new antibiotics and alternative treatment options for bacterial infections.

Malaria

In addition to individuals with bacterial co-infections, mortality during the 1918 influenza pandemic was considerably higher amongst malaria-infected individuals (Langford and Storey, 1992; Afkhami, 2003; Shanks, 2015). Although the underlying mechanism is not fully understood, a malaria-induced procoagulant state could play a role in increasing inflammation and subsequent clinical outcome (Shanks, 2015).

Today, chemopreventive strategies have lowered the disease burden associated with malaria and new eradication strategies are being developed. However, malaria still causes significant worldwide morbidity and mortality, there is ever increasing drug resistance and new malaria vaccines have yet to provide long-lasting benefits at a population level (Ashley et al., 2018). Until effective control measures have been developed and implemented, areas endemic for malaria remain at high risk for increased mortality during the next influenza pandemic.

NON-PHARMACEUTICAL INTERVENTIONS

In 1918, a variety of different approaches were employed to limit the spread of influenza virus and treat infected patients. While many of these methods were of little or no avail, they contain important lessons for influenza pandemic preparedness in the twenty-first century.

Maritime Quarantine

In 1918, when the severity of the second wave of influenza became apparent, many countries imposed strict quarantine measures on all incoming ships to try and prevent the spread of influenza (Johnson, 2006). For the most part, these attempts were unsuccessful (Johnson, 2006). Quarantine measures were either implemented too late and the virus was already present within the country or quarantine was breached by infected individuals who were not yet symptomatic (Crosby, 1976; Tomkins, 1992). Thus, countries such as the U.K. and South Africa dismissed maritime quarantine as impractical and ineffectual (Blakely, 2006; Johnson, 2006). However, Australia imposed the maritime quarantine before any victims of the second wave were reported. All arriving vessels had to be cleared by Commonwealth Quarantine Officials before disembarking. This quarantine protected Australia from the second wave of the pandemic until December 1918 when the quarantine was finally breached. Maritime quarantine thus helped to protect Australia from the worst of the pandemic (Crosby, 2003; Johnson, 2006) and indirectly contributed to protecting certain Pacific Islands that depended on Australian supply ships (Shanks et al., 2018).

The most striking example of this was the mortality difference between American Samoa and Western Samoa. A strict maritime quarantine was imposed in American Samoa by the U.S. Governor in 1918 (Shanks and Brundage, 2013). This quarantine prevented influenza from entering the country, and no deaths from the 1918 influenza were ever recorded in America Samoa (Johnson, 2006; Shanks and Brundage, 2013). This was in sharp contrast to the nearby Western Samoa (located ∼100 km away), which did not practice strict maritime quarantine (Tomkins, 1992; Shanks and Brundage, 2013). As a result, Western Samoa was infected by the New Zealand supply ship, the *Talune*, and it is estimated that influenza killed more than a quarter of the population (Tomkins, 1992).

Global transportation has experienced a major transformation in the last century, with ships being replaced by the faster and more widely used air travel. The rise of commercial air travel helps explains the rapid global spread of the more modern influenza pandemics of 1957, 1968, and 2009 in the absence of major military troop movements (Rvachev and Longini, 1985; Hufnagel et al., 2004; Khan et al., 2009; Bajardi et al., 2011; Lemey et al., 2014). Accordingly, maritime quarantine is unlikely to play a role in limiting the spread of any future influenza pandemic. However, in 2009 authorities tried to limit the spread of influenza by using the modern-day equivalent of maritime quarantine: airport arrival screening. Unfortunately, analysis of arrival passengers at Sydney airport in 2009 suggested that airport screening had only a sensitivity of 6.67% for detecting influenza-infected patients, while costing ∼$50,000 AUD per case detected (Gunaratnam et al., 2014). This limited efficacy likely reflects the fact that individuals infected with influenza virus can be contagious prior to becoming symptomatic (Hollingsworth et al., 2006). Airport arrival screening is therefore unlikely to control the spread of influenza by international air travel. Rather,

advancing modeling (e.g., identifying which travel routes are most vulnerable to disease spread) and a variety of different education campaigns (e.g., raising awareness amongst the general public about the risks of traveling if they have been exposed to an infected individual) are likely to play a more significant role in future pandemic preparedness.

Mass Gatherings

In addition to limiting maritime travel, in 1918 most cities implemented simple non-pharmaceutical interventions to restrict the viral spread. These included imposing restrictions on social gatherings where person-to-person transmission could occur. As a result, schools, theaters, churches, and dance halls were closed, while mass gatherings such as weddings and funerals were banned in order to prevent overcrowding (Frost, 1919; Johnson, 2006; Bootsma and Ferguson, 2007; Hatchett et al., 2007). The peak death rate was lower in cities that rapidly implemented these non-pharmaceutical interventions within a few days after the first local cases were recorded, compared to those which waited a few weeks to respond (Bootsma and Ferguson, 2007; Hatchett et al., 2007). The timing when these interventions were lifted also affected the overall mortality (Bootsma and Ferguson, 2007; Hatchett et al., 2007). Thus, while restrictions on gatherings of people helped reduce influenza virus transmission, as soon as these restrictions were relaxed (typically within 2–8 weeks of their implementation) efficient viral transmission recommenced (Hatchett et al., 2007).

Following the outbreak of the 2009 pandemic influenza virus in Mexico, an 18-day period of mandatory school closure was implemented in the greater Mexico City area (Chowell et al., 2011). This was associated with a 29–37% reduction in influenza transmission (Chowell et al., 2011). Similarly, in Hong Kong there was approximately a 25% reduction influenza virus transmission following secondary schools closures from June 11 to July 10, 2009 (Wu et al., 2010). However, just as in 1918, the duration of these intervention strategies affected their efficacy, and there was a dramatic increase influenza activity in 32 Mexican states in the autumn of 2009, a period which coincided with schools opening for the autumn term (Chowell et al., 2011).

Facemasks and Hygiene

Facemasks were a popular preventative measure employed during the 1918 pandemic. While people were unsure of the etiological agent of the pandemic, the consensus was that it was an airborne disease and wearing a facemask would prevent infections (Crosby, 1976). Accordingly, many cities and regions, including Guatemala City, San Francisco, and certain prefectures of Japan, made wearing a facemask in public places obligatory, and special task forces and education campaigns were established to enforce this regulation (Crosby, 1976; Rice and Palmer, 1993; Rice, 2011). However, in order for a facemask to be at least partially effective against influenza virus it must be (i) worn at all times, (ii) properly made and fitted, and (iii) made of appropriate material. The surgical gauze masks of 1918 often failed to meet these criteria (Crosby, 1976). Thus, the mortality rate of Ontario, Canada (where wearing a mask was voluntary) was not significantly different from Alberta, Canada, (where mask wearing was enforced by law) (MacDougall, 2007). In fact, influenza deaths in Alberta continued to rise even after mask wearing was sanctioned by law, suggesting that in 1918 wearing a facemask was not sufficient to prevent deaths from influenza (World Health Organization Writing Group et al., 2006).

Proper hygiene (e.g., frequent hand washing) would also have helped limiting the spread of the influenza virus during the 1918 pandemic, as influenza viruses are transmissible via hand to face contact (World Health Organization Writing Group et al., 2006; Thomas et al., 2014). Thus, the Japanese traditional attitude to disease and illness might have contributed to a lower national pandemic mortality in 1918–19, as Japanese children are taught to remove their shoes and wash their hands upon re-entering the home (Rice and Palmer, 1993).

In the context of modern influenza pandemics, facemasks and handwashing/hand sanitizers have been used as preventative, non-pharmaceutical interventions. However, during the 2009 influenza pandemic for the most part, the use of facemasks was not obligatory (CDC, 2009). Rather, the CDC only recommended facemasks for individuals at increased risk of severe illness from influenza and/or individuals who were the direct careers of persons infected with the pandemic virus (CDC, 2009). Moreover, the effectiveness of facemasks in preventing the transmission of influenza virus remains unclear (Cowling et al., 2010) and just as was observed in 1918, low public compliance significantly limits the utility of facemasks in a modern pandemic setting (Cowling et al., 2010). Perhaps such interventions will be of greatest relevance to medical personal, who serve in the front line of a pandemic and are at high risk for infection. In contrast, handwashing and the use of hand sanitizers (whether or not in combination with wearing a facemask) had a clear protective effect during the influenza pandemic of 2009 (Larson et al., 2012; Suess et al., 2012; Wong et al., 2014).

These data suggest that non-pharmaceutical interventions such as social distancing, handwashing/hand sanitizers, and facemasks in any future influenza pandemic may buy valuable time before vaccines become widely available. However, the success of these interventions will depend upon their early and continuous implementation and also people's willingness to comply. The 1918 pandemic has shown that measures are most effective when they are voluntary, as people have low tolerance for mandatory health measures (Spinney, 2017). Indeed, a behavioral study showed that individuals were more likely to wear a facemask when they received autonomy-supportive advice as compared to controlled instructions (Chan et al., 2015).

MEDICAL INTERVENTIONS, THERAPIES AND VACCINES: THEN AND NOW

The 1918 influenza pandemic occurred during a period in history when controlling infectious diseases had become a realistic goal of the medical profession (Tomkins, 1992; Johnson, 2006). Public health initiatives had already proven successful in limiting the spread of diseases such as cholera and TB (Hildreth, 1991; Tomkins, 1992; Tognotti, 2003). Thus, there was initially little to suggest that an influenza outbreak could

not be effectively controlled (Hildreth, 1991; Tomkins, 1992; Tognotti, 2003). However, despite the dramatic advances in microbiology in the previous decades, the etiological agent of the 1918 influenza pandemic remained a mystery. In the absence of clear information about the causative agent of the pandemic, a range of different therapeutic and preventative treatments were attempted. People experimented with medications (including Asprin) and homemade remedies such as mustard poultice, quinine, tobacco, beef tea, the inhalation of zinc sulfate, opium, salt water, and alcohol (Rice and Palmer, 1993; Johnson, 2006; Starko, 2009; Keeling, 2010). Traditional eastern medicine, like the Japanese *Kanpo* medicine (consisting of herbal remedies accompanied by green tea) may have had some beneficial effect by stimulating perspiration (helping to reduce fever), improving vitamin C levels and replacing lost fluids (Palmer and Rice, 1992; Rice and Palmer, 1993). Similarly, the use of Traditional Chinese Medicine may have reduced the severity of influenza infections in at least some individuals (Kobayashi et al., 1999; Cheng and Leung, 2007; Chen et al., 2011). However, for the most part little was available in terms of effective therapeutic and/or prophylactic treatments. Nursing care actually proved to have contributed to the recovery of patients, especially those suffering from secondary bacterial pneumonia (Robinson, 1990; Rice and Palmer, 1993). In addition, mortality rates were significantly higher in places deprived from nursing care, e.g., mining compounds (Phimister, 1973; Rice and Palmer, 1993). Unfortunately, during the 1918–19 pandemic many of those who typically performed these duties were either serving overseas or were sick themselves (Crosby, 2003; Keeling, 2010; Shanks et al., 2011b).

Today, the identification of the etiological agent of influenza has dramatically improved diagnostic speed and accuracy. Rapid and highly accurate molecular diagnostic techniques have largely replaced the labor intensive and time consuming "gold standard" cell culture method for diagnosing influenza virus infections (Ellis and Zambon, 2002), which allows for rapid isolation of infected individuals. Furthermore, risk assessment of potentially pandemic viruses has greatly improved by screening the viral genome of human and animal virus isolates for the presence of mutations that increase human adaptation and/or virulence.

In addition, we are able to deploy both anti-viral drugs and vaccines in the case of an influenza virus pandemic. Antivirals (such as the neuraminidase inhibitors oseltamivir and zanamivir) can be used as a therapeutic in severely ill patients, while also being employed prophylactically in outbreak situations (Cooper et al., 2003; Hayden et al., 2004; De Clercq, 2006; Zambon, 2014; Krammer et al., 2018). At present, potentially pandemic influenza viruses (such as avian H7N9 and H5N1 viruses) are sensitive to both oseltamivir and zanamivir (Herfst et al., 2012). However, acquired resistance to oseltamivir has been observed in several H5N1 isolates (De Clercq, 2006). Similarly, oseltamivir resistance is known to emerge in H7N9 viruses within just 2 days from the start of treatment (Hay and Hayden, 2013). These data suggest that in the case of any future influenza virus pandemic, antivirals should be used judiciously, and the emergence of drug-resistant viral variants closely monitored.

Influenza virus vaccines have also played a major role in reducing the morbidity and mortality associated with seasonal influenza. Unfortunately, antibodies elicited by seasonal influenza vaccines do not provide protection in the case of an antigenically distinct influenza virus of a novel subtype, such as A/H5N1 or A/H7N9 (De Jong et al., 2000). Furthermore, current inactivated seasonal influenza vaccines may even prevent the induction of cross-reactive $CD8^+$ T cell responses, which are our primary protection in case of a pandemic outbreak and may therefore prove to be a double-edged sword (Bodewes et al., 2009a,b, 2011b,c). Rapid vaccine production also remains a challenge for future influenza virus pandemics (World Health Organization, 2005; Rockman and Brown, 2010; Pada and Tambyah, 2011). This was particularly apparent during the 2009 pandemic when sufficient amounts of the vaccine against the pandemic virus were only available in October 2009, well and truly after the pandemic had spread globally (Butler, 2010). Vaccine production in a pandemic scenario may be further complicated by the fact that some avian influenza viruses can kill the embryonated chicken eggs needed for vaccine production (Tumpey et al., 2005). Novel vaccines strategies, in combination with alternative vaccine production platforms are needed to accelerate vaccine production and circumvent such problems (Schotsaert and García-Sastre, 2014). However, an influenza vaccine that offers long-lasting, broad-spectrum immunity remains the gold standard for pandemic preparedness. How basic fundamental humoral and cellular biology and human clinical data can be considered for the implementation of a universal influenza vaccine has recently been reviewed (Clemens et al., 2018).

CONCLUDING REMARKS

It is estimated that if a pandemic influenza virus were to re-appear today, with a similar virulence and attack-rate as the 1918 influenza virus, mortality could rise to 21–147 million (Murray et al., 2006; Madhav, 2013). However, the high morbidity and mortality rates associated with the 1918 influenza pandemic resulted from a complex interplay between factors intrinsic to the 1918 virus itself, the host's immune response and the social context in which the pandemic struck. It is thus unlikely that this exact combination of factors would repeat itself in the future. Nevertheless, a comprehensive understanding of the factors that contributed to the severity of the 1918 pandemic plays an important role in our preparedness for the next influenza pandemic (**Figure 2**).

Today, we are better prepared for the next influenza virus pandemic than we were 100 years ago. Global influenza surveillance programs have been established to constantly monitor whether influenza viruses cross the species barrier into the human population (Hay and McCauley, 2018; World Health Organization, 2018a; Ziegler et al., 2018). This has already resulted in improved management strategies (Krammer et al., 2018) and the preventive slaughter of vast numbers of poultry that were infected with potentially pandemic viruses, such as H5N1 and H7N9 viruses (Oi, 2018). In addition, an improved understanding of the host-adaptation of influenza viruses and the existence of pre-existing immunity are likely to

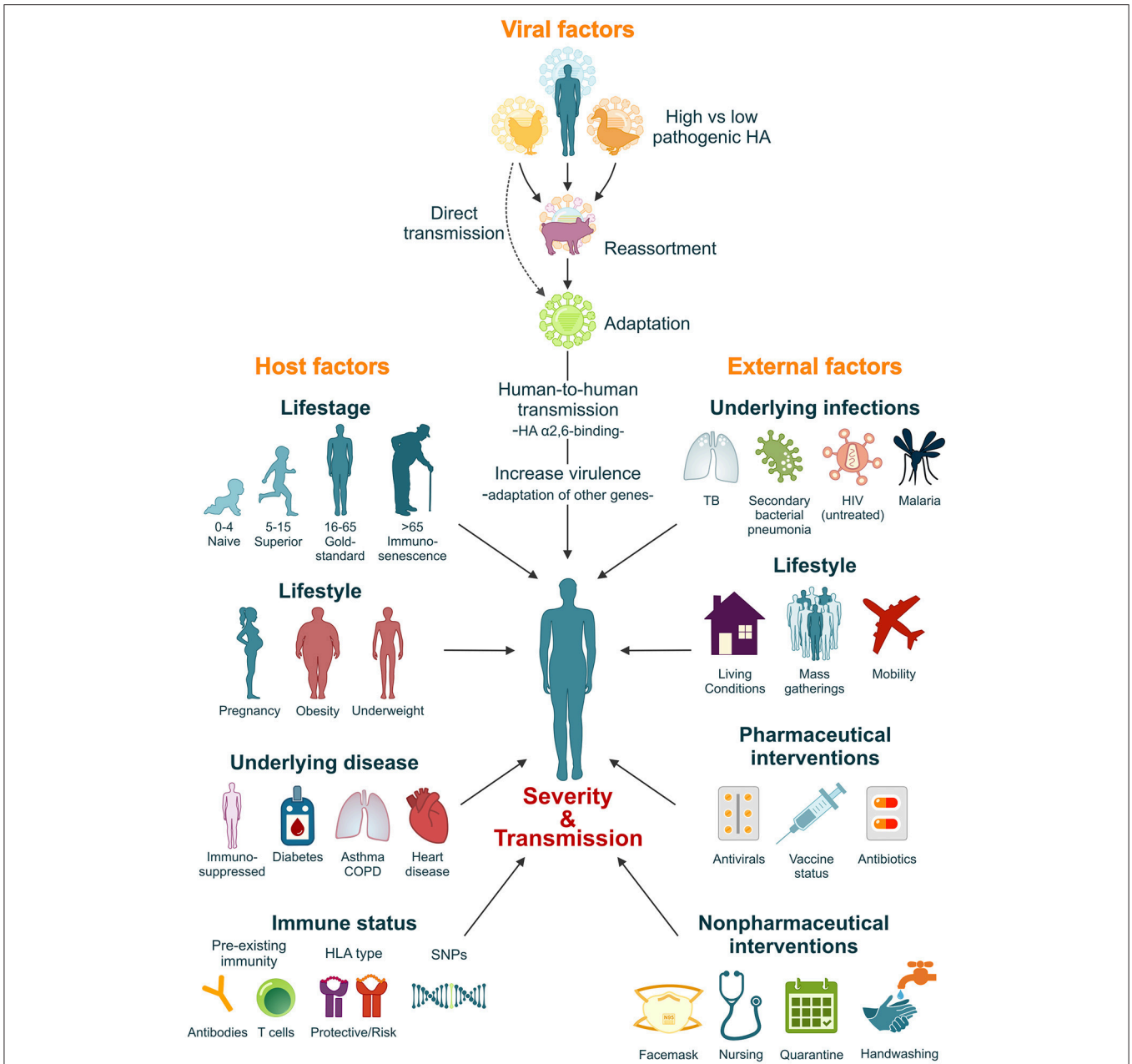


FIGURE 2 | Factors that influence the severity and transmissibility of a pandemic influenza virus. The severity and transmissibility of pandemic influenza viruses are the result of a complex interplay of viral, host, and external factors. We have come a long way since 1918 and pandemic preparedness programs have learned from the 1918 and later pandemic outbreaks. Although unlikely, we cannot exclude the possibility that an influenza pandemic with similar severity will repeat itself in the future. However, lessons learned from the 1918 influenza pandemic will ensure that we are better prepared.

contribute to a more accurate predication of viral severity even before the influenza virus in question becomes established as a pandemic (Kreijtz et al., 2008; Lee et al., 2008; Herfst et al., 2012; Imai et al., 2012; Richard et al., 2013; Quiñones-Parra et al., 2014; van de Sandt et al., 2014; Wang et al., 2015). A better understanding of the human immune response against (pandemic) influenza viruses will eventually aid the development of broad-protective influenza vaccines (Clemens et al., 2018). However, in the interim, the majority of countries have established a pandemic preparedness program, which defines the precautionary measurements needed to be taken in case of an emerging viral pandemic (van Genugten et al., 2001; RIVM, 2018; World Health Organization, 2018b). These programs include surveillance, diagnostics, screening of passengers traveling from a potential outbreak region, quarantine procedures, stockpiling antibiotics, antivirals, bacterial and viral vaccines and the

distribution of medical supplies (Brundage, 2006; Memoli et al., 2008; Mossad, 2009; World Health Organization, 2018b). We have also learnt from the 2009 H1N1 pandemic that it is important to have a somewhat flexible approach to pandemic preparedness, which allows countries to develop and implement their own risk assessments based on the global assessments provided by the WHO (Rudenko et al., 2015). However, good communication between countries and the WHO remains essential. In addition, it will be important for governments and those in authority to gain public trust before the next major pandemic outbreak. This will ensure that the public knows what to expect, how to act and is likely to improve compliance with preventative measures in a pandemic scenario. The internet represents a powerful and effective tool to disperse such information (Little et al., 2015).

Despite the advances that we have made in pandemic preparedness over the last 100 years, there are also several new challenges that we face in the context of twenty-first century (and later) influenza pandemics. Today's population demographic is dramatically different to that of 1918. Today, a large percentage of the world's population is either elderly (Morens et al., 2008; Mossad, 2009; Murray and Chotirmall, 2015) and/or living with one or more chronic medical conditions [such as heart disease, obesity, asthma, chronic obstructive pulmonary disease (COPD), and/or diabetes mellitus] (Morens et al., 2008; Jain et al., 2009; La Ruche et al., 2009; Flint et al., 2010; Hulme et al., 2017). The number of immunosuppressed individuals (due to untreated HIV infection, transplantation or/and chemotherapy) is also increasing (Jain et al., 2009; Kunisaki and Janoff, 2009; Sheth et al., 2011). This changing population demographic is of significance as each one of these host factors is known to increase the severity of even mild influenza virus infections. Mitigating the severity of future influenza pandemics will be further complicated by the prevalence of antibacterial resistance (Memoli et al., 2008), an increasing negative attitudes toward vaccination for other infectious diseases (such as measles and the pneumococcal polysaccharide and Hib vaccine) (Moss and Griffin, 2012; Perry et al., 2014) and an increase in seasonal influenza vaccinations of healthy individuals affecting the cross-reactive immune response otherwise induced by natural influenza virus infections (Bodewes et al., 2009b, 2011b). The high prevalence of underlying infections in less economically developed countries (such as TB and HIV), coupled with an underprepared health care system, places less economically developed countries at particularly high risk of severe morbidity during future influenza virus pandemics (Murray et al., 2006). These effects may even be compounded by the impacts of climate change which will lead to food shortages, famine and migration of climate refugees (van Schaik and Bakker, 2017).

At present, it is impossible to predict which influenza virus strain will cause the next pandemic. However, the growing number of human infections with the avian H7N9 virus represents a point of concern (especially in light of the approximate 40% mortality rate of this virus in humans). Like previous influenza pandemic viruses, human H7N9 virus infections have thus far displayed multiple waves of infections and shown signs of adaptation to human hosts (Zhu et al., 2017). Although this virus has yet to display efficient human-to-human transmission (Chen et al., 2013), it serves as a timely reminder that even though it has been 100 years since the 1918 pandemic, influenza pandemic preparedness remains of a paramount importance.

AUTHOR CONTRIBUTIONS

CvdS planned the review. KS, KK, and CvdS wrote the manuscript.

FUNDING

CvdS has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 792532. KS is supported by an Australian Research Council DECRA (DE180100512). KK is supported by a NHMRC Senior Research Fellowship Level B (GNT#1102792).

REFERENCES

Afkhami, A. (2003). Compromised constitutions: the Iranian experience with the 1918 influenza pandemic. *Bull. Hist. Med.* 77, 367–392. doi: 10.1353/bhm.2003.0049

Ahmed, R., Oldstone, M. B., and Palese, P. (2007). Protective immunity and susceptibility to infectious diseases: lessons from the 1918 influenza pandemic. *Nat. Immunol.* 8, 1188–1193. doi: 10.1038/ni1530

Alexander, J. B. (1919). Cases resembling encephalitis lethargica occurring during the influenza epidemic. *Br. Med. J.* 1, 794–795. doi: 10.1136/bmj.1.3052.794

Ashley, E. A., Pyae Phyo, A., and Woodrow, C. J. (2018). Malaria. *Lancet* 391, 1608–1621. doi: 10.1016/S0140-6736(18)30324-6

Audubon, (2018). *Audubon's Birds and Climate Change Report*. Available online at: http://climate.audubon.org (Accessed May 6, 2018).

Bajardi, P., Poletto, C., Ramasco, J. J., Tizzoni, M., Colizza, V., and Vespignani, A. (2011). Human mobility networks, travel restrictions, and the global spread of 2009 H1N1 pandemic. *PLoS ONE* 6:e16591. doi: 10.1371/journal.pone.0016591

Ballinger, M. N., and Standiford, T. J. (2010). Postinfluenza bacterial pneumonia: host defenses gone awry. *J. Interferon Cytokine Res.* 30, 643–652. doi: 10.1089/jir.2010.0049

Barber, D. L., Andrade, B. B., Sereti, I., and Sher, A. (2012). Immune reconstitution inflammatory syndrome: the trouble with immunity when you had none. *Nat. Rev. Microbiol.* 10, 150–156. doi: 10.1038/nrmicro2712

Barry, J. M. (2004). The site of origin of the 1918 influenza pandemic and its public health implications. *J. Transl. Med.* 2:3. doi: 10.1186/1479-5876-2-3

Barry, J. M., Viboud, C., and Simonsen, L. (2008). Cross-protection between successive waves of the 1918-1919 influenza pandemic: epidemiological evidence from US Army camps and from Britain. *J. Infect. Dis.* 198, 1427–1434. doi: 10.1086/592454

Beck, M. A., Handy, J., and Levander, O. A. (2004). Host nutritional status: the neglected virulence factor. *Trends Microbiol.* 12, 417–423. doi: 10.1016/j.tim.2004.07.007

Belser, J. A., Gustin, K. M., Pearce, M. B., Maines, T. R., Zeng, H., Pappas, C., et al. (2013). Pathogenesis and transmission of avian influenza A (H7N9) virus in ferrets and mice. *Nature* 501, 556–559. doi: 10.1038/nature12391

Beveridge, W. I. (1977). The start of pandemics: site, season and spread. *Dev. Biol. Stand.* 39, 443–444.

Blakely, D. E. (2006). *Mass Mediated Disease: A Case Study Analysis of Three Flu Pandemics and Public Health Policy*. Oxford: Lexington Books.

Bodewes, R., de Mutsert, G., van der Klis, F. R., Ventresca, M., Wilks, S., Smith, D. J., et al. (2011a). Prevalence of antibodies against seasonal influenza A and B viruses in children in Netherlands. *Clin. Vaccine Immunol.* 18, 469–476. doi: 10.1128/CVI.00396-10

Bodewes, R., Fraaij, P. L., Geelhoed-Mieras, M. M., van Baalen, C. A., Tiddens, H. A., and van Rossum, A. M. (2011b). Annual vaccination against influenza virus hampers development of virus-specific $CD8^+$ T cell immunity in children. *J. Virol.* 85, 11995–12000. doi: 10.1128/JVI.05213-11

Bodewes, R., Kreijtz, J. H., Baas, C., Geelhoed-Mieras, M. M., de Mutsert, G., van Amerongen, G. (2009a). Vaccination against human influenza A/H3N2 virus prevents the induction of heterosubtypic immunity against lethal infection with avian influenza A/H5N1 virus. *PLoS ONE* 4:e5538. doi: 10.1371/journal.pone.0005538

Bodewes, R., Kreijtz, J. H., Geelhoed-Mieras, M. M., van Amerongen, G., Verburgh, R. J., van Trierum, S. E., et al. (2011c). Vaccination against seasonal influenza A/H3N2 virus reduces the induction of heterosubtypic immunity against influenza A/H5N1 virus infection in ferrets. *J. Virol.* 85, 2695–2702. doi: 10.1128/JVI.02371-10

Bodewes, R., Kreijtz, J. H., and Rimmelzwaan, G. F. (2009b). Yearly influenza vaccinations: a double-edged sword? *Lancet Infect. Dis.* 9, 784–788. doi: 10.1016/S1473-3099(09)70263-4

Bootsma, M. C., and Ferguson, N. M. (2007). The effect of public health measures on the 1918 influenza pandemic in U.S. cities. *Proc. Natl. Acad. Sci U.S.A.* 104, 7588–7593. doi: 10.1073/pnas.0611071104

Brightman, I. J. (1935). Streptococcus infection occurring in ferrets inoculated with human influenza virus. *Yale J. Biol. Med.* 8, 127–135.

Brundage, J. F. (2006). Interactions between influenza and bacterial respiratory pathogens: implications for pandemic preparedness. *Lancet Infect. Dis.* 6, 303–312. doi: 10.1016/S1473-3099(06)70466-2

Brundage, J. F., and Shanks, G. D. (2008). Deaths from bacterial pneumonia during 1918-19 influenza pandemic. *Emerg. Infect. Dis.* 14, 1193–1199. doi: 10.3201/eid1408.071313

Butler, D. (2010). Portrait of a year-old pandemic. *Nature* 464, 1112–1113. doi: 10.1038/4641112a

CDC (2009). *Interim Recommendations for Facemask and Respirator Use to Reduce 2009 Influenza A (H1N1) Virus Transmission*. Available online at: https://www.cdc.gov/h1n1flu/masks.htm (Accessed May 10, 2018).

Chan, D. K., Yang, S. X., Mullan, B., Du, X., Zhang, X., Chatzisarantis, N. L., et al. (2015). Preventing the spread of H1N1 influenza infection during a pandemic: autonomy-supportive advice versus controlling instruction. *J. Behav. Med.* 38, 416–426. doi: 10.1007/s10865-014-9616-z

Chen, W., Lim, C. E., Kang, H. J., and Liu, J. (2011). Chinese herbal medicines for the treatment of type A H1N1 influenza: a systematic review of randomized controlled trials. *PLoS ONE* 6:e28093. doi: 10.1371/journal.pone.0028093

Chen, Y., Liang, W., Yang, S., Wu, N., Gao, H., Sheng, J., et al. (2013). Human infections with the emerging avian influenza A H7N9 virus from wet market poultry: clinical analysis and characterisation of viral genome. *Lancet* 381, 1916–1925. doi: 10.1016/S0140-6736(13)60903-4

Cheng, K. F., and Leung, P. C. (2007). What happened in China during the 1918 influenza pandemic? *Int. J. Infect. Dis.* 11, 360–364. doi: 10.1016/j.ijid.2006.07.009

Chertow, D. S., and Memoli, M. J. (2013). Bacterial coinfection in influenza: a grand rounds review. *J. Am. Med. Assoc.* 309, 275–282. doi: 10.1001/jama.2012.194139

Chien, Y. W., Klugman, K. P., and Morens, D. M. (2009). Bacterial pathogens and death during the 1918 influenza pandemic. *N. Engl. J. Med.* 361, 2582–2583. doi: 10.1056/NEJMc0908216

Chowell, G., Echevarría-Zuno, S., Viboud, C., Simonsen, L., Tamerius, J., Miller, M. A., et al. (2011). Characterizing the epidemiology of the 2009 influenza A/H1N1 pandemic in Mexico. *PLoS Med.* 8:e1000436. doi: 10.1371/journal.pmed.1000436

Ciancanelli, M. J., Huang, S. X., Luthra, P., Garner, H., Itan, Y., Volpi, S., et al. (2015). Infectious disease. Life-threatening influenza and impaired interferon amplification in human IRF7 deficiency. *Science* 348, 448–453. doi: 10.1126/science.aaa1578

Clemens, E. B., van de Sandt, C., Wong, S. S., Wakim, L. M., and Valkenburg, S. A. (2018). Harnessing the power of T cells, the promising hope for a Universal Influenza vaccine. *Vaccines* 6:E18. doi: 10.3390/vaccines6020018

Cliff, A. D., Haggett, P., and Graham, R. (1983). Reconstruction of diffusion at local scales: the 1846, 1882 and 1904 measles epidemics in northwest Iceland. *J. Hist. Geogr.* 9, 347–368. doi: 10.1016/0305-7488(83)90254-2

Cohen, M. L. (2000). Changing patterns of infectious disease. *Nature* 406, 762–767. doi: 10.1038/35021206

Collins, S. D. (1931). Age and sex incidence of influenza and pneumonia morbidity and mortality in the epidemic of 1928-29 with comparative data for the epidemic of 1918-19: based on surveys of families in certain localities in the united states following the epidemics. *Public Health Rep.* 46, 1909–1937. doi: 10.2307/4580139

Conenello, G. M., Zamarin, D., Perrone, L. A., Tumpey, T., and Palese, P. (2007). A single mutation in the PB1-F2 of H5N1 (HK/97) and 1918 influenza A viruses contributes to increased virulence. *PLoS Pathog.* 3, 1414–1421. doi: 10.1371/journal.ppat.0030141

Cooper, N. J., Sutton, A. J., Abrams, K. R., Wailoo, A., Turner, D., and Nicholson, K. G. (2003). Effectiveness of neuraminidase inhibitors in treatment and prevention of influenza A and B: systematic review and meta-analyses of randomised controlled trials. *BMJ* 326:1235. doi: 10.1136/bmj.326.7401.1235

Cowling, B. J., Zhou, Y., Ip, D. K., Leung, G. M., and Aiello, A. E. (2010). Face masks to prevent transmission of influenza virus: a systematic review. *Epidemiol. Infect.* 138, 449–456. doi: 10.1017/S0950268809991658

Crosby, A. W. (1976). *Epidemic and Peace 1918*. Santa Barbara, CA: Abc-Clio.

Crosby, A. W. (2003). *America's Forgotten Pandemic: the Influenza of 1918*. Cambridge: Cambridge University Press.

Dawood, F. S., Iuliano, A. D., Reed, C., Meltzer, M. I., Shay, D. K, Cheng, P. Y., et al. (2012). Estimated global mortality associated with the first 12 months of 2009 pandemic influenza A H1N1 virus circulation: a modelling study. *Lancet Infect. Dis.* 12, 687–695. doi: 10.1016/S1473-3099(12)70121-4

De Clercq, E. (2006). Antiviral agents active against influenza A viruses. *Nat. Rev. Drug Discov.* 5, 1015–1025. doi: 10.1038/nrd2175

De Jong, J. C., Rimmelzwaan, G. F., Fouchier, R. A., and Osterhaus, A. D. (2000). Influenza virus: a master of metamorphosis. *J. Infect.* 40, 218–228. doi: 10.1053/jinf.2000.0652

de Jong, M. D., Simmons, C. P., Thanh, T. T., Hien, V. M., Smith, G. J., Chau, T. N., et al. (2006). Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. *Nat. Med.* 12, 1203–1207. doi: 10.1038/nm1477

de Vries, R. D., McQuaid, S., van Amerongen, G., Yüksel, S., Verburgh, R. J., Osterhaus, A. D., et al., (2012). Measles immune suppression: lessons from the macaque model. *PLoS Pathog.* 8:e1002885. doi: 10.1371/journal.ppat.1002885

de Wit, E., Siegers, J. Y., Cronin, J. M., Weatherman, S., van den Brand, J. M., Leijten, L. M., et al. (2018). 1918 H1N1 influenza virus replicates and induces proinflammatory cytokine responses in extrarespiratory tissues of ferrets. *J. Infect. Dis.* 217, 1237–1246. doi: 10.1093/infdis/jiy003

Diavatopoulos, D. A., Short, K. R., Price, J. T., Wilksch, J. J., Brown, L. E., Briles, D. E., et al. (2010). Influenza A virus facilitates *Streptococcus pneumoniae* transmission and disease. *FASEB J.* 24, 1789–1798. doi: 10.1096/fj.09-146779

Dowdle, W. R. (1999). Influenza A virus recycling revisited. *Bull. World Health Organ.* 77, 820–828.

Duncan, C. J., Duncan, S. R., and Scott, S. (1997). The dynamics of measles epidemics. *Theor. Popul. Biol.* 52, 155–163. doi: 10.1006/tpbi.1997.1326

Dunham, E. J., Dugan, V. G., Kaser, E. K., Perkins, S. E., Brown, I. H., Holmes, E. C., et al., (2009). Different evolutionary trajectories of European avian-like and classical swine H1N1 influenza A viruses. *J. Virol.* 83, 5485–5494. doi: 10.1128/JVI.02565-08

Ellis, G. T., Davidson, S., Crotta, S., Branzk, N., Papayannopoulos, V., and Wack, A. (2015). $TRAIL^+$ monocytes and monocyte-related cells cause lung damage and thereby increase susceptibility to influenza-*Streptococcus pneumoniae* coinfection. *EMBO Rep.* 16, 1203–1218. doi: 10.15252/embr.201540473

Ellis, J. S., and Zambon, M. C. (2002). Molecular diagnosis of influenza. *Rev. Med. Virol.* 12, 375–389. doi: 10.1002/rmv.370

Epstein, S. L. (2006). Prior H1N1 influenza infection and susceptibility of cleveland family study participants during the H2N2 pandemic of 1957: an experiment of nature. *J. Infect. Dis.* 193, 49–53. doi: 10.1086/498980

Everitt, A. R., Clare, S., Pertel, T., John, S. P., Wash, R. S., Smith, S. E., et al. (2012). IFITM3 restricts the morbidity and mortality associated with influenza. *Nature* 484, 519–523. doi: 10.1038/nature10921

Flint, S. M., Davis, J. S., Su, J. Y., Oliver-Landry, E. P., Rogers, B. A., Goldstein, A., et al. (2010). Disproportionate impact of pandemic (H1N1) 2009 influenza on Indigenous people in the Top End of Australia's Northern Territory. *Med. J. Aust.* 192, 617–622.

Francis, T., and de Torregrosa, M. V. (1945). Combined infection of mice with influenzae, H. and influenza virus by the intranasal route. *J. Infect. Dis.* 76, 70–77.

Fraser, C., Cummings, D. A., Klinkenberg, D., Burke, D. S., and Ferguson, N. M. (2011). Influenza transmission in households during the 1918 pandemic. *Am. J. Epidemiol.* 174, 505–514. doi: 10.1093/aje/kwr122

Frost, W. H. (1919). Public health weekly reports for August 15, 1919. *Public Health Rep.* 34, 1823–1926. doi: 10.2307/4575271

Garten, R. J., Davis, C. T., Russell, C. A., Shu, B., Lindstrom, S., Balish, A., et al. (2009). Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. *Science* 325, 197–201. doi: 10.1126/science.1176225

Georgantopoulos, P., Bergquist, E. P., Knaup, R. C., Anthony, J. R., Bailey, T. C., Williams, M. P., et al., (2009). Importance of routine public health influenza surveillance: detection of an unusual W-shaped influenza morbidity curve. *Am. J. Epidemiol.* 170, 1533–1540. doi: 10.1093/aje/kwp305

Gibbon, J. (1919). Acquired immunity in influenza. *Lancet* 193:583. doi: 10.1016/S0140-6736(01)25706-7

Glaser, L., Stevens, J., Zamarin, D., Wilson, I. A., García-Sastre, A., Tumpey. T. M., et al. (2005). A single amino acid substitution in 1918 influenza virus hemagglutinin changes receptor binding specificity. *J. Virol.* 79:11533–11536. doi: 10.1128/JVI.79.17.11533-11536.2005

Glover, R. E. (1941). Spread of infection from the respiratory tract of the ferret. II. Association of influenza A virus and streptococcus type C. *Br. J. Exp. Pathol.* 22, 98–107.

Goronzy, J. J., and Weyand, C. M. (2013). Understanding immunosenescence to improve responses to vaccines. *Nat. Immunol.* 14, 428–436. doi: 10.1038/ni.2588

Gras, S., Kedzierski, L., Valkenburg, S. A., Laurie, K., Liu, Y. C., Denholm, J. T., et al. (2010). Cross-reactive CD8+ T-cell immunity between the pandemic H1N1-2009 and H1N1-1918 influenza A viruses. *Proc. Natl. Acad. Sci. U.S.A.* 107, 12599–12604. doi: 10.1073/pnas.1007270107

Griffin, D. E. (2010). Measles virus-induced suppression of immune responses. *Immunol. Rev.* 236, 176–189. doi: 10.1111/j.1600-065X.2010.00925.x

Gunaratnam, P. J., Tobin, S., Seale, H., Marich, A., and McAnulty, J. (2014). Airport arrivals screening during pandemic (H1N1) 2009 influenza in New South Wales, Australia. *Med. J. Aust.* 200, 290–292. doi: 10.5694/mja13.10832

Hammond, J. A. B., Rolland, W., and Shore, T. H. G. (1917). Purulent bronchiti. *Lancet* 190, 41–46. doi: 10.1016/S0140-6736(01)56229-7

Hancock, K., Veguilla, V., Lu, X., Zhong, W., Butler, E. N., Sun, H., et al. (2009). Cross-reactive antibody responses to the 2009 pandemic H1N1 influenza virus. *N. Engl. J. Med.* 361, 1945–1952. doi: 10.1056/NEJMoa0906453

Harford, C. G., Smith, M. R., and Wood, W. B. (1946). Sulfonamide chemotherapy of combined infection with influenza virus and bacteria. *J. Exp. Med.* 83, 505–518. doi: 10.1084/jem.83.6.505

Hartmann, B. M., Albrecht, R. A., Zaslavsky, E., Nudelman, G., Pincas, H., Marjanovic., N., et al. (2017). Pandemic H1N1 influenza A viruses suppress immunogenic RIPK3-driven dendritic cell death. *Nat. Commun.* 8:1931. doi: 10.1038/s41467-017-02035-9

Hatchett, R. J., Mecher, C. E., and Lipsitch, M. (2007). Public health interventions and epidemic intensity during the 1918 influenza pandemic. *Proc. Natl. Acad. Sci. U.S.A.* 104, 7582–7587. doi: 10.1073/pnas.0610941104

Hay, A. J., and Hayden, F. G. (2013). Oseltamivir resistance during treatment of H7N9 infection. *Lancet* 381, 2230–2232. doi: 10.1016/S0140-6736(13)61209-X

Hay, A. J., and McCauley, J. W. (2018). The WHO global influenza Surveillance and Response System (GISRS) - a future perspective. *Influenza Other Respir Viruses.* 12, 551–557 doi: 10.1111/irv.12565

Hayden, F. G., Belshe, R., Villanueva, C., Lanno, R., Hughes, C., Small, I., et al. (2004). Management of influenza in households: a prospective, randomized comparison of oseltamivir treatment with or without postexposure prophylaxis. *J. Infect. Dis.* 189, 440–449. doi: 10.1086/381128

Hayward, A. C., Wang, L., Goonetilleke, N., Fragaszy, E. B., Bermingham, A., Copas. A., et al. (2015). Natural T cell-mediated protection against seasonal and pandemic influenza. *Results of the Flu Watch Cohort Study. Am. J. Respir. Crit. Care Med*. 191, 1422–1431. doi: 10.1164/rccm.201411-1988OC

Herfst, S., Schrauwen, E. J., Linster, M., Chutinimitkul, S., de Wit, E., Munster, V. J., et al. (2012). Airborne transmission of influenza A/H5N1 virus between ferrets. *Science* 336, 1534–1541. doi: 10.1126/science.1213362

Hernandez, N., Melki, I., Jing, H., Habib, T., Huang, S. S. Y., Danielson, J., et al., (2018). Life-threatening influenza pneumonitis in a child with inherited IRF9 deficiency. *J Exp Med*. doi: 10.1084/jem.20180628. [Epub ahead of print]

Hildreth, M. L. (1991). The influenza epidemic of 1918-1919 in France: contemporary concepts of aetiology, therapy, and prevention. *Soc. Hist. Med.* 4, 277–294. doi: 10.1093/shm/4.2.277

Hirsch, H. H., Kaufmann, G., Sendi, P., and Battegay, M. (2004). Immune reconstitution in HIV-infected patients.. *Clin. Infect. Dis.* 38, 1159–1166. doi: 10.1086/383034

Hoffman, B. L. (2011). Influenza activity in Saint Joseph, Missouri 1910-1923: evidence for an early wave of the 1918 pandemic. *PLoS Curr.* 2:RRN1287. doi: 10.1371/currents.RRN1287

Hollingsworth, T. D., Ferguson, N. M., and Anderson, R. M. (2006). Will travel restrictions control the international spread of pandemic influenza? *Nat. Med.* 12, 497–499. doi: 10.1038/nm0506-497

Horimoto, T., and Kawaoka, Y. (1994). Reverse genetics provides direct evidence for a correlation of hemagglutinin cleavability and virulence of an avian influenza A virus. *J. Virol.* 68, 3120–3128.

Hrincius, E. R., Liedmann, S., Finkelstein, D., Vogel, P., Gansebom, S., Ehrhardt, C., et al. (2015). Nonstructural protein 1 (NS1)-mediated inhibition of c-Abl results in acute lung injury and priming for bacterial co-infections: insights into 1918 H1N1 pandemic? *J. Infect. Dis.* 211, 1418–1428. doi: 10.1093/infdis/jiu609

Hufnagel, L., Brockmann, D., and Geisel, T. (2004). Forecast and control of epidemics in a globalized world. *Proc. Natl. Acad. Sci. U.S.A.* 101, 15124–15129. doi: 10.1073/pnas.0308344101

Hulme, K. D., Gallo, L. A., and Short, K. R. (2017). Influenza virus and glycemic variability in diabetes: a killer combination? *Front. Microbiol.* 8:861. doi: 10.3389/fmicb.2017.00861

Ikonen, N., Strengell, M., Kinnunen, L., Osterlund, P., Pirhonen, J., Broman, M., et al. (2010). High frequency of cross-reacting antibodies against 2009 pandemic influenza A(H1N1) virus among the elderly in Finland. *Euro Surveill.* 15:19478. doi: 10.2807/ese.15.05.19478-en

Imai, M., Watanabe, T., Hatta, M., Das, S. C., Ozawa, M., Shinya, K., et al. (2012). Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. *Nature* 486, 420–428. doi: 10.1038/nature10831

Jagger, B. W., Memoli, M. J., Sheng, Z. M., Qi, L., Hrabal, R. J., Allen, G. L., et al. (2010). The PB2-E627K mutation attenuates viruses containing the 2009 H1N1 influenza pandemic polymerase. *MBio* 1:e00067-10. doi:10.1128/mBio.00067-10

Jain, S., Kamimoto, L., Bramley, A. M., Schmitz, A. M., Benoit, S. R., Louie, J., et al. (2009). Hospitalized patients with 2009 H1N1 influenza in the United States, April-June 2009. *N. Engl. J. Med.* 361, 1935–1944. doi: 10.1056/NEJMoa0906695

Johnson, N. (2006). *Britain and the 1918-19 Influenza Pandemic: A Dark Epilogue*. Abingdon: Taylor & Francis Ltd.

Johnson, N. P., and Mueller, J. (2002). Updating the accounts: global mortality of the 1918-1920 "Spanish" influenza pandemic. *Bull. Hist. Med.* 76, 105–115. doi: 10.1353/bhm.2002.0022

Joseph, C., Togawa, Y., and Shindo, N. (2013). Bacterial and viral infections associated with influenza. *Influenza Other Respir Viruses* 7(Suppl. 2), 105–113. doi: 10.1111/irv.12089

Kash, J. C., Tumpey, T. M., Proll, S. C., Carter, V., Perwitasari, O., Thomas, M. J., et al. (2006). Genomic analysis of increased host immune and cell death responses induced by 1918 influenza virus. *Nature* 443, 578–581. doi: 10.1038/nature05181

Kash, J. C., Walters, K. A., Davis, A. S., Sandouk, A., Schwartzman, L. M., Jagger, B.W., et al. (2011). Lethal synergism of 2009 pandemic H1N1 influenza virus and *Streptococcus pneumoniae* coinfection is associated with loss of murine lung repair responses. *MBio* 2:e00172–11. doi: 10.1128/mBio.00172-11

Kawaoka, Y., Krauss, S., and Webster, R. G. (1989). Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. *J. Virol.* 63, 4603–4608.

Kawaoka, Y., and Webster, R. G. (1988). Sequence requirements for cleavage activation of influenza virus hemagglutinin expressed in mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.* 85, 324–328. doi: 10.1073/pnas.85.2.324

Keeling, A. W. (2010). Alert to the necessities of the emergency: U.S. nursing during the 1918 influenza pandemic. *Publ. Health Rep*. 125(Suppl. 3), 105–12. doi: 10.1177/00333549101250S313

Khan, K., Arino, J., Hu, W., Raposo, P., Sears, J., Calderon, F., et al. (2009). Spread of a novel influenza A (H1N1) virus via global airline transportation. *N. Engl. J. Med.* 361, 212–214. doi: 10.1056/NEJMc0904559

Klaassen, M., Hoye, B. J., Nolet, B. A., and Buttemer, W. A. (2012). Ecophysiology of avian migration in the face of current global hazards. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 367, 1719–1732. doi: 10.1098/rstb.2012.0008

Kobasa, D., Jones, S. M., Shinya, K., Kash, J. C., Copps, J., Ebihara, H., et al. (2007). Aberrant innate immune response in lethal infection of macaques with the 1918 influenza virus. *Nature* 445, 319–323. doi: 10.1038/nature05495

Kobasa, D., Takada, A., Shinya, K., Hatta, M., Halfmann, P., Theriault, S., et al. (2004). Enhanced virulence of influenza A viruses with the haemagglutinin of the 1918 pandemic virus. *Nature* 431, 703–707. doi: 10.1038/nature02951

Kobayashi, M., Davis, S. M., Utsunomiya, T., Pollard, R. B., and Suzuki, F. (1999). Antiviral effect of gingyo-san, a traditional Chinese herbal medicine, on influenza A2 virus infection in mice. *Am. J. Chin. Med.* 27, 53–62. doi: 10.1142/S0192415X99000082

Krammer, F., Smith, G. J. D., Fouchier, R. A. M., Peiris, M., Kedzierska, K., Doherty, P. C., et al. (2018). Influenza. *Nat. Rev. Dis Prim.* 4:3. doi: 10.1038/s41572-018-0002-y

Kreijtz, J. H., de Mutsert, G., van Baalen, C. A., Fouchier, R. A., Osterhaus, A. D., and Rimmelzwaan, G. F. (2008). Cross-recognition of avian H5N1 influenza virus by human cytotoxic T-lymphocyte populations directed to human influenza A virus. *J. Virol.* 82, 5161–5166. doi: 10.1128/JVI.02694-07

Kunisaki, K. M., and Janoff, E. N. (2009). Influenza in immunosuppressed populations: a review of infection frequency, morbidity, mortality, and vaccine responses. *Lancet Infect. Dis.* 9, 493–504. doi: 10.1016/S1473-3099(09)70175-6

La Ruche, G., Tarantola, A., Barboza, P., Vaillant, L., Gueguen, J., Gastellu-Etchegorry, M., et al. (2009). The 2009 pandemic H1N1 influenza and indigenous populations of the Americas and the Pacific. *Euro Surveill.* 14:19366 doi: 10.2807/ese.14.42.19366-en

Langford, C. M., and Storey, P. (1992). Influenza in Sri Lanka, 1918–1919: the impact of a new disease in a premodern third world setting. *Health Transition Rev.* 2, 97–123.

Larson, E. L., Cohen, B., and Baxter, K. A. (2012). Analysis of alcohol-based hand sanitizer delivery systems: efficacy of foam, gel, and wipes against influenza A (H1N1) virus on hands. *Am. J. Infect. Control* 40, 806–809. doi: 10.1016/j.ajic.2011.10.016

Lee, B., Robinson, K. M., McHugh, K. J., Scheller, E. V., Mandalapu, S., Chen, C., et al. (2015). Influenza-induced type I interferon enhances susceptibility to gram-negative and gram-positive bacterial pneumonia in mice. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 309, L158–L167. doi: 10.1152/ajplung.00338.2014

Lee, L. Y., Ha do, L. A., Simmons, C., de Jong, M. D., Chau, N. V., Schumacher, R., et al. (2008). Memory T cells established by seasonal human influenza A infection cross-react with avian influenza A (H5N1) in healthy individuals. *J. Clin. Invest.* 118, 3478–3490. doi: 10.1172/JCI32460

Lemey, P., Rambaut, A., Bedford, T., Faria, N., Bielejec, F., Baele, G., et al. (2014). Unifying viral genetics and human transportation data to predict the global transmission dynamics of human influenza H3N2. *PLoS Pathog.* 10:e1003932. doi: 10.1371/journal.ppat.1003932

Little, P., Stuart, B., Hobbs, F. D., Moore, M., Barnett, J., Popoola, D., et al. (2015). An internet-delivered handwashing intervention to modify influenza-like illness and respiratory infection transmission (PRIMIT): a primary care randomised trial. *Lancet* 386, 1631–1639. doi: 10.1016/S0140-6736(15)60127-1

Liu, Y., Li, S., Zhang, G., Nie, G., Meng, Z., Mao, D., et al. (2013). Genetic variants in IL1A and IL1B contribute to the susceptibility to 2009 pandemic H1N1 influenza A virus. (2014). *BMC Immunol.* 14:37. doi: 10.1186/1471-2172-14-37

Louie, J. K., Acosta, M., Samuel, M. C., Schechter, R., Vugia, D. J., Harriman, K., et al. (2011). California Pandemic Working, A novel risk factor for a novel virus: obesity and 2009 pandemic influenza A (H1N1). *Clin. Infect. Dis.* 52, 301–312. doi: 10.1093/cid/ciq152

Louria, D. B., Blumenfeld, H. L., Ellis, J. T., Kilbourne, E. D., and Rogers, D. E. (1959). Studies on influenza in the pandemic of 1957–1958. II. Pulmonary complications of influenza. *J. Clin. Invest.* 38(1 Part 2), 213–265. doi: 10.1172/JCI103791

Luk, J., Gross, P., and Thompson, W. W. (2001). Observations on mortality during the 1918 influenza pandemic. *Clin. Infect. Dis.* 33, 1375–1378. doi: 10.1086/322662

MacDougall, H. (2007). Toronto's health department in action: influenza in 1918 and SARS in 2003. *J. Hist. Med. Allied Sci.* 62, 56–89 doi: 10.1093/jhmas/jrl042

Madhav, N. (2013). "Modeling a modern-day spanish flu pandemic," in *Aircurrents*, ed M. J. Markey. Available online at: www.air-worldwide.com/Publications/AIR-Currents/2013/Attachments/Modeling-a-Modern-Day-Spanish-Flu-Pandemic (Accessed May 10, 2018).

Madhi, S. A., Klugman, K. P., and Vaccine Trialist, G. (2004). A role for *Streptococcus pneumoniae* in virus-associated pneumonia. *Nat. Med.* 10, 811–813. doi: 10.1038/nm1077

Mamelund, S. E., Haneberg, B., and Mjaaland, S. (2016). A missed summer wave of the 1918-1919 influenza pandemic, evidence from household surveys in the United States and Norway. *Open Forum Infect. Dis.* 3:ofw040. doi: 10.1093/ofid/ofw040

Mathews, J. D., McBryde, E. S., McVernon, J., Pallaghy, P. K., and McCaw, J. M. (2010). Prior immunity helps to explain wave-like behaviour of pandemic influenza in 1918-9. *BMC Infect. Dis.* 10:128. doi:10.1186/1471-2334-10-128

Mazel-Sanchez, B., Boal Carvalho, I., Silva, F., Dijkman, R., and Schmolke, M. (2018). H5N1 influenza A virus PB1-F2 relieves HAX-1-mediated restriction of avian virus polymerase PA in human lung cells. *J. Virol.* 92. e00425–18 doi:10.1128/JVI.00425-18

McAuley, J. L., Hornung, F., Boyd, K. L., Smith, A. M., McKeon, R., Bennink, J., et al. (2007). Expression of the 1918 influenza A virus PB1-F2 enhances the pathogenesis of viral and secondary bacterial pneumonia. *Cell Host Microbe* 2, 240–249. doi: 10.1016/j.chom.2007.09.001

McCullers, J. A. (2006). Insights into the interaction between influenza virus and pneumococcus. *Clin. Microbiol. Rev.* 19, 571–582. doi: 10.1128/CMR.00058-05

McCullers, J. A., and Bartmess, K. C. (2003). Role of neuraminidase in lethal synergism between influenza virus and *Streptococcus pneumoniae*. *J. Infect. Dis.* 187, 1000–1009. doi: 10.1086/368163

McMichael, A. J., Gotch, F. M., Noble, G. R., and Beare, P. (1983). Cytotoxic T-cell immunity to influenza. *N. Engl. J. Med.* 309, 13–17. doi: 10.1056/NEJM198307073090103

Mehle, A., and Doudna, J. A. (2009). Adaptive strategies of the influenza virus polymerase for replication in humans. *Proc. Natl. Acad. Sci. U.S.A.* 106, 21312–21316. doi: 10.1073/pnas.0911915106

Mehle, A., Dugan, V. G., Taubenberger, J. K., and Doudna, J. A. (2012). Reassortment and mutation of the avian influenza virus polymerase PA subunit overcome species barriers. *J. Virol.* 86, 1750–1757. doi: 10.1128/JVI.06203-11

Memoli, M. J., Morens, D. M., and Taubenberger, J. K. (2008). Pandemic and seasonal influenza: therapeutic challenges. *Drug Discov. Today* 13, 590–595. doi: 10.1016/j.drudis.2008.03.024

Memoli, M. J., Tumpey, T. M., Jagger, B. W., Dugan, V. G., Sheng, Z. M., Qi, L., et al. (2009). An early 'classical' swine H1N1 influenza virus shows similar pathogenicity to the 1918 pandemic virus in ferrets and mice. *Virology* 393, 338–345. doi: 10.1016/j.virol.2009.08.021

Millard, J., Ugarte-Gil, C., and Moore, D. A. (2015). Multidrug resistant tuberculosis. *BMJ* 350:h882. doi: 10.1136/bmj.h882

Mills, I. D. (1986). The 1918-1919 influenza pandemic–the Indian experience. *Indian Econ. Soc. Hist. Rev.* 23, 1–40. doi: 10.1177/001946468602300102

Mina, M. J., Metcalf, C. J., de Swart, R. L., Osterhaus, A. D., and Grenfell, B. T. (2015). Long-term measles-induced immunomodulation increases overall childhood infectious disease mortality. *Science* 348, 694–699. doi: 10.1126/science.aaa3662

Monsalvo, A. C., Batalle, J. P., Lopez, M. F., Krause, J. C., Klemenc, J., Hernandez., J. Z., et al. (2011). Severe pandemic 2009 H1N1 influenza disease due to pathogenic immune complexes. *Nat. Med.* 17, 195–199. doi: 10.1038/nm.2262

Morales, K. F., Paget, J., and Spreeuwenberg, P. (2017). Possible explanations for why some countries were harder hit by the pandemic influenza virus in 2009 - a global mortality impact modeling study. *BMC Infect. Dis.* 17:642. doi: 10.1186/s12879-017-2730-0

Morens, D. M., and Fauci, A. S. (2007). The 1918 influenza pandemic: insights for the 21st century. *J. Infect. Dis.* 195, 1018–1028. doi: 10.1086/511989

Morens, D. M., and Taubenberger, J. K. (2015). A forgotten epidemic that changed medicine: measles in the US Army, 1917-18. *Lancet Infect. Dis.* 15, 852–861. doi: 10.1016/S1473-3099(15)00109-7

Morens, D. M., Taubenberger, J. K., and Fauci, A. S. (2008). Predominant role of bacterial pneumonia as a cause of death in pandemic influenza: implications for pandemic influenza preparedness. *J. Infect. Dis.* 198, 962–970. doi: 10.1086/591708

Morens, D. M., Taubenberger, J. K., and Fauci, A. S. (2009). The persistent legacy of the 1918 influenza virus. *N. Engl. J. Med.* 361, 225–229. doi: 10.1056/NEJMp0904819

Morgan, O. W., Bramley, A., Fowlkes, A., Freedman, D. S., Taylor, T. H., Gargiullo, P., et al. (2010). Morbid obesity as a risk factor for hospitalization and death due to 2009 pandemic influenza A(H1N1) disease. *PLoS ONE* 5:e9694. doi: 10.1371/journal.pone.0009694

Moss, W. J., and Griffin, D. E. (2012). Measles. *Lancet* 379, 153–164. doi: 10.1016/S0140-6736(10)62352-5

Moss, W. J., Ota, M. O., and Griffin, D. E. (2004). Measles: immune suppression and immune responses. *Int. J. Biochem. Cell Biol.* 36, 1380–1385. doi: 10.1016/j.biocel.2004.01.019

Mossad, S. B. (2009). Influenza in long-term care facilities: preventable, detectable, treatable. *Cleve. Clin. J. Med.* 76, 513–521. doi: 10.3949/ccjm.76a.09022

Murray, C. J., Lopez, A. D., Chin, B., Feehan, D., and Hill, K. (2006). Estimation of potential global pandemic influenza mortality on the basis of vital registry data from the 1918-20 pandemic: a quantitative analysis. *Lancet* 368, 2211–2218. doi: 10.1016/S0140-6736(06)69895-4

Murray, M. A., and Chotirmall, S. H. (2015). The Impact of Immunosenescence on pulmonary disease. *Mediators Inflamm.* 2015:692546. doi: 10.1155/2015/692546

Nakamura, S., Davis, K. M., and Weiser, J. N. (2011). Synergistic stimulation of type I interferons during influenza virus coinfection promotes *Streptococcus pneumoniae* colonization in mice. *J. Clin. Invest.* 121, 3657–3665. doi: 10.1172/JCI57762

National Center for Respiratory Diseases, CDC, and Centers for Disease and Prevention (CDC) (2009). Use of influenza A (H1N1) 2009 monovalent vaccine: recommendations of the Advisory Committee on Immunization Practices (ACIP), 2009. *MMWR Recomm Rep*. 58, 1–8.

Navarini, A. A., Recher, M., Lang, K. S., Georgiev, P., Meury, S., Bergthaler, A., et al. (2006). Increased susceptibility to bacterial superinfection as a consequence of innate antiviral responses. *Proc. Natl. Acad. Sci. U.S.A.* 103, 15535–15539. doi: 10.1073/pnas.0607325103

Noymer, A. (2011). The 1918 influenza pandemic hastened the decline of tuberculosis in the United States: an age, period, cohort analysis. *Vaccine* 29(Suppl. 2), B38–B41. doi: 10.1016/j.vaccine.2011.02.053

Noymer, A., and Garenne, M. (2000). The 1918 influenza epidemic's effects on sex differentials in mortality in the United States. *Popul. Dev. Rev.* 26, 565–581. doi: 10.1111/j.1728-4457.2000.00565.x

Oei, W., and Nishiura, H. (2012). The relationship between tuberculosis and influenza death during the influenza (H1N1) pandemic from 1918–19. *Comput. Math. Methods Med.* 2012:124861. doi: 10.1155/2012/124861

Oi, E. (2018). *Avian Influenza Portal, Update On Avian Influenza In Animals (types H5 and H7)*. World Organization for Animal Health. Available online at: http://www.oie.int/animal-health-in-the-world/update-on-avian-influenza/2018/ (Accessed May 12, 2018).

Olson, D. R., Simonsen, L., Edelson, P. J., and Morse, S. S. (2005). Epidemiological evidence of an early wave of the 1918 influenza pandemic in New York City. *Proc. Natl. Acad. Sci. U.S.A.* 102, 11059–11063. doi: 10.1073/pnas.0408290102

Oseasohn, R., Adelson, L., and Kaji, M. (1959). Clinicopathologic study of thirty-three fatal cases of Asian influenza. *N. Engl. J. Med.* 260, 509–518. doi: 10.1056/NEJM195903122601101

Oswald, N. C., Shooter, R. A., and Curwen, M. P. (1958). Pneumonia complicating Asian influenza. *Br. Med. J.* 2, 1305–1311. doi: 10.1136/bmj.2.5108.1305

Oxford, J. S., Lambkin, R., Sefton, A., Daniels, R., Elliot, A., Brown., R., et al. (2005). A hypothesis: the conjunction of soldiers, gas, pigs, ducks, geese and horses in northern France during the Great War provided the conditions for the emergence of the "Spanish" influenza pandemic of 1918-1919. *Vaccine* 23, 940–945. doi: 10.1016/j.vaccine.2004.06.035

Oxford, J. S., Sefton, A., Jackson, R., Innes, W., Daniels, R. S., Johnson, N. P. (2002). World War I may have allowed the emergence of "Spanish" influenza. *Lancet Infect. Dis.* 2, 111–114. doi: 10.1016/S1473-3099(02)00185-8

Oxford, J. S., Sefton, A., Jackson, R., Johnson, N. P, Daniels, R. S. (1999). Who's that lady? *Nat. Med.* 5, 1351–1352.

Pada, S., and Tambyah, P. A. (2011). Overview/reflections on the 2009 H1N1 pandemic. *Microbes Infect.* 13, 470–478. doi: 10.1016/j.micinf.2011.01.009

Palmer, E., and Rice, G. W. (1992). A Japanese physician's response to pandemic influenza, Ijiro Gomibuchi and the "Spanish flu" in Yaita-Cho, 1918-1919. *Bull. Hist. Med.* 66, 560–577.

Pappas, C., Aguilar, P. V., Basler, C. F., Solórzano, A., Zeng, H., Perrone, L. A., et al. (2008). Single gene reassortants identify a critical role for PB1, HA, and NA in the high virulence of the 1918 pandemic influenza virus. *Proc. Natl. Acad. Sci. U. S. A.* 105, 3064–3069. doi: 10.1073/pnas.0711815105

Patterson, K. D., and Pyle, G. F. (1991). The geography and mortality of the 1918 influenza pandemic. *Bull. Hist. Med.* 65, 4–21.

Peltola, V. T., Murti, K. G., and McCullers, J.. A. (2005). Influenza virus neuraminidase contributes to secondary bacterial pneumonia. *J. Infect. Dis.* 192, 249–257. doi: 10.1086/430954

Perry, R. T., Gacic-Dobo, M., Dabbagh, A., Mulders, M. N., Strebel, P. M., Okwo-Bele, J. M., et al., (2014). Centers for disease and prevention, global control and regional elimination of measles, 2000-2012. *MMWR Morb. Mortal. Wkly. Rep.* 63, 103–107.

Phimister, I. R. (1973). The "Spanish" influenza pandemic of 1918 and its impact on the Southern Rhodesian mining industry. *Cent. Afr. J. Med.* 19, 143–148.

Quiñones-Parra, S., Grant, E., Loh, L., Nguyen, T. H., Campbell, K. A., Tong S. Y., et al., (2014). Preexisting $CD8^+$ T-cell immunity to the H7N9 influenza A virus varies across ethnicities. *Proc Natl Acad Sci U.S.A.* 111, 1049–1054 doi: 10.1073/pnas.1322229111

Ravenholt, R. T., and Foege, W. H. (1982). 1918 influenza, encephalitis lethargica, parkinsonism. *Lancet* 2, 860–864. doi: 10.1016/S0140-6736(82)90820-0

Redford, P. S., Mayer-Barber, K. D., McNab, F. W., Stavropoulos, E., Wack, A., Sher, A., et al. (2014). Influenza A virus impairs control of *Mycobacterium tuberculosis* coinfection through a type I interferon receptor-dependent pathway. *J. Infect. Dis.* 209, 270–274. doi: 10.1093/infdis/jit424

Reed, C., and Katz, J. M. (2010). Serological surveys for 2009 pandemic influenza A H1N1. *Lancet* 375, 1062–1063. doi: 10.1016/S0140-6736(09)62194-2

Reid, A. H., Fanning, T. G., Hultin, J. V., and Taubenberger, J. K. (1999). Origin and evolution of the 1918 "Spanish" influenza virus hemagglutinin gene. *Proc. Natl. Acad. Sci. U.S.A.* 96, 1651–1656. doi: 10.1073/pnas.96.4.1651

Reid, A. H., Fanning, T. G., Janczewski, T. A., Lourens, R. M., and Taubenberger, J. K. (2004a). Novel origin of the 1918 pandemic influenza virus nucleoprotein gene. *J. Virol.* 78, 12462–12470. doi: 10.1128/JVI.78.22.12462-12470.2004

Reid, A. H., Janczewski, T. A., Lourens, R. M., Elliot, A. J., Daniels, R. S., Berry, C. L., et al. (2003). 1918 influenza pandemic caused by highly conserved viruses with two receptor-binding variants. *Emerging Infect. Dis.* 9, 1249–1253. doi: 10.3201/eid0910.020789

Reid, A. H., Taubenberger, J. K., and Fanning, T. G. (2004b). Evidence of an absence: the genetic origins of the 1918 pandemic influenza virus. *Nat. Rev. Microbiol.* 2, 909–914. doi: 10.1038/nrmicro1027

Reyes, L., Arvelo, W., Estevez, A., Gray, J., Moir, J. C., Gordillo, B., et al. (2010). Population-based surveillance for 2009 pandemic influenza A (H1N1) virus in Guatemala, 2009. *Influenza Other Respir. Viruses* 4, 129–140. doi: 10.1111/j.1750-2659.2010.00138.x

Rice, G. W. (2011). "Japan and New Zealand in the 1918 influenza pandemic: comparative perspectives on official responses and crisis management," in *Spanish Influenza Pandemic of 1918–1919: New Perspectives,* ed D. Killingray (Melbourne, VIC: Routledge).

Rice, G. W., and Palmer, E. (1993). Pandemic influenza in Japan, 1918–19, mortality patterns and official responses. *J. Japan. Stud.* 19, 389–420. doi: 10.2307/132645

Richard, M., Schrauwen, E. J., de Graaf, M., Bestebroer, T. M., Spronken, M. I., van Boheemen, S., et al. (2013). Limited airborne transmission of H7N9 influenza A virus between ferrets. *Nature* 501, 560–563. doi: 10.1038/nature12476

RIVM (2018). *De Lci-Richtlijnen, Stappenplannen En Draaiboeken Zijn Er Voor En Door Professionals in De Infectieziektebestrijding, Influenza*. RIVM. Available online at: https://lci.rivm.nl/richtlijnen/influenza (Accessed May 05, 2018).

Robertson, L., Caley, J. P., and Moore, J. (1958). Importance of *Staphylococcus aureus* in pneumonia in the 1957 epidemic of influenza A. *Lancet* 2, 233–236. doi: 10.1016/S0140-6736(58)90060-6

Robinson, K. R. (1990). The role of nursing in the influenza epidemic of 1918–1919. *Nurs. Forum* 25, 19–26. doi: 10.1111/j.1744-6198.1990.tb00845.x

Rockman, S., and Brown, L. (2010). Pre-pandemic and pandemic influenza vaccines. *Hum. Vaccin.* 6, 792–801. doi: 10.4161/hv.6.10.12915

Rudenko, L., Sellwood, C., Russell, C., Herfst, S., Gross, D., and Dingwall, R. (2015). Will there ever be a new influenza pandemic and are we prepared? *Vaccine* 33, 7037–7040. doi: 10.1016/j.vaccine.2015.08.045

Rvachev, L. A., and Longini, I. M. (1985). A mathematical-model for the global spread of influenza. *Math. Biosci.* 75, 3–22. doi: 10.1016/0025-5564(85)90064-1

Sauerbrei, A., Langenhan, T., Brandstadt, A., Schmidt-Ott, R., Krumbholz, A., Girschick, H., et al. (2014). Prevalence of antibodies against influenza A and B viruses in children in Germany, 2008 to 2010. *Euro Surveill.* 19.

Schäfer, J. R., Kawaoka, Y., Bean, W. J., Süss, J., Senne, D., Webster, R. G. (1993). Origin of the pandemic 1957 H2 influenza A virus and the persistence of its possible progenitors in the avian reservoir. *Virology* 194, 781–788. doi: 10.1006/viro.1993.1319

Scholtissek, C., Rohde, W., Von Hoyningen, V., and Rott, R. (1978). On the origin of the human influenza virus subtypes H2N2 and H3N2. *Virology* 87, 13–20. doi: 10.1016/0042-6822(78)90153-8

Schotsaert, M., and García-Sastre, A. (2014). Influenza vaccines: a moving interdisciplinary field. *Viruses* 6, 3809–3826. doi: 10.3390/v6103809

Schrauwen, E. J., Herfst, S., Leijten, L. M., van Run, P., Bestebroer, T. M., Linster, M. et al. (2012). The multibasic cleavage site in H5N1 virus is critical for systemic spread along the olfactory and hematogenous routes in ferrets. *J. Virol.* 86, 3975–3984. doi: 10.1128/JVI.06828-11

Shaman, J., and Lipsitch, M. (2013). The El Nino-Southern Oscillation (ENSO)-pandemic influenza connection: coincident or causal? *Proc. Natl. Acad. Sci. U.S.A.* 110(Suppl. 1), 3689–3691. doi: 10.1073/pnas.1107485109

Shanks, G. D. (2015). Synergistic mortality caused by *Plasmodium falciparum* during the 1918 influenza pandemic. *Am. J. Trop. Med. Hyg.* 92, 941–942. doi: 10.4269/ajtmh.14-0792

Shanks, G. D., and Brundage, J. F. (2012). Pathogenic responses among young adults during the 1918 influenza pandemic. *Emerging Infect. Dis.* 18, 201–207. doi: 10.3201/eid1802.102042

Shanks, G. D., and Brundage, J. F. (2013). Pacific islands which escaped the 1918–1919 influenza pandemic and their subsequent mortality experiences. *Epidemiol. Infect.* 141, 353–356. doi: 10.1017/S0950268812000866

Shanks, G. D., Burroughs, S., Sohn, J. D., Waters, N. C., Smith, V. F., Waller, M., et al. (2016b). Variable mortality from the 1918–1919 influenza pandemic during military training. *Mil. Med.* 181, 878–882. doi: 10.7205/MILMED-D-15-00124

Shanks, G. D., Burroughs, S. A., Sohn, J. D., Waters, N. C., Smith, V. F., Waller, M., et al. (2016a). Enhanced risk of illness during the 1918 influenza pandemic after previous influenza-like illnesses in three military populations. *Epidemiol. Infect.* 144, 2043–2048. doi: 10.1017/S0950268816000479

Shanks, G. D., Hu, Z., Waller, M., Lee, S. E., Terfa, D., Howard, A., et al. (2014). Measles epidemics of variable lethality in the early 20th century. *Am. J. Epidemiol.* 179, 413–422. doi: 10.1093/aje/kwt282

Shanks, G. D., Lee, S. E., Howard, A., and Brundage, J. F. (2011a). Extreme mortality after first introduction of measles virus to the polynesian island of Rotuma, 1911. *Am. J. Epidemiol.* 173, 1211–1222. doi: 10.1093/aje/kwq504

Shanks, G. D., Mackenzie, A., McLaughlin, R., Waller, M., Dennis, P., Lee, S. E., et al. (2010). Mortality risk factors during the 1918-1919 influenza pandemic in the Australian army. *J. Infect. Dis.* 201, 1880–1889. doi: 10.1086/652868

Shanks, G. D., MacKenzie, A., Waller, M., and Brundage, J. F. (2011b). Low but highly variable mortality among nurses and physicians during the influenza pandemic of 1918-1919. *Influenza Other Respir. Viruses* 5, 213–219. doi: 10.1111/j.1750-2659.2010.00195.x

Shanks, G. D., Wilson, N., Kippen, R., and Brundage, J. F. (2018). The unusually diverse mortality patterns in the Pacific region during the 1918–21 influenza pandemic: reflections at the pandemic's centenary. *Lancet Infect Dis.* doi: 10.1016/S1473-3099(18)30178-6. [Epub ahead of print].

Sheng, Z. M., Chertow, D. S., Ambroggio, X., McCall, S., Przygodzki, R. M., Cunningham, R. E., et al., (2011). Autopsy series of 68 cases dying before and during the 1918 influenza pandemic peak. *Proc. Natl. Acad. Sci. U.S.A.* 108, 16416–16421. doi: 10.1073/pnas.1111179108

Sheridan, P. A., Paich, H. A., Handy, J., Karlsson, E. A., Hudgens, M. G., Sammon, A. B., et al. (2012). Obesity is associated with impaired immune response to influenza vaccination in humans. *Int. J. Obes.* 36, 1072–1077. doi: 10.1038/ijo.2011.208

Sheth, A. N., Patel, P., and Peters, P. J. (2011). Influenza and HIV: lessons from the 2009 H1N1 influenza pandemic. *Curr. HIV/AIDS Rep.* 8, 181–191. doi: 10.1007/s11904-011-0086-4

Shope, R. E. (1958). Influenza: history, epidemiology, and speculation. *Public Health Rep.* 73, 165–178. doi: 10.2307/4590072

Short, K. R., Habets, M. N., Hermans, P. W., and Diavatopoulos, D. A. (2012a). Interactions between *Streptococcus pneumoniae* and influenza virus: a mutually beneficial relationship? *Future Microbiol.* 7, 609–624. doi: 10.2217/fmb.12.29

Short, K. R., Reading, P. C., Brown, L. E., Pedersen, J., Gilbertson, B., Job, E. R., et al. (2013). Influenza-induced inflammation drives pneumococcal otitis media. *Infect. Immun.* 81, 645–652. doi: 10.1128/IAI.01278-12

Short, K. R., Reading, P. C., Wang, N., Diavatopoulos, D. A., and Wijburg, O. L. (2012b). Increased nasopharyngeal bacterial titers and local inflammation facilitate transmission of *Streptococcus pneumoniae*. *MBio* 3:e00255-12. doi: 10.1128/mBio.00255-12

Shulman, S. T., Shulman, D. L., and Sims, R. H. (2009). The tragic 1824 journey of the Hawaiian king and queen to London: history of measles in Hawaii. *Pediatr. Infect. Dis. J.* 28, 728–733. doi: 10.1097/INF.0b013e31819c9720

Simonsen, L., Chowell, G., Andreasen, V., Gaffey, R., Barry, J., Olson, D., et al. (2018). A review of the 1918 herald pandemic wave: importance for contemporary pandemic response strategies. *Ann. Epidemiol.* 28, 281–288. doi: 10.1016/j.annepidem.2018.02.013

Simonsen, L., Clarke, M. J., Schonberger, L. B., Arden, N. H., Cox, N. J., and Fukuda, K., (1998). Pandemic versus epidemic influenza mortality: a pattern of changing age distribution. *J. Infect. Dis.* 178, 53–60. doi: 10.1086/515616

Slepushkin, A. N. (1959). The effect of a previous attack of A1 influenza on susceptibility to A2 virus during the 1957 outbreak. *Bull. World Health Organ.* 20, 297–301.

Smith, A. M., Adler, F. R., Ribeiro, R. M., Gutenkunst, R. N., McAuley, J. L., McCullers, J.A., et al. (2013). Kinetics of coinfection with influenza A virus and *Streptococcus pneumoniae*. *PLoS Pathog.* 9:e1003238. doi: 10.1371/journal.ppat.1003238

Smith, G. J., Bahl, J., Vijaykrishna, D., Zhang, J., Poon, L. L., Chen, H., et al. (2009a). Dating the emergence of pandemic influenza viruses. *Proc. Natl. Acad. Sci. U.S.A.* 106, 11709–11712. doi: 10.1073/pnas.0904991106

Smith, G. J., Vijaykrishna, D., Bahl, J., Lycett, S. J., Worobey, M., Pybus, O. G., et al. (2009b). Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. *Nature* 459, 1122–1125. doi: 10.1038/nature08182

Smith, W., Andrewes, C. H., and Laidlaw, P. P. (1933). A virus obtained from influenza patients. *Lancet* 222, 66–68. doi: 10.1016/S0140-6736(00)78541-2

Spinney, L. (2017). *Pale Rider: The Spanish Flu of 1918 and How It Changed the World.* New York, NY: Public Affairs.

Sridhar, S., Begom, S., Bermingham, A., Hoschler, K., Adamson, W., Carman, W., et al. (2013). Cellular immune correlates of protection against symptomatic pandemic influenza. *Nat. Med.* 19, 1305–1312. doi: 10.1038/nm.3350

Starko, K. M. (2009). Salicylates and pandemic influenza mortality, 1918–1919 pharmacology, pathology, and historic evidence. *Clin. Infect. Dis.* 49, 1405–1410. doi: 10.1086/606060

Subbarao, E. K., London, W., and Murphy, B. R. (1993). A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. *J. Virol.* 67, 1761–1764.

Subbarao, K., Klimov, A., Katz, J., Regnery, H., Lim, W., Hall, H., et al. (1998). Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. *Science* 279, 393–396. doi: 10.1126/science.279.5349.393

Suess, T., Remschmidt, C., Schink, S. B., Schweiger, B., Nitsche, A., Schroeder, K., et al. (2012). The role of facemasks and hand hygiene in the prevention of influenza transmission in households: results from a cluster randomised trial; Berlin, Germany, 2009–2011. *BMC Infect. Dis.* 12:26. doi: 10.1186/1471-2334-12-26

Suguitan, A. L., Jr., Matsuoka, Y., Lau, Y. F., Santos, C. P., Vogel, L., Cheng., L. I., et al. (2012). The multibasic cleavage site of the hemagglutinin of highly pathogenic A/Vietnam/1203/2004 (H5N1) avian influenza virus acts as a virulence factor in a host-specific manner in mammals. *J. Virol.* 86, 2706–2714. doi: 10.1128/JVI.05546-11

Taubenberger, J. K., Baltimore, D., Doherty, P. C., Markel, H., Morens, D. M., Webster, R. G., et al. (2012). Reconstruction of the 1918 influenza virus: unexpected rewards from the past. *MBio* 3:e00201-12. doi: 10.1128/mBio.00201-12

Taubenberger, J. K., and Morens, D. M. (2006). 1918 Influenza: the mother of all pandemics. *Emerging Infect. Dis.* 12, 15–22. doi: 10.3201/eid1209.05-0979

Taubenberger, J. K., Reid, A. H., Janczewski, T. A., and Fanning, T. G. (2001). Integrating historical, clinical and molecular genetic data in order to explain the origin and virulence of the 1918 Spanish influenza virus. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 356, 1829–1839. doi: 10.1098/rstb.2001.1020

Taubenberger, J. K., Reid, A. H., Krafft, A. E., Bijwaard, K. E., and Fanning, T.. G. (1997). Initial genetic characterization of the 1918 "Spanish" influenza virus. *Science* 275, 1793–1796. doi: 10.1126/science.275.5307.1793

Taubenberger, J. K., Reid, A. H., Lourens, R. M., Wang, R., Jin, G., and Fanning, T. G. (2005). Characterization of the 1918 influenza virus polymerase genes. *Nature* 437, 889–893. doi: 10.1038/nature04230

Thomas, Y., Boquete-Suter, P., Koch, D., Pittet, D., and Kaiser, L. (2014). Survival of influenza virus on human fingers. *Clin. Microbiol. Infect.* 20, O58–O64. doi: 10.1111/1469-0691.12324

To, K. K., Hung, I. F., Li, I. W., Lee, K. L., Koo, C. K., Yan, W. W., et al. (2010). Delayed clearance of viral load and marked cytokine activation in severe cases of pandemic H1N1 2009 influenza virus infection. *Clin. Infect. Dis.* 50, 850–859. doi: 10.1086/650581

To, K. K. W., Zhou, J., Song, Y. Q., Hung, I. F. N., Ip, W. C. T., Cheng, Z. S., et al. (2014). Surfactant protein B gene polymorphism is associated with severe influenza. *Chest* 145, 1237–1243. doi: 10.1378/chest.13-1651

Tognotti, E. (2003). Scientific triumphalism and learning from facts: bacteriology and the "Spanish flu" challenge of 1918. *Soc. Hist. Med.* 16, 97–110. doi: 10.1093/shm/16.1.97

Tomkins, S. M. (1992). The influenza epidemic of 1918–19 in Western Samoa. *J. Pac. Hist.* 27, 181–197. doi: 10.1080/00223349208572706

Tumpey, T. M., Basler, C. F., Aguilar, P. V., Zeng, H., Solórzano, A., Swayne., D. E., et al. (2005). Characterization of the reconstructed 1918 Spanish influenza pandemic virus. *Science* 310, 77–80. doi: 10.1126/science.1119392

Tumpey, T. M., Maines, T. R., Van Hoeven, N., Glaser, L., Solorzano, A., Pappas, C. et al. (2007). A two-amino acid change in the hemagglutinin of the 1918 influenza virus abolishes transmission. *Science* 315, 655–659. doi: 10.1126/science.1136212

Valleron, A. J., Cori, A., Valtat, S., Meurisse, S., Carrat, F., and Boëlle, P. Y. (2010). Transmissibility and geographic spread of the 1889 influenza pandemic. *Proc. Natl. Acad. Sci. U.S.A.* 107, 8778–8781. doi: 10.1073/pnas.1000886107

van de Sandt, C. E, Sagong, K. A., Pronk, M. R., Bestebroer, T. M., Spronken, M. I., Koopmans, M. P. G., et al. (2018b). H1N1pdm09 influenza virus and its descendants lack extra-epitopic amino acid residues associated with reduced recognition by M158-66-specific CD8$^+$ T-cells. *J Infect Dis.* 218, 581–585. doi: 10.1093/infdis/jiy218

van de Sandt, C. E., Hillaire, M. L., Geelhoed-Mieras, M. M., Osterhaus, A. D., Fouchier, R. A., and Rimmelzwaan, G. F. (2015a). Human influenza A virus-specific CD8$^+$ T cell response is long-lived. *J. Infect. Dis.* 212, 81–5. doi: 10.1093/infdis/jiv018

van de Sandt, C. E., Kreijtz, J. H., de Mutsert, G., Geelhoed-Mieras, M. M., Hillaire, M. L., Vogelzang-van Trierum, S. E., et al. (2014). Human cytotoxic T lymphocytes directed to seasonal influenza A viruses cross-react with the newly emerging H7N9 virus. *J. Virol.* 88, 1684–1693. doi: 10.1128/JVI.02843-13

van de Sandt, C. E., Kreijtz, J. H., Geelhoed-Mieras, N. J., Nieuwkoop, M. I., Spronken, M. van de Vijver., D. A., et al. (2015b). Differential recognition of influenza A viruses by M158-66 epitope-specific CD8$^+$ T cells is determined by extraepitopic amino acid residues. *J. Virol.* 90, 1009–1022. doi: 10.1128/JVI.02439-15

van de Sandt, C. E., Pronk, M. R., van Baalen, C. A., Fouchier, R. A. M., and Rimmelzwaan, G. F. (2018a). Variation at extra-epitopic amino acid residues influences suppression of influenza virus replication by M158-66 epitope-specific CD8$^+$ T lymphocytes. *J Virol.* 92:e00232–18. doi: 10.1128/JVI.00232-18

van der Sluijs, K. F., Nijhuis, M., Levels, J. H., Florquin, S., Mellor, A. L., Jansen, H. M., et al. (2006). Influenza-induced expression of indoleamine 2,3-dioxygenase enhances interleukin-10 production and bacterial outgrowth during secondary pneumococcal pneumonia. *J. Infect. Dis.* 193, 214–222. doi: 10.1086/498911

van Genugten, M. L. L., Heijnen, M. L. A., and Jager, J. C. (2001). "Scenario-ontwikkeling zorgvraag bij een influenza pandemie," in *RIVM-Rapport*. RIVM.

Van Kerkhove, M. D., Vandemaele, K. A., Shinde, V., Jaramillo-Gutierrez, G., Koukounari, A., Donnelly, C. A., et al. (2011). Infection, risk factors for severe outcomes following 2009 influenza A (H1N1) infection: a global pooled analysis. *PLoS Med.* 8:e1001053. doi: 10.1371/journal.pmed.1001053

van Schaik, L., and Bakker, T. (2017). "Climate-migration-security: Making the most of a contested relationship," in *Clingendael Policy Brief* (Den Haag: Planetary Security Initiative).

Wahl, B., O'Brien, K. L., Greenbaum, A., Majumder, A., Liu, L., Chu, Y., et al. (2018). Burden of *Streptococcus pneumoniae* and *Haemophilus influenzae* type b disease in children in the era of conjugate vaccines: global, regional, and national estimates for 2000–15. *Lancet Glob. Health* 6, e744–e757. doi: 10.1016/S2214-109X(18)30247-X

Wang, Z., Wan, Y., Qiu, C., Quinones-Parra, S., Zhu, Z., Loh, L., et al. (2015). Recovery from severe H7N9 disease is associated with diverse response mechanisms dominated by CD8$^+$ T cells. (2010). *Nat. Commun.* 6:6833. doi: 10.1038/ncomms7833

Watanabe, T., Watanabe, S., Shinya, K., Kim, J. H., Hatta, M., and Kawaoka, Y. (2009). Viral RNA polymerase complex promotes optimal growth of 1918 virus in the lower respiratory tract of ferrets. *Proc. Natl. Acad. Sci. U.S.A.* 106, 588–592. doi: 10.1073/pnas.0806959106

Watanabe, T., Zhong, G., Russell, C. A., Nakajima, N., Hatta, M., Hanson, A., et al. (2014). Circulating avian influenza viruses closely related to the 1918 virus have pandemic potential. *Cell Host Microbe* 15, 692–705. doi: 10.1016/j.chom.2014.05.006

Wilson, H. E., Saslaw, S., Doan, C. A., Woolpert, O. C., and Schwab, J. L. (1947). Reactions of monkeys to experimental mixed influenza and streptococcus infections: an analysis of the relative roles of humoral and cellular immunity, with the description of an intercurrent nephritic syndrome. *J. Exp. Med.* 85, 199–215. doi: 10.1084/jem.85.2.199

Wong, V. W., Cowling, B. J., and Aiello, A. E. (2014). Hand hygiene and risk of influenza virus infections in the community: a systematic review and meta-analysis. *Epidemiol. Infect.* 142, 922–932. doi: 10.1017/S095026881400003X

World Health Organization (2005). Evolution of H5N1 avian influenza viruses in Asia. *Emerging Infect. Dis.* 11, 1515–21. doi: 10.3201/eid1110.050644

World Health Organization (2018a). *Global Influenza Virological Surveillance*. Available online at: http://www.who.int/gho/epidemic_diseases/influenza/virological_surveillance/en/ (Accessed May 6, 2018).

World Health Organization (2018b). *Pandemic Preparedness*. Available online at: http://www.who.int/influenza/preparedness/pandemic/en/ (Accessed May 12, 2018).

World Health Organization Writing Group, Bell, D., Nicoll, A., Fukuda, K., Horby, P., Monto, A., et al., (2006). Non-pharmaceutical interventions for pandemic influenza, national and community measures. *Emerging Infect. Dis.* 12, 88–94. doi: 10.3201/eid1201.051371

Worobey, M., Han, G. Z., and Rambaut, A. (2014). Genesis and pathogenesis of the 1918 pandemic H1N1 influenza A virus. *Proc Natl Acad Sci U.S.A.* 111, 8107–8112. doi: 10.1073/pnas.1324197111

Wu, J. T., Cowling, B. J., Lau, E. H., Ip, D. K., Ho, L. M., Tsang, T., et al. (2010). School closure and mitigation of pandemic (H1N1) 2009, Hong Kong. *Emerging Infect. Dis.* 16, 538–541. doi: 10.3201/eid1603.091216

Yu, X., Tsibane, T., McGraw, P. A., House, F. S., Keefer, C. J., Hicar., M. D., et al. (2008). Neutralizing antibodies derived from the B cells of 1918 influenza pandemic survivors. *Nature* 455, 532–536. doi: 10.1038/nature07231

Zambon, M. (2014). Developments in the treatment of severe influenza: lessons from the pandemic of 2009 and new prospects for therapy. *Curr. Opin. Infect. Dis.* 27, 560–565. doi: 10.1097/QCO.0000000000000113

Zhang, Y. H., Zhao, Y., Li, N., Peng, Y. C., Giannoulatou, E., Jin, R. H., et al. (2013). Interferon-induced transmembrane protein-3 genetic variant rs12252-C is associated with severe influenza in Chinese individuals. *Nat. Commun.* 4:1418. doi: 10.1038/ncomms2433

Zhou, J., To, K. K., Dong, H., Cheng, Z. S., Lau, C. C., Poon, V. K., et al. (2012). A functional variation in CD55 increases the severity of 2009 pandemic H1N1 influenza A virus infection. *J. Infect. Dis.* 206, 495–503. doi: 10.1093/infdis/jis378

Zhu, H., Wang, D., Kelvin, D. J., Li, L., Zheng, Z., Yoon, S. W., et al., (2013). Infectivity, transmission, and pathology of human H7N9 influenza in ferrets and pigs. *Science* 341:183–186. doi: 10.1126/science.1239844.

Zhu, W., Zhou, J., Li, Z., Yang, L., Li, X., Huang, W., et al. (2017). Biological characterisation of the emerged highly pathogenic avian influenza (HPAI) A(H7N9) viruses in humans, in mainland China, 2016 to 2017. *Euro Surveill.* 22:30533. doi: 10.2807/1560-7917.ES.2017.22.19.30533

Ziegler, T., Mamahit, A., and Cox, N. J. (2018). 65 Years of influenza surveillance by a WHO-coordinated global network. *Influenza Other Respir Viruses* (2018). 12, 558-565 doi: 10.1111/irv.12570

Zumla, A., Raviglione, M., Hafner, R., and von Reyn, C. F. (2013). Tuberculosis. *N. Engl. J. Med.* 368, 745–755. doi: 10.1056/NEJMra1200894

Zúñiga, J., Buendía-Roldán, I., Zhao, Y., Jiménez, L., Torres, D., Romo, J., et al. (2012). Genetic variants associated with severe pneumonia in A/H1N1 influenza infection. *Eur. Respir. J.* 39, 604–610. doi: 10.1183/09031936.00020611

Conflict of Interest Statement: 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 Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 19 February 2019
doi: 10.3389/fcimb.2019.00031

The Combination Vaccine Adjuvant System Alum/c-di-AMP Results in Quantitative and Qualitative Enhanced Immune Responses Post Immunization

Thomas Ebensen†, *Simon Delandre*†, *Blair Prochnow*, *Carlos A. Guzmán*† and *Kai Schulze**†

Department of Vaccinology and Applied Microbiology, Helmholtz Centre for Infection Research, Braunschweig, Germany

OPEN ACCESS

Edited by:
Alberto Moreno,
Emory University School of Medicine,
United States

Reviewed by:
Elizabeth B. Norton,
Tulane University, United States
Yang Zhang,
University of Pennsylvania,
United States

***Correspondence:**
Kai Schulze
kai.schulze@helmholtz-hzi.de

†*These authors have contributed equally to this work*

Specialty section:
This article was submitted to Clinical Microbiology, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *16 August 2018*
Accepted: *31 January 2019*
Published: *19 February 2019*

Citation:
Ebensen T, Delandre S, Prochnow B, Guzmán CA and Schulze K (2019) The Combination Vaccine Adjuvant System Alum/c-di-AMP Results in Quantitative and Qualitative Enhanced Immune Responses Post Immunization. Front. Cell. Infect. Microbiol. 9:31. doi: 10.3389/fcimb.2019.00031

The development of new effective vaccines strongly depends on adjuvants and formulations able to stimulate not only strong humoral responses against a certain pathogen but also effector as well as memory CD4+ and CD8+ T cells (Dubensky et al., 2013). However, the majority of vaccines licensed for human use or currently under clinical investigation fail to stimulate efficient cellular responses. For example, vaccines against hepatitis B virus (HBV), human papillomavirus (HPV), diphtheria, tetanus and influenza are usually administered by intramuscular (i.m.) injection and contain aluminum salts (alum) as adjuvant. Alum has been shown to stimulate Th2 immune cells resulting in increased production of antigen-specific antibodies but to be incapable of stimulating robust Th1 or cytotoxic responses. To overcome such limitations recent research has focused on the development of adjuvant combinations (e.g., MF59, AS03 or AS04) to not only further strengthen antigen-specific immune responses but to also allow their modulation. We have shown previously that bis-(3′,5′)-cyclic dimeric adenosine monophosphate (c-di-AMP) constitutes a promising adjuvant candidate stimulating both effective Th1/Th2 and cytotoxic immune responses when included in mucosal or parenteral vaccine formulations. In the present work we demonstrate that c-di-AMP can be also combined with other adjuvants like alum resulting in increases in not only humoral responses but more striking also in cellular immune responses. This leads to improved vaccine efficacy against intracellular pathogens.

Keywords: adjuvant system, combination, c-di-AMP, alum, humoral, cellular

INTRODUCTION

Today, infectious diseases represent the second leading cause of death worldwide (Global Health Observatory Data Repository, 2016). In order to prevent human illness and death, vaccination is currently the most effective tool. According to the World Health Organization (WHO), vaccination prevents 2 to 3 million deaths each year. Moreover, this number could even reach 6 million, if all children would receive the recommended vaccine schedule (Patil and Shreffler, 2018). Currently, vaccination allows us to control up to 10 major diseases and has resulted in the eradication of the smallpox virus in 1980. Although vaccination has demonstrated its strength to protect against infectious diseases, the emergence of new pathogens,

as well as the increase of antibiotic resistance, reveals the necessity for the development of new vaccines. Presently, the vaccines in use are based on either live-attenuated pathogens, inactivated whole pathogens (virus or bacteria) or only pure microbial components. The latter, so-called subunit vaccines, constitute promising candidates for the development of vaccines showing increased safety profiles. Since subunit vaccines contain no living organism, these vaccines are especially useful for vaccination of immunocompromised individuals. However, the low complexity profile of subunit vaccines makes them less immunogenic. Booster immunizations and/or the inclusion of adjuvants are/is therefore required. In this context, adjuvants are not only used to enhance the stimulated antigen-specific immune responses but also to tailor the immune responses according to the specific clinical needs. In this regard, it is unlikely that a single adjuvant will be able to fulfill all the required properties to be implemented in all foreseeable vaccines. Presumably, different adjuvants are needed that stimulate the immune responses required following different vaccination strategies considering the pathogen, the type of antigen, the immune status and age of the vaccine and the application route. Recent approaches also address the possibility to combine different adjuvants in order to improve vaccine efficacy (Garcon and Di Pasquale, 2017). Especially the stimulation of a cell-mediated immune [T helper 1 (Th1) response and cytotoxic T lymphocytes (CTLs)] is of interest since actual vaccines stimulate predominantly humoral immune responses (Riese et al., 2013; Lee and Nguyen, 2015; Tandrup Schmidt et al., 2016). However, despite significant progress in adjuvant development during the last decades only a limited number of adjuvants are available for human use (Di Pasquale et al., 2015). Therefore, the aim of the present work was to evaluate the potential of the STING agonist c-di-AMP to increase vaccine efficacy when combined with the well-known adjuvant alum. In order to achieve this goal immunization studies were performed using the model antigen beta-galactosidase allowing an in depth dissection of the immune effector mechanisms stimulated by this system. Alum is the most used adjuvant worldwide and represents one of five adjuvants approved in the United States (FDA, 2011). However, alum stimulates only antigen-specific Th2 immune cells resulting in the secretion of IL-4, IL-5, and IL-10 and the subsequent improved antigen-specific antibody production (Brewer et al., 1999). In contrast, the cyclic di-nucleotide c-di-AMP, a second messenger in prokaryotes, exhibits strong immune modulatory properties - stimulating antibody and mixed Th1/Th2 as well as cytotoxic responses when administered by either parenteral or mucosal routes (Ebensen et al., 2007a,b; Ebensen et al., 2017; Schulze et al., 2017a). This renders it very attractive for use in human vaccines, since most adjuvants supporting a Th1-dominated response lack the ability to induce humoral immunity (Libanova et al., 2010; Matos et al., 2017). Moreover, c-di-AMP is also able to promote the stimulation of CTL responses by induction of cross-priming (Lirussi et al., 2017). Therefore, an adjuvant system of alum/c-di-AMP could overcome the limitation of alum and not only further enhance the stimulated antigen-specific humoral response but at the same time promote the stimulation of Th1 and CTL responses.

MATERIALS AND METHODS

Mice

Female BALB/c (H-2d) mice 6–8 weeks of age were purchased from Harlan Germany and kept at the animal facility of the Helmholtz Centre for Infection Research (Germany) under specific pathogen-free conditions as previously described (Schulze et al., 2017a). The animal experiments in this study have been reviewed for ethical compliance by the institutional ethical board and approved by the local government of Lower Saxony (Germany, No. 33.42502-13/1281). All experiments in this study were performed following standard biosecurity and institutional safety guidelines.

Immunization Protocol

Animals ($n = 10$) were immunized 3 times at day 0, 14, and 28 by intramuscular route. Each animal received a dose of 50 µl containing 15 µg of β-Gal protein (Sigma-Aldrich, Germany) as antigen. ß-Gal was either adsorbed to alum [1:1 v/v, aluminum hydroxyphosphate (Adju-Phos®), Brenntag Biosector, Denmark] at pH 7.4 and 25°C or co-administered with c-di-AMP (Biolog, Germany) at a concentration of 5 µg per dose. Fourteen days after the third immunization, spleens of vaccinated mice were collected, immune cells were extracted, pooled and restimulated with β-Gal. The cytokine concentration was measured by cytometric bead array (CBA). Results from one representative out of two independent experiments are shown.

Elisa

β-Gal-specific antibody titers in sera were investigated using ELISA assay as previously described (Schulze et al., 2017a). In brief, high binding protein plates were coated with β-Gal protein (2 µg/ml in 0.05 M carbonate buffer). After blocking unspecific binding sites using 3% bovine serum albumin (BSA) in PBS serial 2-fold dilutions of sera in 3% BSA/PBS were added (100 µl/well). After 1 h incubation at 37°C, plates were washed using 1% BSA/PBS/0.05% Tween 20 and the secondary antibodies were added: biotinylated goat anti-mouse IgG, IgG1, and IgG2a (Sigma, USA), respectively. After 1 h incubation at 37°C, plates were washed and samples were incubated for 1 h at RT in the presence of peroxidase-conjugated streptavidin (BD Pharmingen, USA). Finally, reactions were developed using ABTS [2, 20-azino-bis(3- ethylbenzthiazoline-6-sulfonic acid)] in 0.1 M citrate-phosphate buffer (pH 4.35) containing 0.01% H_2O_2. Endpoint titers are expressed as absolute values of the last dilution giving an optical density (OD405 nm) being two times higher than the values of the negative control after 5 min incubation as previously described (Ebensen et al., 2007b).

ELISpot Assay

The quantity of β-Gal-specific cytokine-producing cells was investigated using an ELISpot assay as previously described (Lirussi et al., 2017; Schulze et al., 2017b). Flat bottomed 96-well plates with a 0.45 µm hydrophobic High Protein Binding Immobilon-P-Membrane (BD Pharmingen) were coated with anti-IFN-γ, anti-IL2, anti-IL4 or anti-IL17 antibodies diluted in PBS and incubated overnight at 4°C. Unspecific binding sites were blocked for 2 h at RT using 200 µl/well of complete

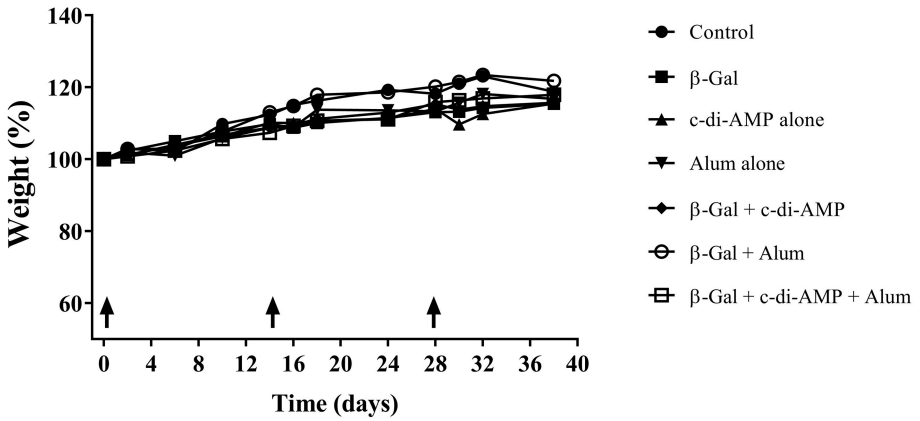


FIGURE 1 | Development of the weight of mice vaccinated with different β-Gal-containing formulations. Animal body weight was monitored throughout the whole experimental setting.

medium. Then, 4×10^5 and 2×10^5 spleen cells/well were added and incubated in the absence (blank, only media added) or presence of the β-Gal protein (5 μg/ml) and the MHC-I immunodominant peptide TPHPARIGL of β-Gal (5 μg/ml), respectively. For positive controls, splenocytes were stimulated with 5 μg/ml of the mitogen concanavalin A. Samples were incubated for 16 (IFN-γ) or 48 h (IL-4) at 37°C. Afterwards, plates were washed and further incubated for 2 h at RT in the presence of appropriate diluted biotinylated detection antibodies. Then, after another washing step samples were incubated for 1 h at RT in the presence of peroxidase-conjugated streptavidin. After a final wash, cytokine-secreting cells were detected by adding AEC substrate (diluted in 0.1 M acetate buffer pH 5.0) mixed with 0.05% H_2O_2 (30%). After stopping the reaction with distilled water, plates were analyzed using the ImmunoSpot Image Analyzer software v3.2 (CTL-Europe GmbH). Results are expressed as Spot Forming Units (SFU) obtained from stimulated cells subtracted of background from non-stimulated cells (Ebensen et al., 2007b).

Proliferation Assay

The ability of immune cells derived from spleen to proliferate upon restimulation with β-Gal as well as their cytokine profile were measured 96 h post restimulation. To this end, cell suspensions were seeded at 5×10^5 cells/well in flat-bottomed 96-well microtiter plates (Nunc) and incubated for 4 days in the presence of 1, 10, 20, and 40 μg/ml of the β-Gal protein. During the final 18 h of culture, 1 μCi of [^{3}H]-thymidine (Amersham International, Freiburg, Germany) was added to each well. Cells were harvested on paper filters (Filtermat A; Wallac, Freiburg, Germany) by using a cell harvester (Inotech, Wohlen, Switzerland) and the number of proliferating cells was indirectly determined by counting [^{3}H]-thymidine events incorporated into the DNA of proliferating cells with a γ-scintillation counter (Wallac 1450, Micro-Trilux) (Lirussi et al., 2017; Schulze et al., 2017b). Results are expressed as ratio of values from stimulated and nonstimulated samples [stimulation index (SI)].

Multiplex Flowcytomix (Cytometric Bead Array)

Supernatants of antigen-restimulated spelenocytes have been used to characterize the stimulated cytokine profiles using the Th1/Th2/Th9/Th17 FlowCytomix immunoassay from Biolegend according to the manufacturer's instructions (Ebensen et al., 2017).

Statistical Analysis

Statistical significance of the observed differences was analyzed using the one-way ANOVA test (Tukey's multiple comparisons test) of the Graph Pad Prism 7 software for Windows (Version 7.04) as previously described (Mittal et al., 2015). Differences were considered significant at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

RESULTS

Adjuvant System C-di-AMP/Alum did not Induce any Signs of Unwanted Side Effects

The aim of this study was to investigate if c-di-AMP in combination with alum can further optimize the immune response against an antigen (β-Gal) when given by parenteral route. In a first attempt, the general behavior and the body weight development was investigated during the course of vaccination. In all vaccinated groups, no changes in spontaneous and provoked behavior, in grooming, feces character and body weight (**Figure 1**) were observed.

Adjuvant System C-di-AMP/Alum Promotes Antigen-Specific Antibody Responses

The β-Gal-specific antibody titers post third immunization with or without the combination of alum and c-di-AMP are displayed as end-point-dilution titer in **Figure 2**. The combination of c-di-AMP with alum led to the stimulation of a stronger humoral immune response. The antigen specific

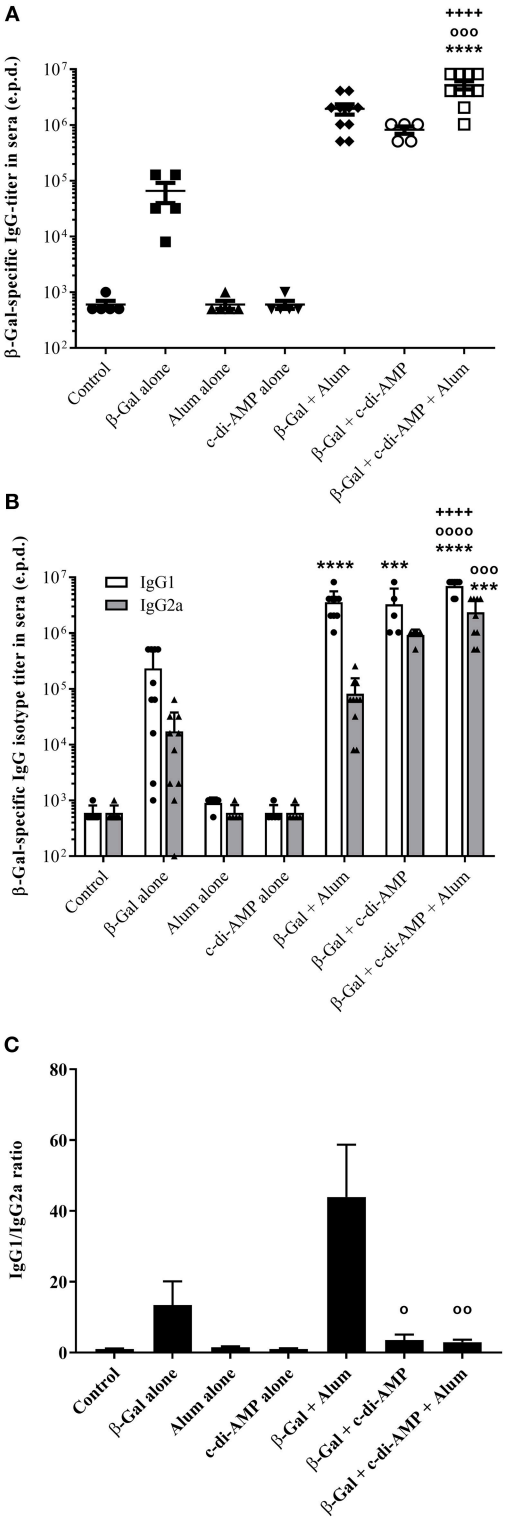


FIGURE 2 | Systemic humoral immune responses induced in mice after three immunizations with β-Gal co-administered with the adjuvant system c-di-AMP/alum via intramuscular route. **(A)** β-Gal–specific IgG titers in sera 13 days after the last immunization. **(B)** β-Gal–specific IgG1 and IgG2a in the sera of immunized mice ($n = 10$). Results are displayed as average of

(Continued)

FIGURE 2 | the last sera dilution (end point dilution, e.p.d) showing the double value (OD 405 nm) of the control background. Each bullet symbol indicates a single animal, whereas horizontal lines represent the mean of animals. **(C)** β-Gal–specific IgG1/IgG2a ratio in the sera of immunized mice. Statistically significant differences were validated using the one-way ANOVA with Tukey's *post hoc* test with $p < 0.001$ (***) and $p < 0.0001$ (****) when compared with β-Gal alone (*), or in combination with alum (o) or c-di-AMP (+), respectively.

antibody titers stimulated by c-di-AMP combined with alum are higher by a factor of 2.5 and 6.4, respectively, than with alum or c-di-AMP alone (**Figure 2A**). Moreover, only the adjuvant combination stimulated β-Gal-specific antibody titers that were statistically significantly higher compared to the titer stimulated by β-Gal alone or in combination with any single adjuvant. The humoral immune response can also give some indications regarding the polarization of the T-lymphocytes by looking at the ratio between IgG1/IgG2a antibody isotypes (**Figures 2B,C**). Taken individually, alum and c-di-AMP evoke the same IgG1 titer (3.6×10^6 and 3.3×10^6, respectively), which was significantly higher compared to those observed in mice receiving β-Gal alone. However, the combination of alum and c-di-AMP resulted in significantly increased IgG1 titers compared to those obtained using single adjuvants (**Figure 2B**). Although not statistically significant, c-di-AMP alone induces an almost 10-fold higher titer of β-Gal-specific IgG2a than alum alone. Again, the combination of the two adjuvants stimulated IgG2a titers significantly increased compared to those stimulated by β-Gal alone or adsorbed to alum. Thus, the addition of c-di-AMP with alum in the formulation somehow restores the ratio between IgG1/IgG2a compared to alum alone (ratio of 2.96 for the combination and 43.21 for alum alone; **Figure 2C**). Moreover, the ratio IgG1/IgG2a between c-di-AMP and the adjuvant combination is unchanged (3.56 and 2.96, respectively). Thus, the alum and c-di-AMP combination leads to not only a higher IgG1 titer but also a better IgG1/IgG2a balance, which could be translated into a better balance between the Th1/Th2 immune response (**Figure 2C**).

Adjuvant System C-di-AMP/Alum Promotes Antigen-Specific Cellular Responses

The strongest proliferative capacity upon antigen restimulation was recorded in mice vaccinated with β-Gal and both adjuvants, as shown by the stimulation index of 13 (**Figure 3**). The proliferative capacity of the splenocytes is not only statistically significantly higher compared to those observed in mice vaccinated with β-Gal alone or in combination with a single adjuvant but also occurs already when restimulated with a very low concentration of antigen (1 μg/ml of β-Gal), indicating a strong activation of antigen-specific immune cells. In contrast, nearly the same stimulation index is obtained only when splenocytes derived from mice immunized with β-Gal plus c-di-AMP were restimulated with 40 μg/ml of β-Gal protein (**Figure 3**).

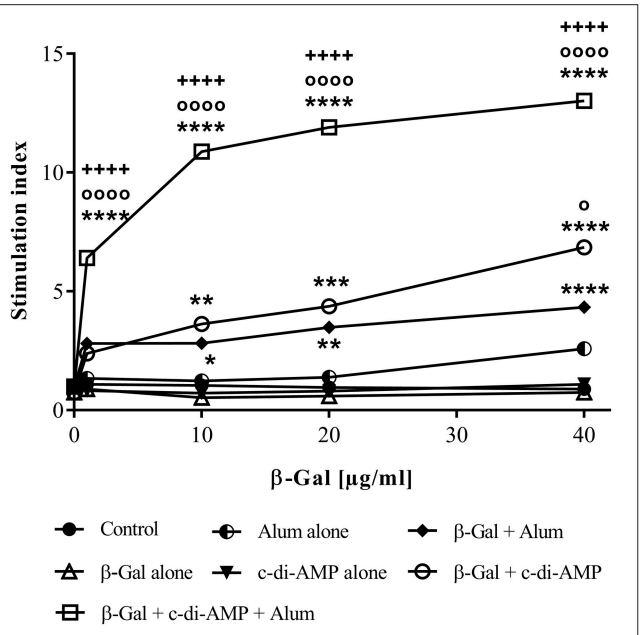


FIGURE 3 | Cellular responses induced in mice after three immunizations with β-Gal co-administered with the adjuvant system c-di-AMP/alum via intramuscular route. Splenocytes from vaccinated animals ($n = 10$) were restimulated with an increased concentration of β-Gal protein (starting from 1 µg/ml to 40 µg/ml). Proliferation of the cells after 96 h was indirectly determined by counting [^{3}H]-thymidine events incorporated into the DNA of proliferating cells. Results are average of quadruplicates and are expressed as stimulation index (SI). Statistically significant differences with respect to β-Gal alone (*), β-Gal with alum (o) or β-Gal with c-di-AMP (+) were validated using one-way ANOVA with Tukey's *post hoc* test, with $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****).

Upon *in vitro* antigen restimulation, lymphocytes of vaccinated animals do not only proliferate, they also secrete cytokines which for some are the hallmark of their polarization. As represented in **Figure 4**, immunization with the combination of alum and c-di-AMP allows the stimulation of a significantly higher number of β-Gal-specific IFN-γ-producing cells than with alum alone. The combination seems also to be more potent than c-di-AMP alone, however, the observed difference wasn't significant. In contrast, when investigating the number of antigen-specific IFN-γ-producing CD8+ T lymphocytes, their number seems to decrease following vaccination with β-Gal using both adjuvants (the average number is comparable to alum alone) as indicated by the levels of β-Gal-specific IFN-γ-producing cells observed when splenocytes were restimulated with the CD8 peptide TPHPARIGL (**Figure 4A**). Thus, the elevated levels of IFN-γ-producing cells observed when splenocytes of mice receiving the adjuvant system c-di-AMP/alum were restimulated with β-Gal protein seem to be CD4+ T cells. The number of IL-2, IL-4 and IL-17 β-Gal-specific-producing cells is also statistically significantly increased in mice immunized with the adjuvant system c-di-AMP/alum with respect to those detected in mice receiving β-Gal alone. This supports the assertion of a more balanced T helper response with respect to the formulation encompassing only single adjuvants (**Figures 4B–D**).

The results obtained analyzing the number of cytokine-producing cells were in line with the levels of the corresponding cytokines detected in the supernatant of β-Gal restimulated splenocytes. Thus, only splenocytes of mice receiving β-Gal with the adjuvant system c-di-AMP/alum secreted statistically significantly higher levels of Th1 (IFN-γ, IL-2), Th2 (IL-4, IL-5, IL-10, IL-13), and Th17 (IL-17A, IL-22) cytokines compared to the group receiving β-Gal alone with $p < 0.05$ (**Figure 5**). However, no significant differences have been observed for TNF-α. Interestingly, the addition of c-di-AMP in the vaccine formulation with alum evokes a Th17 polarization of the lymphocytes following i.m. vaccination while c-di-AMP and alum stimulated only marginal IL-17 production if any at all (**Figure 5**). This is remarkable, since it is known already that c-di-AMP stimulates a strong IL-17 production only when applied by mucosal routes (Ebensen et al., 2011, 2017; Mittal et al., 2015). Taken together, the combination of c-di-AMP and alum seems to not only sustain the Th2 response stimulated by β-Gal but, in addition, strengthen the β-Gal-specifc Th1 and Th17 responses.

DISCUSSION

In contrast to most medications, vaccines are administered to large populations of more or less healthy persons. Moreover, also infants, children and immunocompromised individuals are target subpopulations, making no or only low potential risks or side-effects essential for vaccines (Di Pasquale et al., 2016). In order to increase vaccine safety, recent research has focused on so-called subunit vaccines. In contrast to common vaccine formulations, these vaccines usually consist only of microbial components. Unfortunately, the increased safety profile is at the expense of the vaccine efficacy, as pure antigens typically are only poorly immunogenic. Therefore, subunit vaccines essentially have to include antigen delivery systems or adjuvants (Ebensen and Guzman, 2008).

Adjuvants are key elements in both prophylactic as well as therapeutic vaccines since they can not only improve the strength of antigen-specific immune responses but also modify these responses according to the specific needs (Di Pasquale et al., 2016). However, one of the challenges in adjuvant development is to balance efficacy and safety in order to stimulate immunity (Batista-Duharte et al., 2018).

Besides a handful of adjuvants such as MF59, monophosphoryl lipid A (MPL A), AS03, AF03 or virosomes, only aluminum-based adjuvants (e.g., AS04) continue to be used worldwide since over 90 years. The main forms of aluminum adjuvant used in parenteral administered vaccines are aluminum hydroxide and aluminum phosphate (He et al., 2015). In addition to the beneficial aspects of alum, there are also some limiting effects such as induction of adverse reactions and preferential priming of Th2-type immune responses (He et al., 2015). In this regard, novel adjuvants including also mixtures of adjuvants (adjuvant systems) have opened the door for the development of vaccines with improved safety and efficacy profiles against emerging and/or re-emerging pathogens (Garcon and Di Pasquale, 2017). Thus, adjuvant combinations targeting

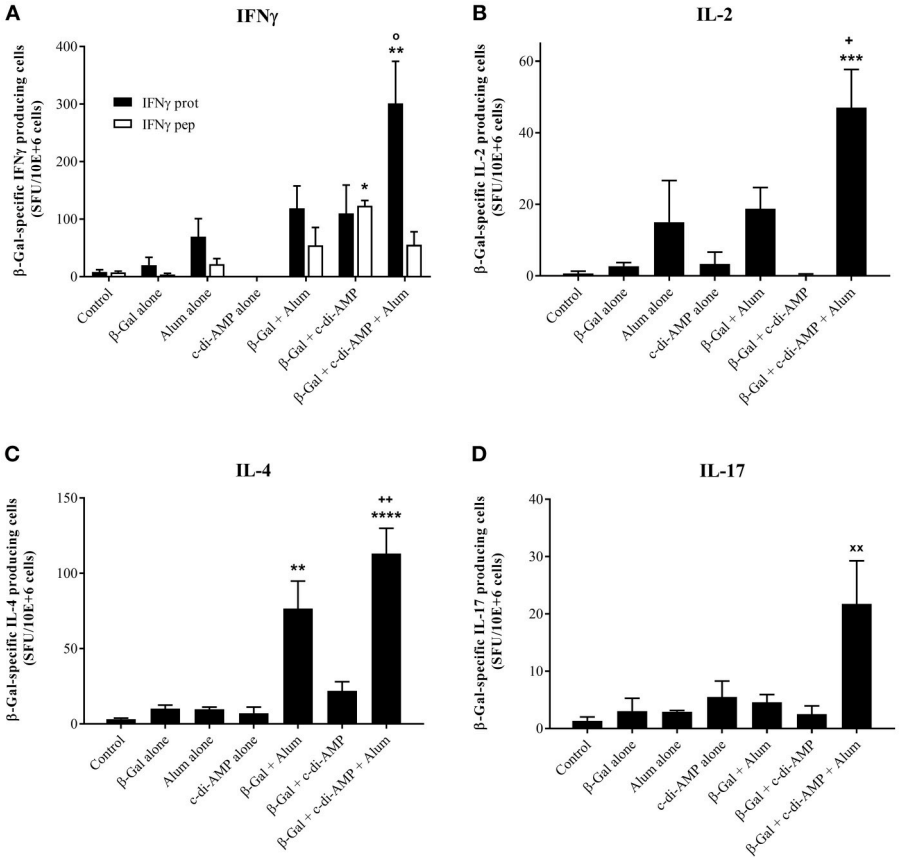


FIGURE 4 | Antigen-specific cytokine-producing cells stimulated by β-Gal co-formulated with different adjuvants. The quantity of β-Gal-specific cytokine-producing splenocytes was determined by ELISpot assay. IFN-γ **(A)** IL-2 **(B)**, IL-4 **(C)**, and IL-17 **(D)** were investigated as indicator cytokines for Th1, Th2, and Th17 biased immune responses. Results obtained restimulating with the TPHPARIGL peptide reflect the number of IFN-γ producing CD8+ T cells while restimulation with β-Gal protein activates CD4+ and by cross-presentation also CD8+ T cells. Results are presented as mean spot-forming units per 10^6 cells above the background values of unstimulated cells. The SD were calculated from triplicates of two cell concentrations each of control, antigen alone, co-administered with c-di-AMP, alum or adjuvant system c-di-AMP/alum. Statistically significant differences with respect to Control (x), β-Gal alone (*), β-Gal with alum (o) or β-Gal with c-di-AMP (+) were validated by one-way ANOVA with Tukey's *post hoc* test, with $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

different pattern-recognition receptors (PRR), both endosomal and intracellular, enhance antigen-specific immune responses and/or direct them toward the response of need (e.g., cytotoxic or mucosal response) (Gutjahr et al., 2016). For example, the combination of the Toll-like receptor (TLR) agonist MPL A adsorbed on aluminum salts (AS04^{TM}, GlaxoSmithKline) results in the stimulation of increased production of antigen-specific antibodies and an enhanced cell-mediated response by causing a local and temporary cytokine response (Reed et al., 2009; Garçon et al., 2011; Del Giudice et al., 2018). Nevertheless, no efficient antigen-specific Th1 and cytotoxic T cell responses are stimulated.

Therefore, the aim of the present work was the exploration of the great potential of c-di-AMP acting as a parenteral adjuvant in an adjuvant system combined with alum and the model antigen β-Gal to overcome the limitations of alum, since c-di-AMP was shown to stimulate strong Th1 and cytotoxic responses even when included in adjuvant combinations (Mittal et al., 2015; Ebensen et al., 2017; Matos et al., 2017; Sanchez Alberti et al., 2017; Schulze et al., 2017a; Temizoz et al., 2018). c-di-AMP binds to the transmembrane protein STING (stimulator of IFN genes) thereby activating the TBK3-IRF3 signaling pathway, subsequently triggering the production of type I IFN and TNF (McWhirter et al., 2009; Burdette et al., 2011; Shu et al., 2012). This in turn, results in strong adaptive immune responses.

In line with previously obtained results, co-administration of β-Gal with either alum or c-di-AMP alone stimulated strong humoral β-Gal-specific immune responses (Ebensen et al., 2011; McKee and Marrack, 2017). However, when immunizing mice with the combination of alum and c-di-AMP, the detected IgG titers were further increased compared to those observed in sera of mice receiving alum or c-di-AMP alone (factor 2.7 and 6.4, respectively). In order to evaluate if the combination of alum and c-di-AMP will also have a beneficial effect on antigen-specific cellular responses, we analyzed the proliferative capacity as well as the cytokine profiles of splenocytes of animals vaccinated i.m. with β-Gal alone or in combination with c-di-AMP, alum or the adjuvant system c-di-AMP/alum. Similar to the

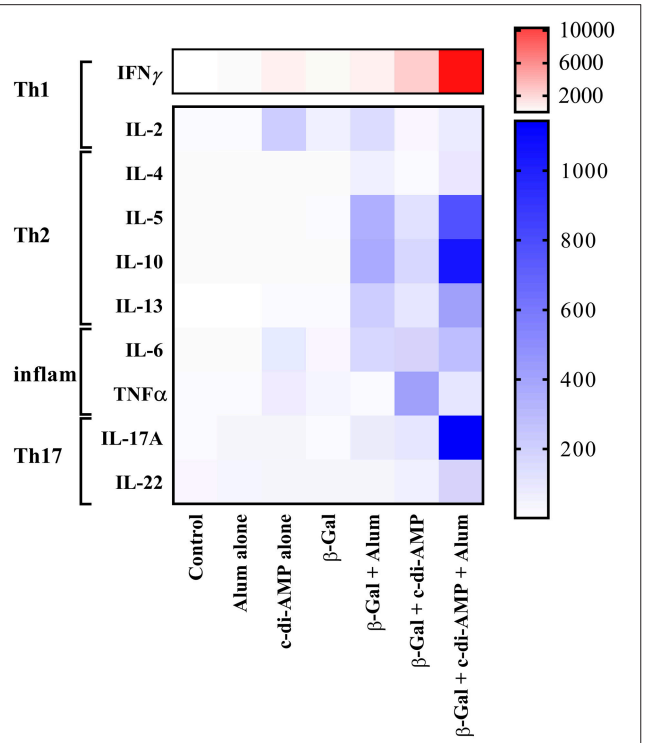


FIGURE 5 | Cytokine profiles stimulated by β-Gal co-formulated with different adjuvants. The presence of mouse IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17A, IL-22, IFN-γ, and TNF-α were determined in an immunoassay using a cytometric bead array according to the manufacturer's instructions (Mouse Th1/Th2/Th9/Th17 13plex Biolegend). Results are presented as a heat map of Th1, Th2, pro-inflammatory, and Th17 cytokines in antigen-restimulated splenocytes derived from mice ($n = 10$) vaccinated with β-Gal co-administered with alum, c-di-AMP or c-di-AMP/alum.

observed humoral responses, the adjuvant system also stimulated increased antigen-specific cellular responses as indicated by the strong proliferative capacity already at relatively low doses of β-Gal antigen compared to the formulations encompassing only a single adjuvant. In addition, the observed cytokine profile stimulated following vaccination reveals the characteristic of cell-mediated effector functions. Thus, splenocytes recovered from mice vaccinated with the adjuvant system c-di-AMP/alum plus β-Gal were found to produce enhanced levels of the Th1 cytokines IFN-γ and IL-2, the Th2 cytokines IL-4, IL-5, IL-10 and IL-13, and the Th17 cytokine IL-17, whereby the Th1/Th2 ratio was shifted toward Th1. In general, groups receiving either c-di-AMP or alum alone as adjuvant gave significantly lower numbers of cytokine-producing cells and cytokine titers compared to the group receiving the adjuvant combination. The observed cellular responses were confirmed by the obtained humoral immune responses, such as IgG titer and IgG subclass profiles. Hence, in line with previous studies, mice immunized with β-Gal co-administered with c-di-AMP showed a balanced production of IgG1 and IgG2a, correlating with a balanced Th1/Th2 response (Libanova et al., 2010; Ebensen et al., 2011; Mittal et al., 2015). The same is true for mice receiving the adjuvant system c-di-AMP/alum which showed a balanced IgG1/IgG2a ratio of approximately three, while alum alone promoted a Th2-biased response visualized by a IgG1/IgG2a ratio greater than 40.

Interestingly, the combination of alum with c-di-AMP also resulted in the stimulation of enhanced levels of IL-10, which was shown to block Th1 responses. Thus, when Oleszycka and co-workers immunized mice using alum as adjuvant, increased IL-10 titers were observed but only limited Th1 responses. In contrast, when they immunized IL-10 deficient mice using alum, increased Th1 responses have been obtained suggesting an inhibitory effect of IL-10 on Th1 cells (Oleszycka et al., 2018). However, previous findings also showed that induction of IL-10, which promotes IgA switch, displays broad anti-inflammatory properties (Lamm and Phillips-Quagliata, 2002) and is involved in self-regulation of Th1 responses (Jankovic et al., 2010). Thus, the strong IL-10 titers observed in mice vaccinated with β-Gal co-administered with the adjuvant system c-di-AMP/alum might simply reflect the necessity of self-regulation in order to restrain the stimulated antigen-specific Th1 immune response and prevent pathology (Ng et al., 2013). Likewise, vaccination using the adjuvant system c-di-AMP/alum also stimulated elevated levels of IL-17. This is in line with previous observations showing that alum-adjuvanted vaccines stimulated efficient immunity based on Th17 responses, which could even be improved when alum was combined with other adjuvants resulting in the induction of Th1/Th17 responses (Ross et al., 2013; Bagnoli et al., 2015). Interestingly, while c-di-AMP has been shown to facilitate Th17 polarization when administered by mucosal routes only marginal, if any at all, IL-17 production is stimulated when c-di-AMP is administered parenteral (Mittal et al., 2015; Ebensen et al., 2017). Therefore, the combination of alum with c-di-AMP applied by i.m. route seems to overcome this confinement.

Nevertheless, although the incorporation of c-di-AMP efficiently stimulates Th1 responses, the adjuvant system c-di-AMP/alum seems to be insufficient in compensating the inadequate stimulation of CD8+ T cells by alum as indicated by similar levels of IFNγ+ CD8-peptide-specific T cells in mice immunized using alum and the combination of alum and c-di-AMP, respectively. Instead, alum seems to inhibit the stimulation of CD8+ T cells by c-di-AMP. Further experiments need to be performed investigating whether higher concentrations of c-di-AMP would invert this effect.

Taken together, functionally distinct effector CD4+ T helper cell subsets are characterized by the secretion of distinct cytokine profiles. By this, our immune system is able to efficiently combat rapidly evolving and spreading pathogens infecting the host (Kara et al., 2014). Thus, while Th2 cells are critical for effective humoral immunity, by facilitating affinity maturation and class switch of the antigen-specific antibodies necessary for eradication of extracellular pathogens, Th1 cells are important for cell-mediated defense mechanisms helping to eliminate intracellular pathogens such as viruses (Chen and Cerutti, 2010). However, research in vaccine development seems to reveal that a single adjuvant will hardly be able to cope with all the foreseeable requirements in the field of infectious diseases. Thus, the development of new adjuvants increasing the portfolio of immunomodulatory molecules which allow the formulation of most effective vaccines tailored for

the specific needs is essential. This tool box for vaccine developers can be even extended by combining adjuvants with different mechanisms of action. Moreover, adjuvant combination can also increase the safety profile of a certain vaccine, as in the case of alum, for example, the likelihood of connected adverse side effects is reduced by restraining immune reactions and diminishing the risk of immuno-pathological outcomes (Didierlaurent et al., 2009).

In this regard, our results demonstrate that the adjuvant system c-di-AMP/alum generates conditions sufficient for stimulating both humoral and cellular immune responses. Moreover, the combination of alum with c-di-AMP not only strengthens the stimulated antigen-specific immune responses but also modulates them in the direction of a Th1 response.

ETHICS STATEMENT

All animal experiments in this study were approved by the institutional ethical board and have been performed with ethical agreement by the local government of Lower Saxony (Germany) with the No. 33.42502 13/1281.

DISCLOSURE

CAG and TE are named as inventors in a patent application covering the use of c-di-AMP as adjuvant (PCT/EP 2006010693). This does not alter our adherence to the Frontier Science policies on sharing data.

AUTHOR CONTRIBUTIONS

TE and KS conceived and designed the study. TE, KS, and SD performed the experiments. analyzed and interpreted the data, and wrote the manuscript. BP contributed to the writing and proofreading of the manuscript. CG gave scientific advice and supervised the work.

ACKNOWLEDGMENTS

The work was supported in part by the European Commission under the project Univax (contract No. 601738) and TRANSVAC (contract No. 228403). Furthermore, we are particularly grateful to Elena Reinhard for her outstanding technical help.

REFERENCES

Bagnoli, F., Fontana, M. R., Soldaini, E., Mishra, R. P., Fiaschi, L., Cartocci, E., et al. (2015). Vaccine composition formulated with a novel TLR7-dependent adjuvant induces high and broad protection against Staphylococcus aureus. *Proc. Natl. Acad. Sci. U.S.A.* 112, 3680–3685. doi: 10.1073/pnas.1424924112

Batista-Duharte, A., Martinez, D. T., and Carlos, I. Z. (2018). Efficacy and safety of immunological adjuvants. Where is the cut-off? *Biomed. Pharmacother.* 105, 616–624. doi: 10.1016/j.biopha.2018.06.026

Brewer, J. M., Conacher, M., Hunter, C. A., Mohrs, M., Brombacher, F., and Alexander, J. (1999). Aluminium hydroxide adjuvant initiates strong antigen-specific Th2 responses in the absence of IL-4- or IL-13-mediated signaling. *J. Immunol.* 163, 6448–6454.

Burdette, D. L., Monroe, K. M., Sotelo-Troha, K., Iwig, J. S., Eckert, B., Hyodo, M., et al. (2011). STING is a direct innate immune sensor of cyclic di-GMP. *Nature* 478, 515–518. doi: 10.1038/nature10429

Chen, K., and Cerutti, A. (2010). Vaccination strategies to promote mucosal antibody responses. *Immunity* 33, 479–491. doi: 10.1016/j.immuni.2010.09.013

Del Giudice, G., Rappuoli, R., and Didierlaurent, A. M. (2018). Correlates of adjuvanticity: a review on adjuvants in licensed vaccines. *Semin. Immunol.* 39, 14–21. doi: 10.1016/j.smim.2018.05.001

Di Pasquale, A., Bonanni, P., Garcon, N., Stanberry, L. R., El-Hodhod, M., and Tavares Da Silva, F. (2016). Vaccine safety evaluation: practical aspects in assessing benefits and risks. *Vaccine* 34, 6672–6680. doi: 10.1016/j.vaccine.2016.10.039

Di Pasquale, A., Preiss, S., Tavares Da Silva, F., and Garcon, N. (2015). Vaccine Adjuvants: from 1920 to 2015 and Beyond. *Vaccines (Basel)* 3, 320–343. doi: 10.3390/vaccines3020320

Didierlaurent, A. M., Morel, S., Lockman, L., Giannini, S. L., Bisteau, M., Carlsen, H., et al. (2009). AS04, an aluminum salt- and TLR4 agonist-based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity. *J. Immunol.* 183, 6186–6197. doi: 10.4049/jimmunol.0901474

Dubensky, T. W. Jr., Kanne, D. B., and Leong, M. L. (2013). Rationale, progress and development of vaccines utilizing STING-activating cyclic dinucleotide adjuvants. *Ther. Adv. Vaccines* 1, 131–143. doi: 10.1177/2051013613501988

Ebensen, T., Debarry, J., Pedersen, G. K., Blazejewska, P., Weissmann, S., Schulze, K., et al. (2017). Mucosal administration of cycle-Di-nucleotide-adjuvanted virosomes efficiently induces protection against influenza H5N1 in mice. *Front. Immunol.* 8:1223. doi: 10.3389/fimmu.2017.01223

Ebensen, T., and Guzman, C. A. (2008). Immune modulators with defined molecular targets: cornerstone to optimize rational vaccine design. *Hum. Vaccin.* 4, 13–22. doi: 10.1007/978-1-4419-1132-2_13

Ebensen, T., Libanova, R., Schulze, K., Yevsa, T., Morr, M., and Guzman, C. A. (2011). Bis-(3',5')-cyclic dimeric adenosine monophosphate: strong Th1/Th2/Th17 promoting mucosal adjuvant. *Vaccine* 29, 5210–5220. doi: 10.1016/j.vaccine.2011.05.026

Ebensen, T., Schulze, K., Riese, P., Link, C., Morr, M., and Guzman, C. A. (2007a). The bacterial second messenger cyclic diGMP exhibits potent adjuvant properties. *Vaccine* 25, 1464–1469. doi: 10.1016/j.vaccine.2006.10.033

Ebensen, T., Schulze, K., Riese, P., Morr, M., and Guzman, C. A. (2007b). The bacterial second messenger cdiGMP exhibits promising activity as a mucosal adjuvant. *Clin. Vaccine Immunol.* 14, 952–958. doi: 10.1128/cvi.00119-07

FDA (2011). *Common Ingredients in U.S. Licensed Vaccines* [Online]. U.S. Food and Drug Administration. Available online at: http://www.fda.gov/BiologicsBloodVaccines/SafetyAvailability/VaccineSafety/ucm187810.htm [Accessed 2011].

Garcon, N., and Di Pasquale, A. (2017). From discovery to licensure, the Adjuvant System story. *Hum. Vaccin. Immunother.* 13, 19–33. doi: 10.1080/21645515.2016.1225635

Garçon, N., Morel, S., Didierlaurent, A., Descamps, D., Wettendorff, M., and Van Mechelen, M. (2011). Development of an AS04-adjuvanted HPV vaccine with the adjuvant system approach. *BioDrugs* 25, 217–226. doi: 10.2165/11591760-000000000-00000

Global Health Observatory Data Repository (2016). *Global Health Estimates 2016: DALYs by Cause Globally*. Geneva: World Health Organization.

Gutjahr, A., Tiraby, G., Perouzel, E., Verrier, B., and Paul, S. (2016). Triggering intracellular receptors for vaccine adjuvantation. *Trends Immunol.* 37, 573–587. doi: 10.1016/j.it.2016.07.001

He, P., Zou, Y., and Hu, Z. (2015). Advances in aluminum hydroxide-based adjuvant research and its mechanism. *Hum. Vaccin. Immunother.* 11, 477–488. doi: 10.1080/21645515.2014.1004026

Jankovic, D., Kugler, D. G., and Sher, A. (2010). IL-10 production by CD4+ effector T cells: a mechanism for self-regulation. *Mucosal Immunol.* 3, 239–246. doi: 10.1038/mi.2010.8

Kara, E. E., Comerford, I., Fenix, K. A., Bastow, C. R., Gregor, C. E., McKenzie, D. R., et al. (2014). Tailored immune responses: novel effector helper T cell subsets in protective immunity. *PLoS Pathog.* 10:e1003905. doi: 10.1371/journal.ppat.1003905

Lamm, M. E., and Phillips-Quagliata, J. M. (2002). Origin and homing of intestinal IgA antibody-secreting cells. *J. Exp. Med.* 195, F5–F8. doi: 10.1084/jem.20011910

Lee, S., and Nguyen, M. T. (2015). Recent advances of vaccine adjuvants for infectious diseases. *Immune Netw.* 15, 51–57. doi: 10.4110/in.2015.15.2.51

Libanova, R., Ebensen, T., Schulze, K., Bruhn, D., Norder, M., Yevsa, T., et al. (2010). The member of the cyclic di-nucleotide family bis-(3', 5')-cyclic dimeric inosine monophosphate exerts potent activity as mucosal adjuvant. *Vaccine* 28, 2249–2258. doi: 10.1016/j.vaccine.2009.12.045

Lirussi, D., Ebensen, T., Schulze, K., Trittel, S., Duran, V., Liebich, I., et al. (2017). Type I IFN and not TNF, is essential for cyclic Di-nucleotide-elicited CTL by a cytosolic cross-presentation pathway. *EBioMedicine* 22, 100–111. doi: 10.1016/j.ebiom.2017.07.016

Matos, M. N., Cazorla, S. I., Schulze, K., Ebensen, T., Guzman, C. A., and Malchiodi, E. L. (2017). Immunization with Tc52 or its amino terminal domain adjuvanted with c-di-AMP induces Th17+Th1 specific immune responses and confers protection against Trypanosoma cruzi. *PLoS Negl. Trop. Dis.* 11:e0005300. doi: 10.1371/journal.pntd.0005300

McKee, A. S., and Marrack, P. (2017). Old and new adjuvants. *Curr. Opin. Immunol.* 47, 44–51. doi: 10.1016/j.coi.2017.06.005

McWhirter, S. M., Barbalat, R., Monroe, K. M., Fontana, M. F., Hyodo, M., Joncker, N. T., et al. (2009). A host type I interferon response is induced by cytosolic sensing of the bacterial second messenger cyclic-di-GMP. *J. Exp. Med.* 206, 1899–1911. doi: 10.1084/jem.20082874

Mittal, A., Schulze, K., Ebensen, T., Weissmann, S., Hansen, S., Guzman, C. A., et al. (2015). Inverse micellar sugar glass (IMSG) nanoparticles for transfollicular vaccination. *J Control Release* 206, 140–152. doi: 10.1016/j.jconrel.2015.03.017

Ng, T. H., Britton, G. J., Hill, E. V., Verhagen, J., Burton, B. R., and Wraith, D. C. (2013). Regulation of adaptive immunity; the role of interleukin-10. *Front. Immunol.* 4:129. doi: 10.3389/fimmu.2013.00129

Oleszycka, E., McCluskey, S., Sharp, F. A., Munoz-Wolf, N., Hams, E., Gorman, A. L., et al. (2018). The vaccine adjuvant alum promotes IL-10 production that suppresses Th1 responses. *Eur. J. Immunol.* 48, 705–715. doi: 10.1002/eji.201747150

Patil, S. U., and Shreffler, W. G. (2018). Novel vaccines: technology and development. *J. Allergy Clin. Immunol.* doi: 10.1016/j.jaci.2018.05.021. [Epub ahead of print].

Reed, S. G., Bertholet, S., Coler, R. N., and Friede, M. (2009). New horizons in adjuvants for vaccine development. *Trends Immunol.* 30, 23–32. doi: 10.1016/j.it.2008.09.006

Riese, P., Schulze, K., Ebensen, T., Prochnow, B., and Guzman, C. A. (2013). Vaccine adjuvants: key tools for innovative vaccine design. *Curr. Top. Med. Chem.* 13, 2562–2580. doi: 10.2174/15680266113136660183

Ross, P. J., Sutton, C. E., Higgins, S., Allen, A. C., Walsh, K., Misiak, A., et al. (2013). Relative contribution of Th1 and Th17 cells in adaptive immunity to Bordetella pertussis: towards the rational design of an improved acellular pertussis vaccine. *PLoS Pathog.* 9:e1003264. doi: 10.1371/journal.ppat.1003264

Sanchez Alberti, A., Bivona, A. E., Cerny, N., Schulze, K., Weissmann, S., Ebensen, T., et al. (2017). Engineered trivalent immunogen adjuvanted with a STING agonist confers protection against Trypanosoma cruzi infection. *N.P. J. Vaccines* 2, 9. doi: 10.1038/s41541-017-0010-z

Schulze, K., Ebensen, T., Babiuk, L. A., Gerdts, V., and Guzman, C. A. (2017a). Intranasal vaccination with an adjuvanted polyphosphazenes nanoparticle-based vaccine formulation stimulates protective immune responses in mice. *Nanomedicine* 13, 2169–2178. doi: 10.1016/j.nano.2017.05.012

Schulze, K., Ebensen, T., Chandrudu, S., Skwarczynski, M., Toth, I., Olive, C., et al. (2017b). Bivalent mucosal peptide vaccines administered using the LCP carrier system stimulate protective immune responses against Streptococcus pyogenes infection. *Nanomedicine* 13, 2463–2474. doi: 10.1016/j.nano.2017.08.015

Shu, C., Yi, G., Watts, T., Kao, C. C., and Li, P. (2012). Structure of STING bound to cyclic di-GMP reveals the mechanism of cyclic dinucleotide recognition by the immune system. *Nat. Struct. Mol. Biol.* 19, 722–724. doi: 10.1038/nsmb.2331

Tandrup Schmidt, S., Foged, C., Korsholm, K. S., Rades, T., and Christensen, D. (2016). Liposome-based adjuvants for subunit vaccines: formulation strategies for subunit antigens and immunostimulators. *Pharmaceutics* 8:E7. doi: 10.3390/pharmaceutics8010007

Temizoz, B., Kuroda, E., and Ishii, K. J. (2018). Combination and inducible adjuvants targeting nucleic acid sensors. *Curr. Opin. Pharmacol.* 41, 104–113. doi: 10.1016/j.coph.2018.05.003

Conflict of Interest Statement: 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 Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 01 March 2019
doi: 10.3389/fcimb.2019.00045

Oral Vaccination With a Formulation Combining *Rhipicephalus microplus* Subolesin With Heat Inactivated *Mycobacterium bovis* Reduces Tick Infestations in Cattle

Marinela Contreras[1], *Paul D. Kasaija*[1,2], *Octavio Merino*[3], *Ned I. de la Cruz-Hernandez*[3], *Christian Gortazar*[1] *and José de la Fuente*[1,4]*

[1] SaBio, Instituto de Investigación en Recursos Cinegéticos IREC-CSIC-UCLM-JCCM, Ciudad Real, Spain, [2] National Livestock Resources Research Institute (NaLIRRI/NARO), Tororo, Uganda, [3] Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma de Tamaulipas, Ciudad Victoria, Mexico, [4] Department of Veterinary Pathobiology, Center for Veterinary Health Sciences, Oklahoma State University, Stillwater, OK, United States

OPEN ACCESS

Edited by:
Alberto Moreno,
Emory University School of Medicine,
United States

Reviewed by:
Riti Sharan,
Texas A&M Health Science Center,
United States
Adriana Seixas,
Federal University of Health Sciences
of Porto Alegre, Brazil

***Correspondence:**
José de la Fuente
jose_delafuente@yahoo.com

Specialty section:
This article was submitted to Clinical Microbiology, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: 16 November 2018
Accepted: 13 February 2019
Published: 01 March 2019

Citation:
Contreras M, Kasaija PD, Merino O, de la Cruz-Hernandez NI, Gortazar C and de la Fuente J (2019) Oral Vaccination With a Formulation Combining Rhipicephalus microplus Subolesin With Heat Inactivated Mycobacterium bovis Reduces Tick Infestations in Cattle. Front. Cell. Infect. Microbiol. 9:45. doi: 10.3389/fcimb.2019.00045

Vaccines are an environmentally friendly alternative to acaracides for the control of tick infestations, to reduce the risk for tick-borne diseases affecting human and animal health worldwide, and to improve animal welfare and production. Subolesin (SUB, also known as 4D8) is the functional homolog of Akirin2 involved in the regulation of development and innate immune response, and a proven protective antigen for the control of ectoparasite infestations and pathogen infection. Oral vaccination combining protein antigens with immunostimulants has proven efficacy with increased host welfare and safety, but has not been used for the control of tick infestations. Here we describe the efficacy of oral vaccination with a formulation combining *Rhipicephalus microplus* SUB and heat inactivated *Mycobacterium bovis* (IV) on cattle tick infestations and fertility. The levels of IgG antibody titers against SUB and *M. bovis* P22, and the expression of selected immune response genes were determined and analyzed as possible correlates of protection. We demonstrated that oral immunization with the SUB+IV formulation resulted in 51% reduction in the number of female ticks and 30% reduction in fertility with an overall efficacy of 65% in the control of *R. microplus* infestations by considering the cumulative effect on reducing tick survival and fertility in cattle. The *akr2*, *IL-1β,* and *C3* mRNA levels together with antibody levels against SUB correlated with vaccine efficacy. The effect of the oral immunization with SUB+IV in cattle on tick survival and fertility is essential to reduce tick infestations, and extended previous results on the effect of *R. microplus* SUB for the control of cattle tick infestations. These results support the development of oral vaccines formulations for the control of tick infestations and the incidence of tick-borne diseases.

Keywords: subolesin, cattle tick, vaccine, oral vaccination, *Rhipicephalus microplus*, heat inactivated *Mycobacterium bovis*

INTRODUCTION

Ticks are arthropod vectors of pathogens affecting human and animal health as well as animal welfare and production worldwide (Jongejan and Uilenberg, 2004; de la Fuente et al., 2008; Rashid et al., 2018). The cattle tick *Rhipicephalus microplus* Canestrini (Acari: Ixodidae) are economically important as parasites of a variety of livestock species with an impact on cattle industry in tropical and subtropical regions of the world (Rashid et al., 2018).

Despite the use of traditional cattle tick control methods such as the use of chemical acaricides, habitat management, and genetic selection of animals with higher resistance to ticks, tick prevalence continues to be a major economic problem for the cattle industry (de la Fuente et al., 2017; Rashid et al., 2018). This persistent problem is due to several factors including acaracide resistance in ticks and safety issues associated with these chemicals, which support the development of vaccines as an effective and environmentally sound approach for the control of tick infestations (de la Fuente and Contreras, 2015; de la Fuente et al., 2016b; 2017; de la Fuente, 2018). The commercial vaccines based on the *R. microplus* BM86 or BM95 recombinant antigens proved their efficacy for the control of cattle tick infestations and the reduction in the prevalence of certain tick-borne pathogens (de la Fuente et al., 2007, 2017; de la Fuente and Contreras, 2015; Rodríguez-Mallon, 2016; de la Fuente, 2018).

Tick Subolesin (SUB, also known as 4D8) is the functional ortholog of Akirin2 and is involved in the regulation of different biological processes including development and innate immune response (Artigas-Jerónimo et al., 2018). SUB was discovered as a tick protective antigen (Almazán et al., 2010), and since then it has shown vaccination efficacy for the control of infestations by different arthropod ectoparasite species and pathogen infection and transmission (recently reviewed by de la Fuente and Contreras, 2015; Artigas-Jerónimo et al., 2018).

Recent advances in tick vaccine research have resulted in the identification of new protective antigens for the control of tick infestations (recently reviewed by de la Fuente and Contreras, 2015; de la Fuente et al., 2016b, 2017; de la Fuente, 2018). However, research aimed at improving tick vaccine efficacy and safety by combining protective antigens and oral formulations is still to be done. Oral or intranasal vaccine formulations are easier to administer, and have proven efficacy with increased host welfare and safety by reducing stress and the risk of contamination or infection at the injection site and pathogen mechanical transmission (Wang et al., 2015; Lawan et al., 2018). However, orally delivered protein vaccines have a relatively low immunogenicity and antigen stability after immunization that require vaccine formulations with selected combinations of antigens and immunostimulants, and needleless delivery systems (Fry et al., 2012; Wang et al., 2015). In this context, the heat inactivated *Mycobacterium bovis* (IV) has been shown to activate the innate immune response-mediated trained immunity through complement component 3 (C3) to reduce mycobacterial infection and tuberculosis-like lesions in cattle, deer, pig, and zebrafish orally or systemically vaccinated with IV (Beltrán-Beck et al., 2014; de la Fuente et al., 2016a; Juste et al., 2016; Thomas et al., 2017; López et al., 2018, 2019; Risalde et al., 2018). Therefore, IV appears as a good immunostimulant candidate for oral vaccine formulations (de la Fuente et al., 2016a).

As a proof of concept of oral tick vaccine formulations, in this study we orally vaccinated cattle via needleless syringe using a formulation combining *R. microplus* SUB with IV for the control of cattle tick infestation. The results showed an effect of the oral vaccination on the reduction in the number of female ticks and fertility. Additionally, the *akirin2* (*akr2*), *interleukin-1beta* (*IL-1β*), and *C3* mRNA levels together with antibody levels against SUB correlated with vaccine efficacy. These results support research for the development of oral vaccines formulations for the control of tick infestations and the incidence of tick-borne diseases.

MATERIALS AND METHODS

Ticks

The *R. microplus* (Susceptible Media Joya strain, CENAPA, Mexico) ticks were obtained from a laboratory colony maintained at the University of Tamaulipas, Mexico (Merino et al., 2011). Tick larvae were fed on cross-bred *Bos taurus* cattle and collected after repletion to allow for oviposition and hatching in humidity chambers at 12 h light:12 h dark photoperiod, 22–25°C and 95% relative humidity (RH). Larvae were used for infestations at 15 days after hatching from eggs.

Antigen Production and Vaccine Formulations

The synthetic *R. microplus* histidine-tag recombinant SUB (Genbank accession number GQ456170) with optimized codon usage for *Escherichia coli* was produced in *E. coli* BL21 and purified to >95% purity (**Figure 1**) by Ni affinity chromatography using 1 ml HisTrap FF columns mounted on an AKTA–FPLC system (GE Healthcare, Piscataway, NJ, USA) in the presence of 7 M urea lysis buffer as previously described (Almazán et al., 2010; Contreras et al., 2015). Protein concentration was measured using the Pierce® BCA protein assay kit (Thermo Scientific, Rockford, IL, USA).

The IV was prepared from *M. bovis* field isolate Neiker 1403 (spoligotype SB0339 originally isolated from a naturally infected wild boar) IV at Neiker-Tecnalia (Derio, Spain) under good manufacturing practices as previously described (Beltrán-Beck et al., 2014; Juste et al., 2016; Risalde et al., 2018). Briefly, the isolate was cultivated for 2–3 weeks in Middlebrook 7H9 medium enriched with OADC growth supplement (Sigma-Aldrich, St. Louis, MI, USA), and inactivated in a shaking water bath at 81–83°C for 40 min. The inactivated inoculum was cultured in BACTEC Mycobacterial Growth Indicator Tubes (Becton Dickinson; Franklin Lakes, NJ, USA) and onto OADC agar solidified 7H9 plates in triplicate (100 μl each) to confirm the absence of viable mycobacteria. The final *M. bovis* IV preparation contained approximately the equivalent of 10^7 colony forming units (cfu) in 0.2 ml of PBS.

For vaccine formulation, purified recombinant SUB protein (200 μg) was mixed with 6 x 10^6 cfu IV in 18 ml PBS for SUB+IV group, or the same amount of IV was placed in 18 ml PBS

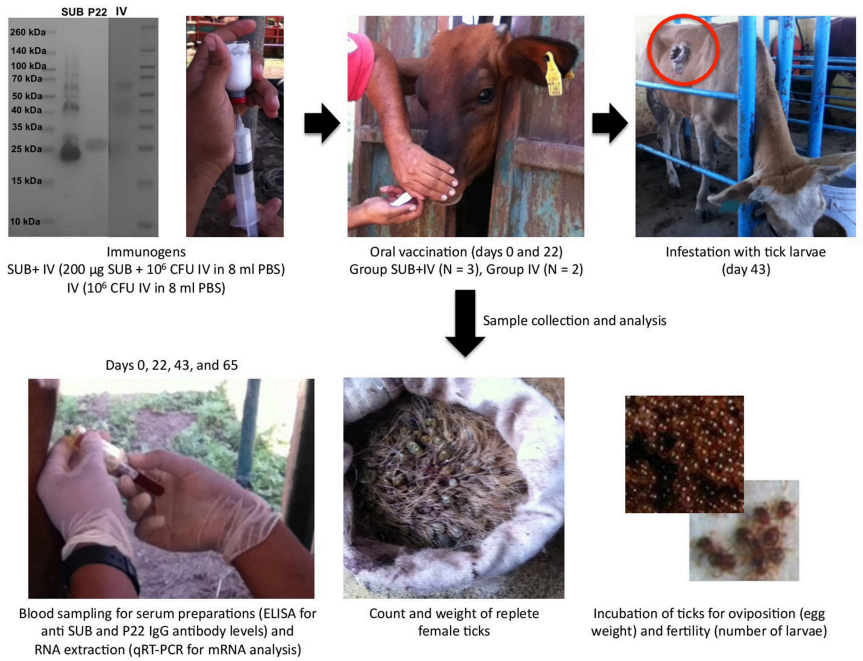


FIGURE 1 | Experimental design. The antigens used in the study included recombinant *R. microplus* SUB (>95% purity), heat inactivated *M. bovis* IV proteins and *M. bovis* P22 (as an example, a Coomassie-blue stained SDS-12% polyacrilamide gel is shown with the three antigens and spectra multicolor molecular weight markers from Thermo Scientific). Cattle were orally vaccinated via needleless syringe on days 0 and 22 using a formulation combining *R. microplus* SUB antigen with IV as immunostimulator (SUB+IV group) or IV alone (IV group) for comparative analyses between both groups. After vaccination, cattle were infested with *R. microplus* larvae on day 43 for the analysis of vaccine efficacy on the control of tick development and fertility, and the identification of possible correlates of protection and immune mechanisms based on the host antibody response and the mRNA levels for selected immune response genes.

for control IV group. Vaccine formulations were stored at 4°C until used.

Cattle Vaccination and Tick Infestation

Three and two 10-month-old European crossbred calves were randomly assigned to SUB+IV and IV vaccinated groups. Vaccines were administered via needleless syringe in the lateral region of the mouth followed by slightly raising the head of the calves (Jones et al., 2016). Cattle were vaccinated at days 0 and 22, and then infested at day 43 with 500 *R. microplus* larvae in single cells glued on the back of the calves. Adult engorged female ticks dropping from cattle were daily collected, counted, and weighted. All the collected adult female ticks were assessed for oviposition (egg mass weight/tick) and egg fertility (number of larvae/tick) to calculate vaccine efficacy (E) as previously reported (Merino et al., 2011, 2013) using only parameters with significant differences between groups (**Table 1**). The personnel vaccinating cattle and collecting the ticks were "blinded" as to which group animals belonged. Data were analyzed statistically to compare results between individuals fed on SUB+IV and IV vaccinated calves by Student's *t*-test with unequal variance ($p = 0.05$).

Analysis of Cattle IgG Antibody Response by ELISA

Serum samples were prepared from blood samples collected from each calf before each immunization (days 0 and 22), tick infestation (day 43) and at the end of the experiment (day 65), and stored at −20°C until analysis. An indirect ELISA test was performed to detect IgG antibodies against *R. microplus* SUB and *M. bovis* P22 proteins as described previously (Merino et al., 2011, 2013; Infantes-Lorenzo et al., 2018). High absorption capacity polystyrene microtiter plates were coated with 100 µl (0.01 µg/ml solution of purified recombinant proteins) per well in carbonate-bicarbonate buffer

TABLE 1 | Results of the oral vaccination on cattle tick infestations.

Group (cattle no.)	No. female ticks	Female tick weight (mg)	Oviposition egg mass/tick (mg)	Fertility (No. larvae/tick)
SUB+IV (1)	36	233 ± 92	98 ± 61	592 ± 490
SUB+IV (3)	59	210 ± 68	116 ± 37	961 ± 620
SUB+IV (5)	73	234 ± 47	104 ± 38	819 ± 610
Average ± S.D.	56 ± 19*	226 ± 14	106 ± 9	791 ± 186**
Control IV (2)	113	206 ± 53	114 ± 40	1,118 ± 571
Control IV (4)	116	207 ± 54	114 ± 39	1,157 ± 558
Average ± S.D.	115 ± 2	207 ± 1	114 ± 0	1,138 ± 28

*Results are shown for each vaccinated and infested cattle (N = 3 for SUB+IV and N = 2 for IV). Data was analyzed statistically to compare results between ticks fed on SUB+IV and IV vaccinated cattle (*p = 0.02, **p = 0.04; Student's t-test with unequal variance). Vaccine efficacy (E = 65%) was calculated as E = 100 x [1-(DT x DF)], where DT = No. female ticks in SUB+IV vaccinated calves/female ticks in IV vaccinated calves and DF = No. larvae/tick in SUB+IV vaccinated calves/No. larvae/tick in IV vaccinated calves.*

(Sigma-Aldrich). After an overnight incubation at 4°C, coated plates were blocked with 100 μl/well of blocking solution (5% skim milk in PBS). Serum samples or PBS as negative control were diluted (1:1000, 1:5000 and 1:10000, v/v) in blocking solution and 100 μl/well were added into duplicate wells of the antigen-coated plates. After an overnight incubation at 4°C, the plates were washed three times with a washing solution (PBS containing 0.05% Tween 20) and then incubated with 1:20,000 rabbit anti-bovine immunoglubolin G (IgG)–horseradish peroxidase conjugate (Sigma-Aldrich) for 1 h at 37°C. After three washes with washing solution, 100 μl/well of substrate solution (Fast OPD, Sigma-Aldrich) was added. Finally, the reaction was stopped with 50 μl/well of 2 N H_2SO_4 and the optical density (OD) was measured in a spectrophotometer at 450 nm. Antibody titers in SUB+IV and IV vaccinated cattle were expressed as the $O.D_{450nm}$ ($O.D_{cattlesera}$ – $O.D_{PBScontrol}$) and compared between groups by ANOVA test ($p = 0.05$; https://www.socscistatistics.com/tests/anova/Default2.aspx).

Analysis of Gene Expression by qRT-PCR

Total RNA was extracted from blood samples collected from each calf before each immunization (days 0 and 22), tick infestation (day 43) and at the end of the experiment (day 65) using TRI Reagent BD (Sigma-Aldrich, St. Louis, MI, USA) following the manufacturer's instructions. Gene expression profiles from selected genes involved in immunity *C3* (NM_001040469.2), tumor necrosis factor alpha (*TNF-α*; AF348421), *IL-1β* (NM174093), interleukin 2 (*IL-2*; NM180997), *akr2* (NM_001110087.1), and interleukin 12 (*IL-12*; U11815.1) were analyzed by qRT-PCR using the following gene-specific forward (F) and reverse (R) 5′- 3′ primers and annealing temperature (*C3*, F: ATTGCCAGGTTCTTGTACGG and R: GTCACTGCCTGATTGCAAGA, 56°C; *TNF-α*, F: CCTC ACCCACACCATCAG, and R: GCGATCTCCCTTCTCCAG, 54°C; *IL-1β*, F: TCAGAATGGAAACCCTCTCTC and R: GCATGGATCAGACAACAGTG, 56°C; *IL-12*, F: AAGT GAAGTCATTGCTGCTG and R: TGTCCATTGAATCCTTG ATCTC, 54°C; *akr2*, F: CATTTATGGGCTGCCTTGTT and R: TGCACAGCTTCTACCACGAC, 54°C; *IL-12*, F: TCTGG ACACTTCACCTGCTG and R: TGCACAGCTTCTACCA CGAC, 58°C) and a Quantitect SYBR Green RT-PCR Kit in a Rotor Gene Q thermocycler (Qiagen, Inc. Valencia, CA, USA) following manufacturer's recommendations. A dissociation curve was run at the end of the reaction to ensure that only one amplicon was formed and that the amplicon denatured consistently in the same temperature range for every sample (Ririe et al., 1997). The mRNA Ct values were normalized against *Bos taurus ß-actin* (AY141970.1, F: GGCCGAGCGGAAATCG and R: GCCATCTCCTGCTCG AAGTC, 52°C) using the genNorm ddCT method (Livak and Schmittgen, 2001). The normalized mRNA levels (mean of duplicated and normalized Ct values) were compared between SUB+IV and IV vaccinated groups by ANOVA test ($p = 0.05$; https://www.socscistatistics.com/tests/anova/Default2.aspx).

Correlation Analysis

Two different Spearman's Rho correlation analyses ($p = 0.05$; https://www.socscistatistics.com/tests/spearman/Default2.aspx) were conducted for the identification of putative correlates of vaccine efficacy. The analysis was conducted in individual SUB+IV and IV vaccinated cattle. The first analysis was conducted between the number of female ticks or larvae/tick and antibody titers ($O.D_{450nm}$) or the normalized mRNA levels at the time of tick infestations (day 43) and at the end of the experiment (day 65). The second analysis was conducted between the normalized mRNA levels and antibody titers ($O.D_{450nm}$) at the time of tick infestations (day 43) and the end of the experiment (day 65).

RESULTS AND DISCUSSION

Rationale and Experimental Design

Despite the proven efficacy of *R. microplus* BM86 and BM95 based vaccines for the control of cattle tick infestations (de la Fuente et al., 2007, 2017; de la Fuente and Contreras, 2015; Rodríguez-Mallon, 2016; de la Fuente, 2018) and recent advances in the identification of new tick protective antigens (de la Fuente and Contreras, 2015; de la Fuente et al., 2016b, 2017; de la Fuente, 2018), research is needed for the development of vaccine formulations with higher efficacy and safety for the control of tick infestations and tick-borne diseases (de la Fuente, 2018). To address this challenge, the objective of this study was to provide a proof of concept for oral vaccine formulations for the control of cattle tick infestations, and the identification of candidate correlates of vaccine efficacy. Oral vaccine formulations are easier to administer and offer the possibility of reducing stress and vaccination-associated risks while increasing protective efficacy with effective immunostimulants (Wang et al., 2015; Lawan et al., 2018).

The experimental design for this study included cattle vaccination and *R. microplus* infestations for the analysis of vaccine efficacy on the control of tick development and fertility (**Figure 1**). Cattle were orally vaccinated via needleless syringe using a formulation combining *R. microplus* SUB antigen with proven efficacy in the control of ectoparasite infestations (de la Fuente and Contreras, 2015; Artigas-Jerónimo et al., 2018) and IV with immunostimulatory activity (Beltrán-Beck et al., 2014; de la Fuente et al., 2016a; Juste et al., 2016; Thomas et al., 2017; López et al., 2018, 2019; Risalde et al., 2018) (SUB+IV group) or IV alone (IV group) for comparative analyses between both groups. An untreated group was not included because the study focused at comparing the effect of the SUB+IV combined formulation with any background of the IV immunostimulant alone. The immune markers selected to characterize possible correlates of vaccine protection included IgG antibodies, which have been shown to mediate SUB protective response (Merino et al., 2011, 2013), and the innate immune response-mediated trained immunity markers *akr2*, *IL-1β*, *IL-2*, *IL-12*, *TNF-α*, and *C3* involved in protective response to IV oral or systemic vaccination in different species (Beltrán-Beck et al., 2014; de la Fuente et al., 2016a; Juste et al., 2016; Thomas et al., 2017; López et al., 2018, 2019; Risalde et al., 2018).

Vaccine Efficacy for the Control of Cattle Tick Infestations

The results of the vaccination trial showed a 51% reduction in the number of engorged female ticks obtained in the SUB+IV group when compared to the IV control ($p = 0.02$; **Table 1**). This reduction in the number of ticks completing feeding was similar in all three cattle vaccinated with SUB+IV (**Table 1**). Additionally and despite animal-to-animal variations, a 30% reduction in tick fertility was observed in the eggs from ticks fed on SUB+IV vaccinated calves when compared to the IV control ($p = 0.04$; **Table 1**). The effect of tick vaccines on the reduction in the number of female ticks completing feeding and fertility has been identified as a critical parameter for the control of cattle tick infestations (Schetters et al., 2016). However, no differences were observed in the weight of engorged ticks or the oviposition (**Table 1**), a result that differs from previous vaccination trials with systemic SUB (Merino et al., 2011, 2013; Artigas-Jerónimo et al., 2018).

In a previous experiment (Contreras et al., 2015), sera from pigs orally immunized with SUB, SUB-Major surface protein 1a (MSP1a) *E. coli* membrane-bound chimera or PBS as control were used for *R. microplus* capillary feeding to evaluate tick weigh. The results of this preliminary trial showed no effect of anti-SUB antibodies but a 55% reduction in tick weight increase after feeding on SUB-MSP1a serum (Contreras et al., 2015). Bacterial membranes with the surface exposed SUB-MSP1a chimeric antigen were shown to enhance SUB immunogenicity and protective capacity, thus providing supporting evidences for the inclusion of bacterial-derived immunostimulants for SUB-based oral vaccine formulations (Contreras et al., 2015). The discrepancies on the effect of vaccination on tick weight may be attributable to different factors including differences in the immune response to various SUB antigens and between systemic and oral vaccine formulations.

Correlates of Vaccine Efficacy and Immune Mechanisms of Protection

To address the putative protective mechanisms of oral SUB+IV vaccination in cattle, the IgG antibody response against both SUB and IV (P22) were first characterized in all animals included in the study. The antibody response against SUB has been correlated before with vaccine efficacy in cattle (Merino et al., 2011, 2013). The P22 antigen is composed by various *M. bovis* proteins that have been validated before for the analysis of the antibody response in IV vaccinated hosts (Infantes-Lorenzo et al., 2017, 2018; López et al., 2018; Risalde et al., 2018).

Despite animal-to-animal differences in the IgG antibody response to both antigens (**Figure 2A**), the anti-SUB but not anti-P22 IgG antibody response was higher in SUB+IV vaccinated cattle when compared to IV vaccinated group ($p = 0.01$; **Figure 2B**). Differences in the animal-to-animal response could be attributed to variations in vaccine administration via

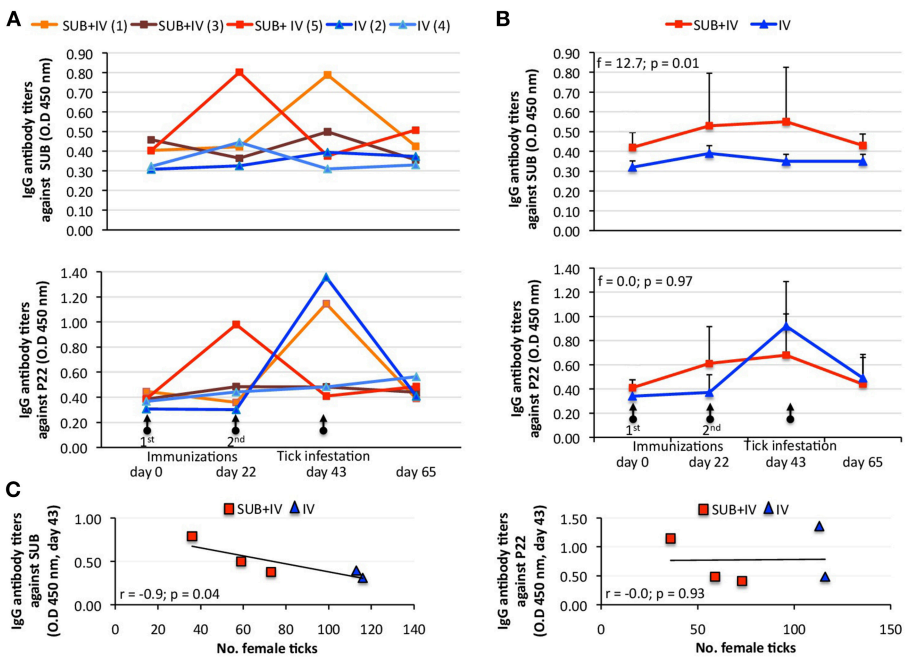


FIGURE 2 | Effect of oral vaccination and infestation with *R. microplus* larvae on the cattle IgG antibody response. Serum samples were collected before 1st (day 0) and 2nd (day 22) immunizations and tick infestation (day 43) with *R. microplus* larvae, and at the end of the experiment (day 65). **(A)** IgG antibody titers were determined by ELISA with serum samples (1:5,000) from individual vaccinated cattle against SUB or P22 proteins. **(B)** Antibody titers in vaccinated cattle were expressed as the average + S.D. $O.D_{450nm}$ ($O.D_{cattlesera} - O.D_{PBScontrol}$) and compared between SUB+IV and IV groups by ANOVA test ($p < 0.05$). **(C)** The Spearman's Rho correlation analyses ($p < 0.05$) were conducted to correlate the IgG antibody response to SUB or P22 at time of tick infestations (day 43) with the vaccine effect on tick. The linear correlation coefficients (r) and *p*-value are shown ($N = 5$).

needleless syringe. Furthermore, a correlation analysis between vaccine efficacy on the reduction in the number of female ticks and anti-SUB or P22 IgG antibody levels was conducted as one of the proposed correlates of protection in tick vaccine trials (de la Fuente and Contreras, 2015). As in previous tick SUB vaccination trials (Merino et al., 2011, 2013), a negative correlation ($r = -0.9$; $p = 0.04$) was obtained between the number of engorged female ticks and IgG antibody titers against SUB but not P22 in vaccinated cattle at day 43 (**Figure 2C**), showing a correlation between the effect of oral SUB+IV vaccination (IgG antibodies) and vaccine efficacy on tick development.

The protective mechanism associated with IV vaccination has been attributed to the activation of the innate immune response-mediated trained immunity through C3 pathway (Beltrán-Beck et al., 2014; de la Fuente et al., 2016a; Juste et al., 2016). Therefore, C3 pathway components and other immune response genes were selected for the analysis of mRNA levels in vaccinated cattle. The results showed a tendency toward an increase in mRNA levels in response to vaccination/infestation in SUB+IV vaccinated cattle (**Figure 3A**). In IV-vaccinated cattle, the tendency was a decrease in mRNA levels at days 22 and 65 following first vaccination and tick infestation, respectively, and an increase at day 43 after second vaccination (**Figure 3A**). Nevertheless, a positive correlation ($r = 0.9$; $p = 0.04$) was obtained only between *TNF-α* mRNA levels and IgG antibody titers against SUB but not P22 at day 43 (**Figure 3B**). Additionally, for C3 pathway immune response *akr2*, *IL-1β*, and *C3* genes a negative correlation ($r = -0.9$ to -1.0; $p = 0.005$ to 0.04) was observed between mRNA levels and the number of engorged female ticks at day 65 (**Figure 4**). Finally, *TNF-α* mRNA levels negatively correlated ($r = -0.9$; $p = 0.04$) with tick fertility (number of larvae per tick) at day 65 (**Figure 4**).

Recently, we proposed that the mechanism behind systemic SUB vaccine protective capacity is based on anti-SUB antibodies that could enter into tick cells by still unknown mechanisms where they can interact with cytosolic SUB to prevent its translocation to the nucleus and/or SUB-protein interactions necessary to exert its regulatory functions (de la Fuente et al., 2011) (**Figure 4**). SUB exerts its function through physical and/or functional interactions with other proteins that have not been fully characterized, but include some candidate proteins involved in the regulation of developmental processes potentially affecting tick feeding (Artigas-Jerónimo et al., 2018). Herein, the IgG antibody response against SUB also correlated with oral vaccination efficacy, therefore suggesting a similar protective mechanism in orally and systemically vaccinated cattle (**Figure 4**).

Considering the negative correlation between anti-SUB IgG antibody and C3 pathway gene mRNA levels and the number of female ticks, a protective mechanism for SUB+IV oral vaccination was proposed (**Figure 4**). Once the vaccine reaches the oral mucosa-associated lymphoid tissue underlying the epithelium, antigen uptake, processing and presentation occurs via microfold/membraneous cells (M) by antigen-presenting cells

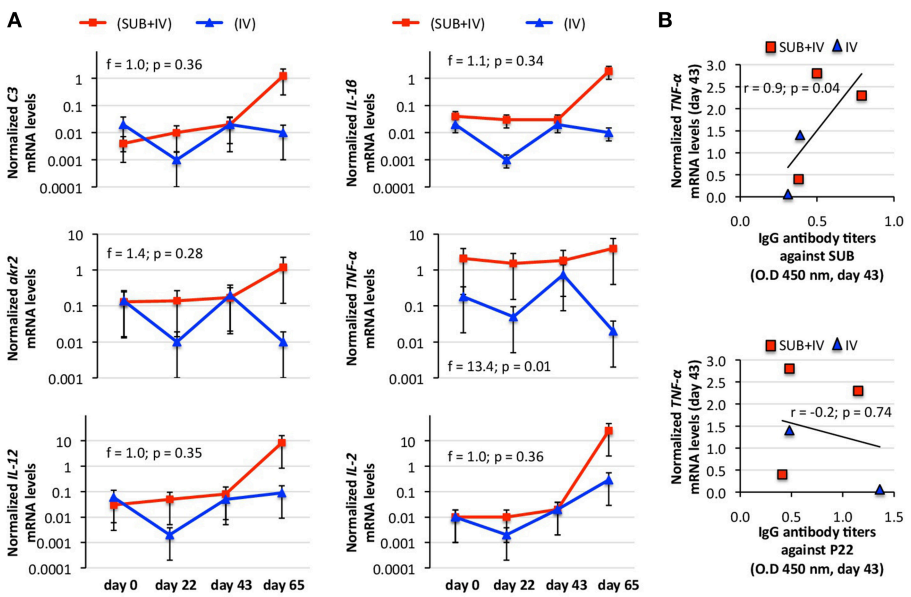


FIGURE 3 | Effect of oral vaccination and infestation with *R. microplus* larvae on the cattle mRNA levels for immune response genes. Blood mRNA was obtained from samples collected before 1st (day 0) and 2nd (day 22) immunizations and tick infestation (day 43) with *R. microplus* larvae, and at the end of the experiment (day 65). **(A)** The mRNA levels for immune response genes *C3*, *TNF-α*, *IL-1β*, *IL-2*, *akr2*, and *IL-12* were determined by qRT-PCR in vaccinated cattle, normalized against *B. taurus ß-actin* and normalized mRNA levels (mean of duplicated and normalized Ct values) compared between SUB+IV and IV groups by ANOVA test ($p < 0.05$). **(B)** The Spearman's Rho correlation analyses ($p < 0.05$; https://www.socscistatistics.com/tests/spearman/Default2.aspx) were conducted to correlate the IgG antibody response to mRNA levels at time of tick infestations (day 43). The normalized *TNF-α* mRNA levels were the only that positively correlated with anti-SUB but not P22 IgG antibody levels. The linear correlation coefficients (r) and *p*-value are shown ($N = 5$).

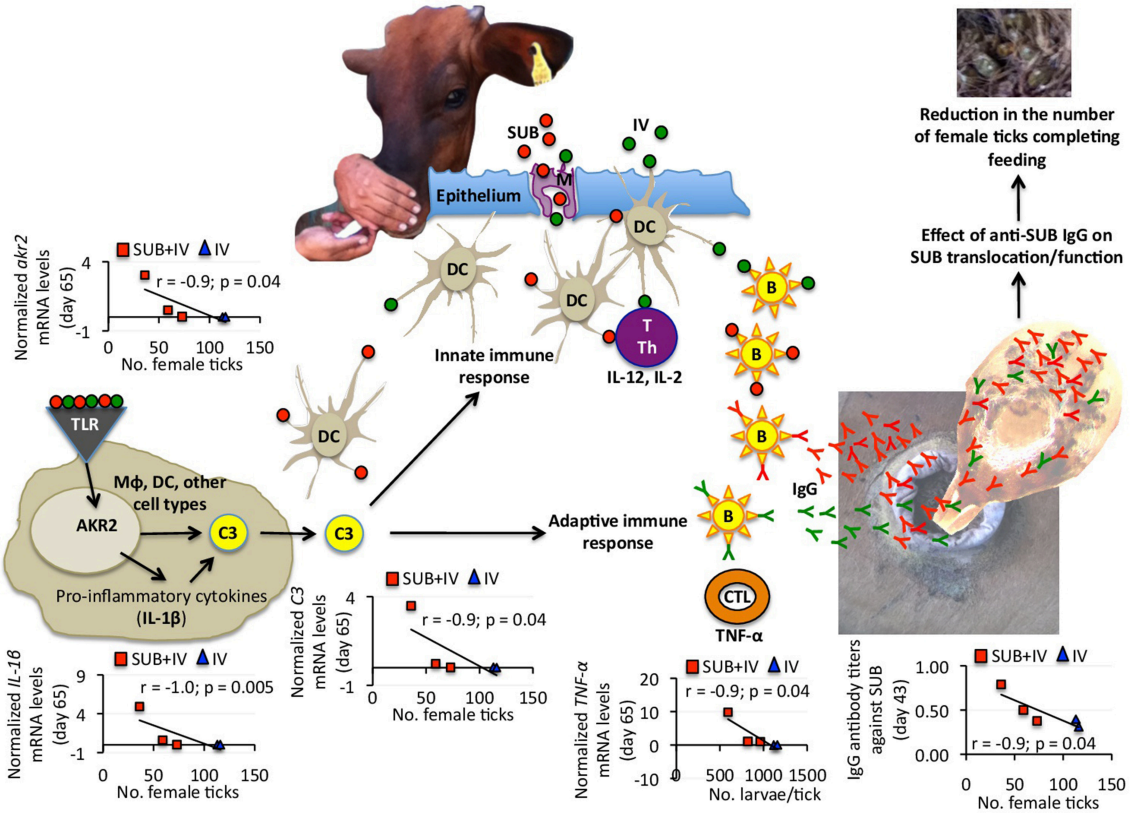


FIGURE 4 | Proposed mechanism of protection for oral SUB+IV vaccine against tick infestations in cattle. Based on the negative correlation between anti-SUB IgG antibody and C3 pathway gene mRNA levels and the number of female ticks, a protective mechanism for SUB+IV oral vaccination was proposed. After the vaccine reaches the oral mucosa-associated lymphoid tissue underlying the epithelium, antigen uptake, processing, and presentation occurs via microfold/membraneous cells (M) by antigen-presenting cells (APCs) such as macrophages (Mϕ), dendritic cells (DCs) and B cells (B). The C3 pathway activated in DCs and other cell types bridge the innate and adaptive immune systems for the initiation of antigen-specific response by activated T cells (T), T helper cells (Th), cytotoxic T lymphocytes (CTLs) and B cells. Activated adaptive immune response cells secrete cytokines such as IL-12, IL-2, and TNF-α to realize the systemic immune protection. The C3 pathway was activated after oral vaccination with SUB+IV to mediate the production of IgG antibodies affecting SUB translocation/function after ingestion with blood meal for the reduction of tick infestations.

(APCs) such as macrophages (Mϕ), dendritic cells (DCs) and B cells (B) (Wang et al., 2015) (**Figure 4**). The key APCs, DCs bridge the innate and adaptive immune systems for the initiation of antigen-specific response by activated T cells (T), T helper cells (Th), cytotoxic T lymphocytes (CTLs) and B cells (Holmgren and Czerkinsky, 2005; Peng et al., 2006; Lawson et al., 2011; Wang et al., 2015) (**Figure 4**). The C3 pathway mediates bridging of the innate and adaptive immune responses (Lubbers et al., 2017) (**Figure 4**). Activated adaptive immune response cells secrete cytokines such as IL-12, IL-2, and TNF-α, which mRNA levels positively correlated with anti-SUB antibody titers (**Figure 3B**), and then migrate to and multiply through the common mucosal immune system to realize the systemic immune protection (Seder and Hill, 2000; Wang et al., 2015). As also shown in this study, the production of neutralizing antibodies is vital for protective humoral immune response in reducing tick infestations (de la Fuente et al., 1998, 2011; Merino et al., 2011, 2013; Contreras and de la Fuente, 2016, 2017). In this process, the C3 pathway that was activated after oral vaccination with SUB+IV has been shown to mediate antibody response (Rutemark et al., 2011), a finding supported here by the positive correlation between *akr2*, *IL-1β*, and *C3* mRNA levels and the number of female ticks (**Figure 4**). Additionally, the proinflammatory cytokine IL-1β has been reported to induce antigen-specific serum IgG and lymphocyte proliferative responses with intranasally administered with soluble protein antigens (Staats and Ennis, 1999).

Finally, the negative correlation between *TNF-α* mRNA levels and the number of larvae per tick suggested that T-mediated response after SUB+IV oral vaccination may have a role in the reduction of tick fertility. As discussed above and supported in this study, antigen-specific IgG antibody response plays a key role in the protective response to tick vaccines (de la Fuente et al., 1998, 2011; Merino et al., 2011, 2013; Contreras and de la Fuente, 2016, 2017). However, the possible role of the complement, T immune response and other effector mechanisms has been considered before as part of tick vaccine efficacy (Tellam et al., 1992). The development of specific antibody- or T-mediated

immunologic responses and the activation of mucosally-induced immunity depend on complex sets of immunologic events, including the antigen-induced activation of different populations of B, T, and APC, and the expression of proinflammatory and immunoregulatory cytokines (Dinarello, 2000; Ogra et al., 2001). In particular, the proinflammatory cytokine TNF-α has been associated with DC maturation in mucosal immune response (Ogra et al., 2001). The results obtained here suggested that future experiments should address this component of the protective immune response to both systemic and oral vaccination with tick antigens. Additionally, other events and cytokines such as IL-4, IL-5, IL-6, IL-10, colony-stimulating factor (CSF), interferon gamma (IFN-γ) and transforming growth factor beta (TGF-β) reported to be involved in mucosal immune responses together with variations in APC, B, and T cell subsets should be addressed to provide additional support for the proposed protective mechanisms elicited by oral vaccination with SUB+IV.

CONCLUSIONS

The results of this study confirmed the efficacy of tick SUB for the control of cattle tick infestations, and expanded these results to oral vaccination with this antigen in combination with the IV immunostimulant. Furthermore, the identification of correlates of oral SUB+IV vaccine protection provided markers and targets for future experiments with larger number of animals aimed at conducting analyses to provide additional support for the protective immune mechanisms, the duration of the protective response, and optimizing vaccine formulation, delivery and efficacy. An optimal oral vaccine formulation requires the appropriate combination of antigens, immunostimulants and delivery carriers to induce a series of protective immune responses as shown here by the activation of the C3 pathway and the production of IgG antibodies and relevant cytokines (**Figure 4**). Research focused on oral tick vaccines would have a positive impact on the control of tick infestations and tick-borne diseases in humans, farm and companion animals through reducing stress and systemic vaccination-associated risks while increasing protective efficacy.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

ETHICS STATEMENT

The study was conducted in accordance with standards specified in the Guide for Care and Use of Laboratory Animals for the University of Tamaulipas (UAT), Mexico. The protocol was approved by the ethics committee of the UAT (No. CBBA_17_0).

AUTHOR CONTRIBUTIONS

MC, OM, CG, and JF conceived the study and designed the experiments. MC, PK, OM, and NdlC-H performed the experiments. MC and JF performed data analysis. MC, PK, CG, and JF wrote the manuscript. All authors approved and contributed to the final version of the manuscript.

FUNDING

This research was financially supported by the Ministerio de Economía, Industria y Competitividad (Spain) grant BFU2016-79892-P and the Fondo Sectorial de Investigación para la Educación (SEP-CONACYT, Mexico) project CB-2015-01-255205 (DICB/C1000/3284/2016).

ACKNOWLEDGMENTS

We thank Jenifer Naomi Gueta Spain and Justo Carrizales Zapata (UAT, Mexico) for technical assistance during vaccination trial.

REFERENCES

Almazán, C., Lagunes, R., Villar, M., Canales, M., Rosario-Cruz, R., Jongejan, F., et al. (2010). Identification and characterization of *Rhipicephalus (Boophilus) microplus* candidate protective antigens for the control of cattle tick infestations. *Parasitol. Res.*106, 471–479. doi: 10.1007/s00436-009-1689-1

Artigas-Jerónimo, S., Villar, M., Cabezas-Cruz, A., Valdés, J. J., Estrada-Peña, A., Alberdi, P., et al. (2018). Functional evolution of Subolesin/Akirin. *Front. Physiol.* 9:1612. doi: 10.3389/fphys.2018.01612

Beltrán-Beck, B., de la Fuente, J., Garrido, J. M., Aranaz, A., Sevilla, I., Villar, M., et al. (2014). Oral vaccination with heat inactivated *Mycobacterium bovis* activates the complement system to protect against tuberculosis. *PLoS ONE* 9:e98048. doi: 10.1371/journal.pone.0098048

Contreras, M., and de la Fuente, J. (2016). Control of *Ixodes ricinus* and *Dermacentor reticulatus* tick infestations in rabbits vaccinated with the Q38 Subolesin/Akirin chimera. *Vaccine* 34, 3010–3013. doi: 10.1016/j.vaccine.2016.04.092

Contreras, M., and de la Fuente, J. (2017). Control of infestations by *Ixodes ricinus* tick larvae in rabbits vaccinated with aquaporin recombinant antigens. *Vaccine* 35, 1323–1328. doi: 10.1016/j.vaccine.2017.01.052

Contreras, M., Moreno-Cid, J. A., Domingos, A., Canales, M., Díez-Delgado, I., Pérez de la Lastra, J. M., et al. (2015). Bacterial membranes enhance the immunogenicity and protective capacity of the surface exposed tick Subolesin-*Anaplasma marginale* MSP1a chimeric antigen. *Ticks Tick Borne Dis.* 6, 820–828. doi: 10.1016/j.ttbdis.2015.07.010

de la Fuente, J. (2018). Controlling ticks and tick-borne diseases...looking forward. *Ticks Tick Borne Dis.* 9, 1354–1357. doi: 10.1016/j.ttbdis.2018.04.001

de la Fuente, J., Almazán, C., Canales, M., Pérez de la Lastra, J. M., Kocan, K. M., and Willadsen, P. (2007). A ten-year review of commercial vaccine performance for control of tick infestations on cattle. *Anim. Health Res. Rev.* 8, 23–28. doi: 10.1017/S1466252307001193

de la Fuente, J., and Contreras, M. (2015). Tick vaccines: current status and future directions. *Expert Rev. Vaccines* 14, 1367–1376. doi: 10.1586/14760584.2015.1076339

de la Fuente, J., Contreras, M., Estrada-Peña, A., and Cabezas-Cruz, A. (2017). Targeting a global health problem: vaccine design and

challenges for the control of tick-borne diseases. *Vaccine* 35, 5089–5094. doi: 10.1016/j.vaccine.2017.07.097

de la Fuente, J., Estrada-Peña, A., Venzal, J. M., Kocan, K. M., and Sonenshine, D. E. (2008). Overview: ticks as vectors of pathogens that cause disease in humans and animals. *Front. Biosci.* 13, 6938–6946.

de la Fuente, J., Gortázar, C., and Juste, R. (2016a). Complement component 3: a new paradigm in tuberculosis vaccine. *Expert Rev. Vaccines* 15, 275–277. doi: 10.1586/14760584.2016.1125294

de la Fuente, J., Kopáček, P., Lew-Tabor, A., and Maritz-Olivier, C. (2016b). Strategies for new and improved vaccines against ticks and tick-borne diseases. *Parasite Immunol.* 38, 754–769. doi: 10.1111/pim.12339

de la Fuente, J., Moreno-Cid, J. A., Canales, M., Villar, M., Pérez de la Lastra, J. M., Kocan, K. M., et al. (2011). Targeting arthropod subolesin/akirin for the development of a universal vaccine for control of vector infestations and pathogen transmission. *Vet. Parasitol.* 181, 17–22. doi: 10.1016/j.vetpar.2011.04.018

de la Fuente, J., Rodríguez, M., Redondo, M., Montero, C., García-García, J. C., Méndez, L., et al. (1998). Field studies and cost-effectiveness analysis of vaccination with Gavac^{TM} against the cattle tick *Boophilus microplus*. *Vaccine* 16, 366–373. doi: 10.1016/S0264-410X(97)00208-9

Dinarello, C. A. (2000). Proinflammatory cytokines. *Chest* 118, 503–508. doi: 10.1378/chest.118.2.503

Fry, T., Van Dalen, K., Hurley, J., and Nash, P. (2012). Mucosal adjuvants to improve wildlife rabies vaccination. *J. Wildl. Dis.* 48, 1042–1046. doi: 10.7589/2011-11-331

Holmgren, J., and Czerkinsky, C. (2005). Mucosal immunity and vaccines. *Nat. Med.* 11, S45–S53. doi: 10.1038/nm1213

Infantes-Lorenzo, J. A., Moreno, I., Risalde, M. A., Roy, A., Villar, M., Romero, B., et al. (2017). Proteomic characterisation of bovine and avian purified protein derivatives and identification of specific antigens for serodiagnosis of bovine tuberculosis. *Clin. Proteomics* 14:36. doi: 10.1186/s12014-017-9171-z

Infantes-Lorenzo, J. A., Whitehead, C. E., Moreno, I., Bezos, J., Roy, A., Domínguez, L., et al. (2018). Development and evaluation of a serological assay for the diagnosis of tuberculosis in alpacas and llamas. *Front. Vet. Sci.* 5:189. doi: 10.3389/fvets.2018.00189

Jones, G. J., Steinbach, S., Sevilla, I. A., Garrido, J. M., Juste, R., and Vordermeier, H. M. (2016). Oral vaccination of cattle with heat inactivated *Mycobacterium bovis* does not compromise bovine TB diagnostic tests. *Vet. Immunol. Immunopathol.* 182, 85–88. doi: 10.1016/j.vetimm.2016.10.010

Jongejan, F., and Uilenberg, G. (2004). The global importance of ticks. *Parasitology* 129, S3–S14. doi: 10.1017/S0031182004005967

Juste, R. A., Alonso-Hearn, M., Garrido, J. M., Abendaño, N., Sevilla, I. A., Gortazar, C., et al. (2016). Increased lytic efficiency of bovine macrophages trained with killed mycobacteria. *PLoS ONE* 11:e0165607. doi: 10.1371/journal.pone.0165607

Lawan, A., Jesse, F. F. A., Idris, U. H., Odhah, M. N., Arsalan, M., Muhammad, N. A., et al. (2018). Mucosal and systemic responses of immunogenic vaccines candidates against enteric *Escherichia coli* infections in ruminants: a review. *Microb. Pathog.* 117, 175–183. doi: 10.1016/j.micpath.2018.02.039

Lawson, L. B., Norton, E. B., and Clements, J. D. (2011). Defending the mucosa: adjuvant and carrier formulations for mucosal immunity. *Curr. Opin. Immunol.* 23, 414–420. doi: 10.1016/j.coi.2011.03.009

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real time quantitative PCR and the 2(-Delta Delta CT) method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

López, V., Risalde, M. A., Contreras, M., Mateos-Hernández, L., Vicente, J., Gortázar, C., et al. (2018). Heat-inactivated *Mycobacterium bovis* protects zebrafish against mycobacteriosis. *J. Fish Dis.* 41, 1515–1528. doi: 10.1111/jfd.12847

López, V., van der Heijden, E., Villar, M., Michel, A., Alberdi, P., Gortázar, C., et al. (2019). Comparative proteomics identified immune response proteins involved in response to vaccination with heat-inactivated *Mycobacterium bovis* and mycobacterial challenge in cattle. *Vet. Immunol. Immunopathol.* 206, 54–64. doi: 10.1016/j.vetimm.2018.10.013

Lubbers, R., van Essen, M. F., van Kooten, C., and Trouw, L. A. (2017). Production of complement components by cells of the immune system. *Clin. Exp. Immunol.* 188, 183–194. doi: 10.1111/cei.12952

Merino, M., Antunes, S., Mosqueda, J., Moreno-Cid, J. A., Pérez de la Lastra, J. M., Rosario-Cruz, R., et al. (2013). Vaccination with proteins involved in tick-pathogen interactions reduces vector infestations and pathogen infection. *Vaccine* 31, 5889–5896. doi: 10.1016/j.vaccine.2013.09.037

Merino, O., Almazán, C., Canales, M., Villar, M., Moreno-Cid, J. A., Estrada-Peña, A., et al. (2011). Control of *Rhipicephalus (Boophilus) microplus* infestations by the combination of subolesin vaccination and tick autocidal control after subolesin gene knockdown in ticks fed on cattle. *Vaccine* 29, 2248–2254. doi: 10.1016/j.vaccine.2011.01.050

Ogra, P. L., Faden, H., and Welliver, R. C. (2001). Vaccination strategies for mucosal immune responses. *Clin. Microbiol. Rev.* 14, 430–445. doi: 10.1128/CMR.14.2.430-445.2001

Peng, Q., Li, K., Patel, H., Sacks, S. H., and Zhou, W. (2006). Dendritic cell synthesis of C3 is required for full T cell activation and development of a Th1 phenotype. *J. Immunol.* 176, 3330–3341. doi: 10.4049/jimmunol.176.6.3330

Rashid, M., Rashid, M. I., Akbar, H., Ahmad, L., Hassan, M. A., Ashraf, K., et al. (2018). A systematic review on modelling approaches for economic losses studies caused by parasites and their associated diseases in cattle. *Parasitology* 2, 1–13. doi: 10.1017/S0031182018001282

Ririe, K. M., Rasmussen, R. P., and Wittwer, C. T. (1997). Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Anal. Biochem.* 245, 154–160. doi: 10.1006/abio.1996.9916

Risalde, M. A., López, V., Contreras, M., Mateos-Hernández, L., Gortázar, C., and de la Fuente, J. (2018). Control of mycobacteriosis in zebrafish (*Danio rerio*) mucosally vaccinated with heat-inactivated *Mycobacterium bovis*. *Vaccine* 36, 4447–4453. doi: 10.1016/j.vaccine.2018.06.042

Rodríguez-Mallon, A. (2016). Developing anti-tick vaccines. *Methods Mol. Biol.* 1404, 243–259. doi: 10.1007/978-1-4939-3389-1_17

Rutemark, C., Alicot, E., Bergman, A., Ma, M., Getahun, A., Ellmerich, S., et al. (2011). Requirement for complement in antibody responses is not explained by the classic pathway activator IgM. *Proc. Natl. Acad. Sci. U.S.A.* 108, E934–E942. doi: 10.1073/pnas.1109831108

Schetters, T., Bishop, R., Crampton, M., Kopáček, P., Lew-Tabor, A., Maritz-Olivier, C., et al. (2016). Cattle tick vaccine researchers join forces in CATVAC. *Parasit. Vectors* 9:105. doi: 10.1186/s13071-016-1386-8

Seder, R. A., and Hill, A. V. (2000). Vaccines against intracellular infections requiring cellular immunity. *Nature* 406, 793–798. doi: 10.1038/35021239

Staats, H. F., and Ennis, F. A. Jr. (1999). IL-1 is an effective adjuvant for mucosal and systemic immune responses when coadministered with protein immunogens. *J. Immunol.* 162, 6141–6147.

Tellam, R. L., Dmith, D., Kemp, D. H., and Willadsen, P. (1992). "Vaccination against ticks," in *Animal Parasite Control Utilizing Biotechnology*, ed W. K. Yong (Boca Raton, FL: CRC Press), 303–331.

Thomas, J., Risalde, M. A., Serrano, M., Sevilla, I., Geijo, M. V., Ortíz, J. A., et al. (2017). The response of red deer to oral administration of heat-inactivated *Mycobacterium bovis* and challenge with a field strain. *Vet. Microbiol.* 208, 195–202. doi: 10.1016/j.vetmic.2017.08.007

Wang, S., Liu, H., Zhang, X., and Qian, F. (2015). Intranasal and oral vaccination with protein-based antigens: advantages, challenges and formulation strategies. *Protein Cell.* 6, 480–503. doi: 10.1007/s13238-015-0164-2

Conflict of Interest Statement: 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 Cellular and Infection Microbiology

MINI REVIEW
published: 03 April 2019
doi: 10.3389/fcimb.2019.00091

Toward DNA-Based T-Cell Mediated Vaccines to Target HIV-1 and Hepatitis C Virus: Approaches to Elicit Localized Immunity for Protection

*Zelalem A. Mekonnen[1], Branka Grubor-Bauk[1], Makutiro G. Masavuli[1], Ashish C. Shrestha[1], Charani Ranasinghe[2], Rowena A. Bull[3], Andrew R. Lloyd[3], Eric J. Gowans[1] and Danushka K. Wijesundara[1]**

[1] Virology Laboratory, Basil Hetzel Institute for Translational Health Research, Discipline of Surgery, University of Adelaide, Adelaide, SA, Australia, [2] Molecular Mucosal Vaccine Immunology Group, The John Curtin School of Medical Research, The Australian National University, Canberra, ACT, Australia, [3] Viral Immunology Systems Program, The Kirby Institute, The University of New South Wales, Sydney, NSW, Australia

OPEN ACCESS

Edited by:
Alberto Moreno,
Emory University School of Medicine,
United States

Reviewed by:
Leo J. Swadling,
University College London,
United Kingdom
Antonella Folgori,
ReiThera Srl, Italy

***Correspondence:**
Danushka K. Wijesundara
danushka.wijesundara@
adelaide.edu.au

Specialty section:
This article was submitted to Clinical Microbiology, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: 23 November 2018
Accepted: 14 March 2019
Published: 03 April 2019

Citation:
Mekonnen ZA, Grubor-Bauk B, Masavuli MG, Shrestha AC, Ranasinghe C, Bull RA, Lloyd AR, Gowans EJ and Wijesundara DK (2019) Toward DNA-Based T-Cell Mediated Vaccines to Target HIV-1 and Hepatitis C Virus: Approaches to Elicit Localized Immunity for Protection. Front. Cell. Infect. Microbiol. 9:91. doi: 10.3389/fcimb.2019.00091

Human immunodeficiency virus (HIV)-1 and hepatitis C virus (HCV) are major contributors to the global disease burden with many experts recognizing the requirement of an effective vaccine to bring a durable end to these viral epidemics. The most promising vaccine candidates that have advanced into pre-clinical models and the clinic to eliminate or provide protection against these chronic viruses are viral vectors [e.g., recombinant cytomegalovirus, Adenovirus, and modified vaccinia Ankara (MVA)]. This raises the question, is there a need to develop DNA vaccines against HIV-1 and HCV? Since the initial study from Wolff and colleagues which showed that DNA represents a vector that can be used to express transgenes durably *in vivo*, DNA has been regularly evaluated as a vaccine vector albeit with limited success in large animal models and humans. However, several recent studies in Phase I-IIb trials showed that vaccination of patients with recombinant DNA represents a feasible therapeutic intervention to even cure cervical cancer, highlighting the potential of using DNA for human vaccinations. In this review, we will discuss the limitations and the strategies of using DNA as a vector to develop prophylactic T cell-mediated vaccines against HIV-1 and HCV. In particular, we focus on potential strategies exploiting DNA vectors to elicit protective localized $CD8^+$ T cell immunity in the liver for HCV and in the cervicovaginal mucosa for HIV-1 as localized immunity will be an important, if not critical component, of an efficacious vaccine against these viral infections.

Keywords: DNA vaccine, hepatitis C, human immunodeficiency virus, HIV/AIDS, HCV, tissue-resident memory, T cell immunity

INTRODUCTION

Human immunodeficiency virus (HIV)-1 and hepatitis C virus (HCV) are significant contributors to the global disease burden with ∼36.9 million people living with HIV-1 and at least 71 million people persistently infected with HCV (WHO, 2017; UNAIDS, 2018). Anti-retroviral therapy (ART) and direct acting anti-virals (DAAs) have contributed significantly to prolonging the lifespan and curing of HIV-1- and HCV-infected individuals, respectively (Cihlar and Fordyce, 2016; Zhang, 2016), but the annual HIV-1 and HCV incidences are still rising by millions. Furthermore, only 17 million (<50%) people have access to ART (Cihlar and Fordyce, 2016) and only 20% of patients are diagnosed for HCV (WHO, 2017). Additional issues involving drug resistance, reactogenicity associated with life-long ART and the lack of universal access to testing and cost-subsidized therapies minimize the ability of effective anti-viral drugs to end the HIV-1 and HCV epidemics. Thus, there is an urgent need to develop effective prophylactic vaccines to control the number of new infections and reduce the burden of supplying ART and DAA therapies to patients (Shin, 2016; Stone et al., 2016).

HIV-1 and HCV are rapidly mutating RNA viruses that exhibit considerable genetic diversity (nine subtypes in the major group of HIV-1 (German Advisory Committee Blood SAoPTbB., 2016) and 8 genotypes (gt1-8) of HCV which include at least 67 subtypes (Borgia et al., 2018) making immunity that develops during natural infection mostly ineffective. The lack of immune correlates of protection and convenient animal models permissive to infection make vaccine design and testing extremely challenging, and have also contributed to the fact that there is still no licensed vaccine for either HIV-1 or HCV (Wang et al., 2015; Bailey et al., 2019). HIV-1 and HCV co-infections represent an additional obstacle (Platt et al., 2016) although a recent clinical study suggests that co-administration of HIV-1 and HCV vaccines in humans can elicit robust HIV-1- and HCV-specific T cell responses without perturbing the immunodominance hierarchies of T cells responding against the vaccine encoded HIV-1 or HCV antigens (Hartnell et al., 2018).

DNA vaccines have been investigated for nearly three decades and are essentially bacteria-derived plasmids genetically engineered to encode immunogens under the control of promoters that facilitate robust expression of DNA in mammalian cells to induce adaptive immunity (Ferraro et al., 2011). DNA vaccines are inexpensive, easily constructed, stable at room temperature, replication defective in transfected mammalian cells and have minimum side effects which simplifies handling and distribution such that even developing countries can benefit from DNA vaccines (Jorritsma et al., 2016). Furthermore, plasmid DNA can be more easily used in multi-dose regimens unlike recombinant virus vectors that suffer from anti-vector immunity (Frahm et al., 2012). Recent seminal studies described therapeutic DNA vaccination against human papillomavirus (HPV) which resulted in histological regression and/or eliminated persistent HPV infection and HPV-related cervical lesions (Kim et al., 2014; Trimble et al., 2015). More recently, a DNA vaccine was developed that induced protective neutralizing antibodies (NAb) to Zika virus (ZIKV) in mice (Larocca et al., 2016) and rhesus macaques (Abbink et al., 2017) leading to the development of safe and immunogenic ZIKV DNA vaccines for humans (Tebas et al., 2017; Gaudinski et al., 2018). Thus, the many advantages of using plasmid DNA to develop vaccines and the recent developments of DNA vaccines in eliciting protective immunity in humans and higher animal models warrant further examination as to how DNA vaccines can be harnessed in vaccination regimens to target HIV-1 and HCV.

IMMUNE TARGETS FOR HIV-1 AND HCV PROPHYLACTIC VACCINE DEVELOPMENT

It is imperative that vaccines take into account the virus tropism, transmission routes, pathogenesis and immune responses that provide effective resistance against infections to elicit protective immunity against HIV-1 and/or HCV.

It is now established that mucosal tissues, mainly the gentio-rectal tissues and gastrointestinal tract, are the major sites of HIV-1 entry and pathogenesis, respectively (Belyakov and Ahlers, 2012). Induction of robust HIV-specific immune responses at these sites will be necessary to prevent HIV-1 infection or at the very least control viraemia during the acute phase of infection thus reducing the viral set point (McMichael and Koff, 2014) and infection-induced microbial translocation which can result in diversion of immune responses to counteract dysbiosis (Vujkovic-Cvijin et al., 2013). Furthermore, a prophylactic HIV-1 vaccine will likely be delivered using an active immunization strategy and attempt to mimic immune responses reported to be protective in macaques against simian immunodeficiency virus (SIV) and/or provide resistance against natural HIV-1 infections (Pontesilli et al., 1998; Saez-Cirion et al., 2007; Hansen et al., 2011; Haynes et al., 2012; Barouch et al., 2015, 2018; Ackerman et al., 2016; Borducchi et al., 2016). In this regard, the most protective immune responses reported to date involve T cell-mediated immunity (CMI) (Pontesilli et al., 1998; Saez-Cirion et al., 2007; Hansen et al., 2011; Borducchi et al., 2016), polyfunctional antibody responses (Barouch et al., 2015, 2018; Ackerman et al., 2016), antibody-dependent cellular cytotoxicity (Haynes et al., 2012), and broadly neutralizing antibodies (bNAb) (Burton and Hangartner, 2016). Although potent bNAb represent a blueprint for HIV-1 vaccine design, these antibodies are unlikely to be as effective in preventing cell to cell transmission compared to neutralizing cell free virus (Parsons et al., 2017). Consequently, a highly effective prophylactic HIV-1 vaccine will likely also rely on CMI to target highly conserved viral proteins such as Gag and Pol (Rolland et al., 2007) and/or non-neutralizing antibodies to broadly target the virus Envelope to prevent cell-cell transmission of the virus.

Unlike HIV-1 which has a relatively broad tropism, HCV is a bloodborne virus that primarily infects and replicates in hepatocytes. In primary hepatitis C infection, ∼25% of patients naturally clear the virus and although reinfection occurs in many individuals (Grebely et al., 2012), it is evident that repeated infection is associated with a reduced magnitude and duration of viraemia, and a greater likelihood of clearance (Sacks-Davis et al., 2015). Thus, characterizing and eliciting the naturally-protective

immune responses during primary infection and reinfection provide a rational path for the design of a prophylactic HCV vaccine (Grebely et al., 2012). The immune responses that correlate best with natural protection include robust and broad CMI to conserved HCV non-structural (NS) proteins (NS3, NS4, and NS5) (Smyk-Pearson et al., 2008; Baumert et al., 2014) and NAb targeting conserved regions of the viral envelope (E1E2) proteins (Houghton, 2011; Osburn et al., 2014; Bailey et al., 2017). Although CMI will not prevent infection, clinical data suggest that T cell responses could prevent the development of persistent infection in individuals who naturally clear the virus, which is an acceptable outcome given that primary infection is often asymptomatic and not associated with severe disease outcomes (Baumert et al., 2014). After two decades of unsuccessful pre-clinical studies and Phase I HCV vaccine trials, the current lead prophylactic candidate is in an NIH-sponsored Phase IIb, placebo-controlled trial (ClinicalTrials.gov Identifier: NCT01436357) in high risk people who inject drugs (PWID) (Swadling et al., 2014). The candidate vaccination regimen being tested utilizes a chimpanzee adenovirus (ChAd) prime and a modified vaccinia Ankara (MVA) boost to elicit systemic T cell immunity to gt1 NS antigens (Swadling et al., 2014). However, it is not clear if this vaccination can induce robust intrahepatic T cell immunity and sufficient multi-genotypic immunity to result in significant protection in vaccinated individuals especially given the increased prevalence of multiple genotypes in HCV endemic regions.

T CELL-MEDIATED DNA VACCINES AGAINST HIV-1 AND HCV IN THE CLINIC

DNA vaccines against HCV have been routinely tested in small and large animals including non-human primates (Latimer et al., 2014; Gummow et al., 2015; Grubor-Bauk et al., 2016; Wijesundara et al., 2018). Some candidates have also progressed in phase I/II clinical trials, but none have progressed to a large-scale efficacy trial in humans. A promising DNA vaccine that included a cocktail of four plasmids with each plasmid encoding codon optimized NS3/4A, NS4B, NS5A, or NS5B sequences from gt1a/b virus was used to prime/boost vaccinate macaques by electroporation (Latimer et al., 2014). In this study, the vaccine induced $CD4^+$ and $CD8^+$ T cells against each of the NS proteins encoded in the DNA cocktail which has resulted in the testing of the DNA cocktail in a phase I clinical trial (ClinicalTrials.gov Identifier: NCT02027116) although the results are yet to be disclosed.

A DNA vaccine has been tested for therapeutic vaccination against HCV. 12 hepatitis C patients suffering from chronic disease received three doses of a DNA vaccine encoding codon optimized NS3/4A from gt1a virus via electroporation on the deltoid muscle which induced NS3-specific CMI and a transient decrease in viral RNA levels (Weiland et al., 2013). The vaccine was also tested in eight patients who received interferon and ribavirin treatment of which six patients were completely cured of the infection (Weiland et al., 2013). Thus, DNA vaccines could be exploited in therapeutic settings against HCV, but this is unlikely to occur in the future given the success of using DAA to cure hepatitis C patients.

DNA vaccines against HIV-1 have been tested in different pre-clinical models and some have been tested in phase I/II clinical trials (Okuda et al., 1997; Cafaro et al., 2001; Tomusange et al., 2016). The first human clinical trial of a DNA vaccine, encoding *env* and *rev* genes, against HIV-1 was conducted in 1998 (MacGregor et al., 1998). Following vaccination of HIV-1 positive, treatment naïve individuals, no significant changes were observed in $CD4^+$ and $CD8^+$ T cell responses as well as in plasma HIV RNA. In another phase I clinical trial a DNA vaccine that encoded *env* and *rev* was shown to induce $CD4^+$ T cell and poor $CD8^+$ T cells responses in HIV-1 seronegative individuals (MacGregor et al., 2002). Similarly, low $CD8^+$ T cell responses were observed in another phase I clinical trial following prime/boost vaccination with a DNA vaccine that encoded *gag* and *pol* genes (Tavel et al., 2007). More robust HIV-specific T cell responses have been elicited when DNA vaccines are used to prime and recombinant viral vectors are used to boost immune responses (Kibuuka et al., 2010; Bakari et al., 2011; Churchyard et al., 2011; Hayton et al., 2014; Moyo et al., 2017). However, prime/boost vaccinations with DNA vaccines alone can be optimized to elicit robust immune responses in humans against HIV-1. For instance, a retrospective study evaluating the immunogenicity of 10 HIV-1 DNA vaccine trials that used DNA vaccines in the absence of viral vectors or adjuvants suggest that the use of DNA delivery devices (e.g., electroporators and biojectors), and increasing the number of vaccine doses and dosage could more reproducibly elicit $CD4^+$ and $CD8^+$ T cell responses (Jin et al., 2015).

The main limitation associated with DNA vaccines is their inability to induce long-term immune responses following a single or a few vaccinations (Abbink et al., 2017). Furthermore, DNA vaccines are poorly effective and not well-optimized in eliciting immunity in the liver, gut or genito-rectal mucosa which warrant further refinements of DNA-based vaccination regimens in order to elicit durable protection against HIV-1 and/or HCV.

THE POTENTIAL OF TISSUE-RESIDENT MEMORY T CELLS FOR CONTROLLING HIV-1 AND HCV INFECTIONS

Since the initial discovery of highly cytotoxic memory T cells residing in tissues (Masopust et al., 2001), several studies have shown that $CD8^+$ tissue-resident memory T (T_{RM}) cells residing in the female reproductive tract, the gut, the lung and the liver form a formidable frontline defense against various pathogen infections (Mueller and Mackay, 2016; Rosato et al., 2017). The protective role of $CD8^+$ T_{RM} cells is primarily due to their ability to (1) maintain a stable and durable population following their formation in tissues even in the absence of cognate antigen encounter following their formation (Gebhardt et al., 2009; MacKay et al., 2012; Beura et al., 2018; Park et al., 2018), and (2) produce anti-viral cytokines and/or exert cytotoxic functions to reduce the number of pathogen-infected cells and to recruit other immune cells (e.g., circulating memory T cells) rapidly to

the site of infection (Schenkel et al., 2013; Muruganandah et al., 2018; Park et al., 2018). Furthermore, $CD8^+$ T_{RM} cells respond more rapidly, produce greater amounts of anti-viral/cytotoxic molecules (i.e., in the liver) and appear to be crucial for protection against liver tropic pathogens and pathogens exposed in the vagina and the female reproductive tract compared to circulating memory T cells (Cuburu et al., 2012, 2015; Shin and Iwasaki, 2012; Fernandez-Ruiz et al., 2016; Beura et al., 2018). The greater frequency of intrahepatic $CD8^+$ T_{RM} cells ($CD69^+$ $CD103^+$) amongst the total $CD8^+$ T cell population correlated with partial control of viraemia in Hepatitis B Virus (HBV)-infected patients (Pallett et al., 2017), providing further encouragement that intrahepatic HCV-specific $CD8^+$ T_{RM} cells will likely be protective against HCV.

Despite HIV-1 and HCV being highly mutable with a complex and evolving quasispecies, several studies have revealed that only one or few variants, referred to as transmitted/founder (T/F) viruses, establish infection following transmission reflecting a strong genetic bottleneck (Bull et al., 2011; Joseph et al., 2015). T/F viruses will be exposed in the genito-rectal mucosa (i.e., the vagina and the rectum) during the vast majority (>80%) of HIV transmission and in the liver during HCV transmission. Thus, eliciting HIV- and HCV-specific $CD8^+$ T_{RM} cells in the genito-rectal mucosa and the liver, respectively, following vaccination is also an attractive strategy to circumvent issues associated with viral diversity and eliminate these viruses shortly after transmission/exposure. Several vaccine vectors such as radiation attenuated sporozoites (RAS), protein loaded nanoparticles (NP), adenovirus (Ad) vectors, adeno-associated virus (AAV), and HPV pseudovirus (HPV PsV) have been developed to elicit localized protection and in some instances elicit $CD8^+$ T_{RM} cells in the liver or the vagina (**Figure 1**) (Cuburu et al., 2012, 2015, 2018; Fernandez-Ruiz et al., 2016; Ishizuka et al., 2016; Gola et al., 2018). This provides hope that a vaccine to elicit intravaginal or intrahepatic $CD8^+$ T_{RM} cells can be developed to potentially provide protection against HIV-1 or HCV, respectively.

A recent study suggests that strategies that can induce interleukin (IL)-15 and/or inflammation in the liver can be effective in recruiting circulating effector $CD8^+$ T cells to differentiate into $CD8^+$ T_{RM} cells in the liver (Holz et al., 2018). Systemic immunization strategies that promote up-regulation of gut homing molecules such as α4β7 on antigen-primed $CD8^+$ T cells in secondary lymphoid organs can be efficiently recruited to establish residency in the gut (Masopust et al., 2010). Although these studies and others suggest that local antigen deposition and antigen encounter are not essential to elicit $CD8^+$ T_{RM} cells, it is evident that this process leads to the formation of greatest densities of $CD8^+$ T_{RM} cells especially at sites such as the liver and the vagina (Cuburu et al., 2012; Fernandez-Ruiz et al., 2016; Davies et al., 2017). Intravenous delivery of vaccine vectors appears to be most efficient route to facilitate local, intrahepatic expression of vaccine encoded antigens (Nganou-Makamdop et al., 2012; Tay et al., 2014; Gola et al., 2018), to elicit high numbers of intrahepatic $CD8^+$ T_{RM} cells, and protection against hepatotropic pathogens compared to intradermal and intramuscular vaccine delivery routes (**Figure 1**) (Epstein et al., 2011; Fernandez-Ruiz et al., 2016; Ishizuka et al., 2016; Gola et al., 2018). In the vagina, several studies suggest that intravaginal delivery of vaccine vectors [HPV PsV, and Ad serotypes 26 (Ad26) and 35 (Ad35)] is the most efficient route to express vaccine-encoded antigens in vaginal tissues and elicit cervicovaginal $CD8^+$ T_{RM} cells (**Figure 1**) (Cuburu et al., 2015, 2018; Fernandez-Ruiz et al., 2016). Furthermore, topical application of chemokine ligands (CXCL9 and CXCL10) in the vagina have been reported to "pull" systemically primed effector $CD8^+$ T cells into the vagina and allow these cells to differentiate into $CD8^+$ T_{RM} cells (Shin and Iwasaki, 2012).

CAN WE EXPLOIT DNA VACCINES TO ELICIT TISSUE-RESIDENT MEMORY T CELLS FOR PROTECTION AGAINST HIV-1 OR HCV?

There has been much research and progress made to improve the immunogenicity of DNA vaccines with respect to the choice of adjuvants, route of vaccine delivery, codon optimization of genes, method of delivery (e.g., electroporation and gene gun), etc. These aspects have been reviewed extensively elsewhere (Nagata et al., 1999; Garmory et al., 2003; Jechlinger, 2006; Vanniasinkam et al., 2006; Jorritsma et al., 2016) and the resulting refinements have led to DNA vaccines being more effectively exploited for use in Phase I and II clinical trials especially in the context of cancer (Kim et al., 2014; Trimble et al., 2015). However, vast majority of the studies including those progressing to the clinic have delivered DNA vaccines using intradermal or intramuscular routes. These routes may not be as effective compared to intravenous route to elicit intrahepatic $CD8^+$ T_{RM} cells or the intravaginal route to elicit cervicovaginal $CD8^+$ T_{RM} cells (**Figure 1**).

As mentioned above, it is important that a vaccination regimen designed to elicit $CD8^+$ T_{RM} cells facilitate local antigen presentation to naïve and antigen experienced precursors of $CD8^+$ T_{RM} cells, which is best achieved by the local expression of vaccine-encoded antigens and/or promoting local inflammation (**Figure 1**). Manual massaging (Liu et al., 2004), hydrodynamic injections (Yu et al., 2014), and liposome complexes (Kawakami et al., 2000) are some commonly used techniques to transfect hepatocytes *in vivo* following intravenous delivery of DNA. The expression of vaccine-encoded antigens in hepatocytes is a common hallmark of studies that have elicited intrahepatic $CD8^+$ T_{RM} cells (Fernandez-Ruiz et al., 2016; Ishizuka et al., 2016), but none of these delivery strategies have led to a licensed vaccine for use in humans. Furthermore, it not known whether any of these strategies can elicit intrahepatic $CD8^+$ T_{RM} cells in humans mainly owing to the difficulties of isolating liver biopsies in healthy patients although fine needle aspirates may be used to less invasively sample liver-resident T cells (Gill et al., 2018a,b) and the lack of biomarkers (i.e., in the blood) that can accurately predict the formation of $CD8^+$ T_{RM} cells in the liver and other tissues/organs. In the vagina, a proof of concept study has shown that DNA can be expressed following submucosal intravaginal delivery of DNA in mice (Sun et al., 2015). However, the same study

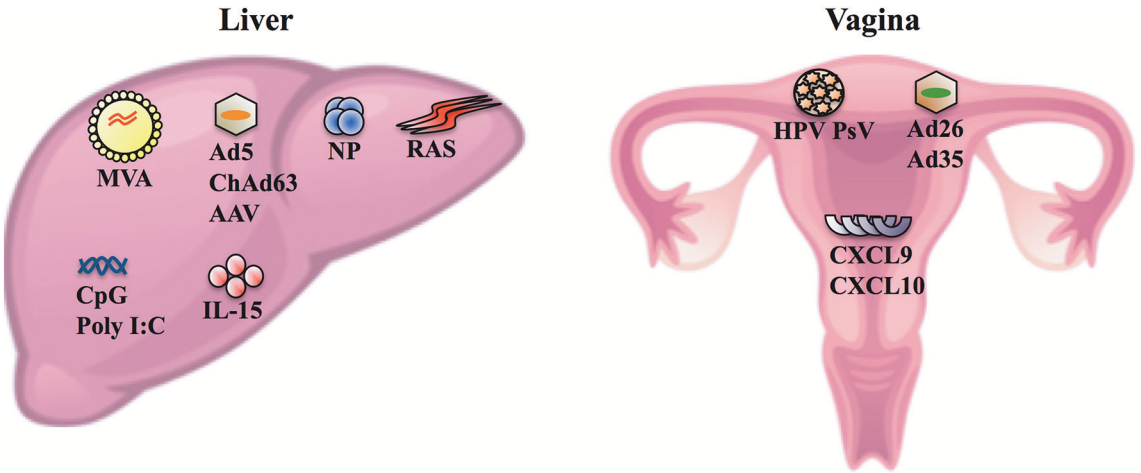


FIGURE 1 | Vaccine vectors and agents that can be used to elicit and/or enhance the formation of CD8$^+$ T_{RM} cells in the liver and the vagina. Following intravenous delivery, MVA, Ad serotype 5 (Ad5), ChAd serotype 63 (ChAd63), AAV, NP, and RAS can enter hepatocytes and/or cells surrounding the hepatic tissues (e.g., Kupffer cells) (Nganou-Makamdop et al., 2012; Tay et al., 2014; Gola et al., 2018). Intravenous delivery of these vectors in multi-dose prime/trap (Fernandez-Ruiz et al., 2016; Olsen et al., 2018), prime/target (Gola et al., 2018) or homologous prime/boost (Ishizuka et al., 2016) regimens elicits protection following *P. berghei* sporozoite challenge in mice or controlled *P. falciparum* infections in humans, and elicits intrahepatic CD8$^+$ T_{RM} cells (Fernandez-Ruiz et al., 2016; Ishizuka et al., 2016; Gola et al., 2018). The prime/trap approaches involve priming CD8$^+$ T cells systemically using antibodies that deliver a peptide antigen to cross-presenting dendritic cells (Fernandez-Ruiz et al., 2016) or using gene gun delivery of DNA encoding the cognate antigen (Olsen et al., 2018). The primed CD8$^+$ T cells are then recruited to the liver and differentiate into T_{RM} cells (i.e., trapped) following intravenous delivery of AAV (Fernandez-Ruiz et al., 2016) or RAS (Olsen et al., 2018) that enter cells in the liver and express the relevant cognate antigen which are recognized by the primed CD8$^+$ T cells. The prime/target approach is an adaptation of the prime/trap approach and essentially involves priming naïve CD8$^+$ T cells with Ad5 or ChAd63 vaccine vector delivered via the intramuscular route and recruiting the primed CD8$^+$ T cells to the liver following intravenous delivery of Ad5, NP, MVA, or ChAd63 vaccine vector (Gola et al., 2018). The same study showed that intravenous delivery was more efficient than intramuscular delivery of vaccine vectors to elicit high numbers of intrahepatic CD8$^+$ T_{RM} cells and protection against *P. berghei* sporozoite challenge in the prime/target approach (Gola et al., 2018). IL-15 appears to be crucial for activated CD8$^+$ T cells to differentiate into T_{RM} cells in the liver and inflammatory signals (e.g., CpG and Poly I:C) can enhance the formation of intrahepatic CD8$^+$ T_{RM} cells *in vivo* (Holz et al., 2018). In the vagina, intravaginal delivery of HPV PsV, and more recently Ad26 and Ad35 have been shown to transduce cervicovaginal epithelial cells and elicit HPV-specific CD8$^+$ T_{RM} cells in the cervicovaginal mucosa (Cuburu et al., 2012, 2015, 2018). In the absence of a vaccine vector, topical application of CXCL9 and CXCL10 in the vagina can be used to recruit/pull CXCR3$^+$ effector CD8$^+$ T cells into the vagina which subsequently differentiate into cervicovaginal T_{RM} cells (Shin and Iwasaki, 2012).

reported poorly immunogenic responses in mice and noted that electroporation was required to improve the immunogenicity of intravaginally delivered DNA which could be difficult to exploit in humans.

DNA can be used as a vector to prime high numbers of circulating antigen-specific T cells (Gummow et al., 2015; Wijesundara et al., 2018) which can then be recruited to the liver using vectors that efficiently enter cells in the hepatic tissues or the vagina using chemokine ligands or vectors that transduce vaginal epithelial cells (**Figure 1**). Furthermore, given the poor transfection efficiency and immunogenicity of DNA when delivered into the vagina or the liver, it is more feasible to exploit DNA as an immune priming agent in a vaccination regimen to elicit HIV-1- or HCV-specific CD8$^+$ T_{RM} cells in the vagina or the liver, respectively. Furthermore, analogous strategies using protein-based T cell priming agents in prime/pull (Shin and Iwasaki, 2012), prime/trap (Fernandez-Ruiz et al., 2016), or prime/target (Gola et al., 2018) regimens have been used to elicit protective cervicovaginal or intrahepatic CD8$^+$ T_{RM} cells. A caveat in this case is to determine whether the primed T cells express adequate levels of chemokine receptors (e.g., CXCR3 and CXCR6) necessary to home to the liver (Sato et al., 2005; Tse et al., 2014; Gola et al., 2018; Olsen et al., 2018) or the vagina (Shin and Iwasaki, 2012) following DNA immunization. Even if not obligatory, the expression of the relevant homing receptors could be required to ensure that high densities of primed CD8$^+$ T cells are recruited to the cervicovaginal mucosa or the liver following introduction of a vaccine vector or an agent (**Figure 1**) to facilitate the formation of CD8$^+$ T_{RM} cells. Several studies have shown that the number of CD8$^+$ T_{RM} cells is a crucial parameter that dictates the collective ability of these cells to confer protection against pathogens exposed in the skin, liver, or the vagina with greater numbers favoring protective outcomes (Cuburu et al., 2012; Shin and Iwasaki, 2012; Fernandez-Ruiz et al., 2016; Park et al., 2018).

CONCLUDING REMARKS

DNA has recently re-emerged as an effective vaccination platform in humans, but its use in developing a T cell-based vaccine will likely rely on its ability to be exploited in a regimen that can elicit robust immunity in the vagina and the gut in the context of HIV-1, or the liver in the context of HCV. In this regard, we have highlighted the importance of eliciting cervicovaginal or intrahepatic CD8$^+$ T_{RM} cells against these viruses and also reviewed strategies as well as caveats associated with using DNA to elicit localized CD8$^+$ T_{RM} cells as a frontline defense against HIV-1 and HCV.

AUTHOR CONTRIBUTIONS

DW and ZM conceived the initial drafts of the manuscript. BG-B, MM, AS, CR, RB, AL, and EG revised many parts of the manuscript and contributed to finalize the manuscript.

FUNDING

The following grants have supported the work conducted in our laboratories and cited in the manuscript: From the National Health and Medical Research Council (NHMRC): grants APP1026293 (EG), APP525431 (CR), APP543139 (EG), and APP543143 (EG). From the Australian Centre for HIV and Hepatitis Virology Research, CR and EG have received an EOI grant. From The Hospital Research Foundation (THRF) and the Channel 7 Children's Research Foundation, DW received a project grant.

ACKNOWLEDGMENTS

THRF have provided early career fellowships for DW and AS. AL is supported by a Fellowship from the NHMRC (NHMRC; No. 1043067).

REFERENCES

Abbink, P., Larocca, R. A., Visitsunthorn, K., Boyd, M., De La Barrera, R. A., Gromowski, G. D., et al. (2017). Durability and correlates of vaccine protection against Zika virus in rhesus monkeys. *Sci. Transl. Med.* 9:eaao4163. doi: 10.1126/scitranslmed.aao4163

Ackerman, M. E., Mikhailova, A., Brown, E. P., Dowell, K. G., Walker, B. D., Bailey-Kellogg, C., et al. (2016). Polyfunctional HIV-specific antibody responses are associated with spontaneous HIV control. *PLoS Pathog.* 12:e1005315. doi: 10.1371/journal.ppat.1005315

Bailey, J. R., Barnes, E., and Cox, A. L. (2019). Approaches, progress, and challenges to hepatitis C vaccine development. *Gastroenterology* 156, 418–4430. doi: 10.1053/j.gastro.2018.08.060

Bailey, J. R., Flyak, A. I., Cohen, V. J., Li, H., Wasilewski, L. N., Snider, A. E., et al. (2017). Broadly neutralizing antibodies with few somatic mutations and hepatitis C virus clearance. *JCI Insight* 2:e92872. doi: 10.1172/jci.insight.92872

Bakari, M., Aboud, S., Nilsson, C., Francis, J., Buma, D., Moshiro, C., et al. (2011). Broad and potent immune responses to a low dose intradermal HIV-1 DNA boosted with HIV-1 recombinant MVA among healthy adults in Tanzania. *Vaccine* 29, 8417–8428. doi: 10.1016/j.vaccine.2011.08.001

Barouch, D. H., Alter, G., Broge, T., Linde, C., Ackerman, M. E., Brown, E. P., et al. (2015). Protective efficacy of adenovirus/protein vaccines against SIV challenges in rhesus monkeys. *Science* 349, 320–324. doi: 10.1126/science.aab3886

Barouch, D. H., Tomaka, F. L., Wegmann, F., Stieh, D. J., Alter, G., Robb, M. L., et al. (2018). Evaluation of a mosaic HIV-1 vaccine in a multicentre, randomised, double-blind, placebo-controlled, phase 1/2a clinical trial (APPROACH) and in rhesus monkeys (NHP 13-19). *Lancet* 392, 232–243. doi: 10.1016/S0140-6736(18)31364-3

Baumert, T. F., Fauvelle, C., Chen, D. Y., and Lauer, G. M. (2014). A prophylactic hepatitis C virus vaccine: a distant peak still worth climbing. *J. Hepatol.* 61(1 Suppl.), S34–S44. doi: 10.1016/j.jhep.2014.09.009

Belyakov, I. M., and Ahlers, J. D. (2012). Mucosal immunity and HIV-1 infection: applications for mucosal AIDS vaccine development. *Curr. Top. Microbiol. Immunol.* 354:157–179. doi: 10.1007/82_2010_119

Beura, L. K., Mitchell, J. S., Thompson, E. A., Schenkel, J. M., Mohammed, J., Wijeyesinghe, S., et al. (2018). Intravital mucosal imaging of CD8(+) resident memory T cells shows tissue-autonomous recall responses that amplify secondary memory. *Nat. Immunol.* 19, 173–182. doi: 10.1038/s41590-017-0029-3

Borducchi, E. N., Cabral, C., Stephenson, K. E., Liu, J., Abbink, P., Ng'ang'a, D., et al. (2016). Ad26/MVA therapeutic vaccination with TLR7 stimulation in SIV-infected rhesus monkeys. *Nature* 540, 284–287. doi: 10.1038/nature20583

Borgia, S. M., Hedskog, C., Parhy, B., Hyland, R. H., Stamm, L. M., Brainard, D. M., et al. (2018). Identification of a novel hepatitis C virus genotype from Punjab, India: expanding classification of hepatitis C virus into 8 genotypes. *J. Infect. Dis.* 218, 1722–1729. doi: 10.1093/infdis/jiy401

Bull, R. A., Luciani, F., McElroy, K., Gaudieri, S., Pham, S. T., Chopra, A., et al. (2011). Sequential bottlenecks drive viral evolution in early acute hepatitis C virus infection. *PLoS Pathog.* 7:e1002243. doi: 10.1371/journal.ppat.1002243

Burton, D. R., and Hangartner, L. (2016). Broadly neutralizing antibodies to HIV and their role in vaccine design. *Annu. Rev. Immunol.* 34, 635–659. doi: 10.1146/annurev-immunol-041015-055515

Cafaro, A., Titti, F., Fracasso, C., Maggiorella, M. T., Baroncelli, S., Caputo, A., et al. (2001). Vaccination with DNA containing tat coding sequences and unmethylated CpG motifs protects cynomolgus monkeys upon infection with simian/human immunodeficiency virus (SHIV89.6P). *Vaccine* 19, 2862–2877. doi: 10.1016/S0264-410X(01)00002-0

Churchyard, G. J., Morgan, C., Adams, E., Hural, J., Graham, B. S., Moodie, Z., et al. (2011). A phase IIA randomized clinical trial of a multiclade HIV-1 DNA prime followed by a multiclade rAd5 HIV-1 vaccine boost in healthy adults (HVTN204). *PLoS ONE* 6:e21225. doi: 10.1371/journal.pone.0021225

Cihlar, T., and Fordyce, M. (2016). Current status and prospects of HIV treatment. *Curr. Opin. Virol.* 18:50–56. doi: 10.1016/j.coviro.2016.03.004

Cuburu, N., Graham, B. S., Buck, C. B., Kines, R. C., Pang, Y. Y., Day, P. M., et al. (2012). Intravaginal immunization with HPV vectors induces tissue-resident CD8+ T cell responses. *J. Clin. Invest.* 122, 4606–4620. doi: 10.1172/jci63287

Cuburu, N., Khan, S., Thompson, C. D., Kim, R., Vellinga, J., Zahn, R., et al. (2018). Adenovirus vector-based prime-boost vaccination via heterologous routes induces cervicovaginal CD8(+) T cell responses against HPV16 oncoproteins. *Int. J. Cancer* 142, 1467–1479. doi: 10.1002/ijc.31166

Cuburu, N., Wang, K., Goodman, K. N., Pang, Y. Y., Thompson, C. D., Lowy, D. R., et al. (2015). Topical herpes simplex virus 2 (HSV-2) vaccination with human papillomavirus vectors expressing gB/gD ectodomains induces genital-tissue-resident memory CD8+ T cells and reduces genital disease and viral shedding after HSV-2 challenge. *J. Virol.* 89, 83–96. doi: 10.1128/jvi.02380-14

Davies, B., Prier, J. E., Jones, C. M., Gebhardt, T., Carbone, F. R., Mackay, L. K. (2017). Cutting edge: tissue-resident memory T cells generated by multiple immunizations or localized deposition provide enhanced immunity. *J. Immunol.* 198, 2233–2237. doi: 10.4049/jimmunol.1601367

Epstein, J. E., Tewari, K., Lyke, K. E., Sim, B. K., Billingsley, P. F., Laurens, M. B., et al. (2011). Live attenuated malaria vaccine designed to protect through hepatic CD8(+) T cell immunity. *Science* 334, 475–480. doi: 10.1126/science.1211548

Fernandez-Ruiz, D., Ng, W. Y., Holz, L. E., Ma, J. Z., Zaid, A., Wong, Y. C., et al. (2016). Liver-resident memory CD8(+) T cells form a front-line defense against malaria liver-stage infection. *Immunity* 45, 889–902. doi: 10.1016/j.immuni.2016.08.011

Ferraro, B., Morrow, M. P., Hutnick, N. A., Shin, T. H., Lucke, C. E., and Weiner, D. B. (2011). Clinical applications of DNA vaccines: current progress. *Clin. Infect. Dis.* 53, 296–302. doi: 10.1093/cid/cir334

Frahm, N., DeCamp, A. C., Friedrich, D. P., Carter, D. K., Defawe, O. D., Kublin, J. G., et al. (2012). Human adenovirus-specific T cells modulate HIV-specific T cell responses to an Ad5-vectored HIV-1 vaccine. *J. Clin. Invest.* 122, 359–367. doi: 10.1172/jci60202

Garmory, H. S., Brown, K. A., and Titball, R. W. (2003). DNA vaccines: improving expression of antigens. *Genet. Vaccines Ther.* 1:2. doi: 10.1186/1479-0556-1-2

Gaudinski, M. R., Houser, K. V., Morabito, K. M., Hu, Z., Yamshchikov, G., Rothwell, R. S., et al. (2018). Safety, tolerability, and immunogenicity of two Zika virus DNA vaccine candidates in healthy adults:

randomised, open-label, phase 1 clinical trials. *Lancet* 391, 552–562. doi: 10.1016/S0140-6736(17)33105-7

Gebhardt, T., Wakim, L. M., Eidsmo, L., Reading, P. C., Heath, W. R., and Carbone, F. R. (2009). Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus. *Nat. Immunol.* 10, 524–530. doi: 10.1038/ni.1718

German Advisory Committee Blood SAoPTbB. (2016). Human Immunodeficiency Virus (HIV). *Transfus. Med. Hemother.* 43, 203–222. doi: 10.1159/000445852

Gill, U. S., Pallett, L. J., Kennedy, P. T. F., and Maini, M. K. (2018a). Liver sampling: a vital window into HBV pathogenesis on the path to functional cure. *Gut* 67, 767–775. doi: 10.1136/gutjnl-2017-314873

Gill, U. S., Pallett, L. J., Thomas, N., Burton, A. R., Patel, A. A., Yona, S., et al. (2018b). Fine needle aspirates comprehensively sample intrahepatic immunity. *Gut*. 1-11 doi: 10.1136/gutjnl-2018-317071

Gola, A., Silman, D., Walters, A. A., Sridhar, S., Uderhardt, S., Salman, A. M., et al. (2018). Prime and target immunization protects against liver-stage malaria in mice. *Sci. Transl. Med.* 10:eaap9128. doi: 10.1126/scitranslmed.aap9128

Grebely, J., Prins, M., Hellard, M., Cox, A. L., Osburn, W. O., Lauer, G., et al. (2012). Hepatitis C virus clearance, reinfection, and persistence, with insights from studies of injecting drug users: towards a vaccine. *Lancet Infect. Dis.* 12, 408–414. doi: 10.1016/s1473-3099(12)70010-5

Grubor-Bauk, B., Yu, W., Wijesundara, D., Gummow, J., Garrod, T., Brennan, A. J., et al. (2016). Intradermal delivery of DNA encoding HCV NS3 and perforin elicits robust cell-mediated immunity in mice and pigs. *Gene Ther.* 23, 26–37. doi: 10.1038/gt.2015.86

Gummow, J., Li, Y., Yu, W., Garrod, T., Wijesundara, D., Brennan, A. J., et al. (2015). A multiantigenic DNA vaccine that induces broad hepatitis C virus-specific T-cell responses in mice. *J. Virol.* 89, 7991–8002. doi: 10.1128/jvi.00803-15

Hansen, S. G., Ford, J. C., Lewis, M. S., Ventura, A. B., Hughes, C. M., Coyne-Johnson, L., et al. (2011). Profound early control of highly pathogenic SIV by an effector memory T-cell vaccine. *Nature* 473, 523–527. doi: 10.1038/nature10003

Hartnell, F., Brown, A., Capone, S., Kopycinski, J., Bliss, C., Makvandi-Nejad, S., et al. (2018). A novel vaccine strategy employing serologically different chimpanzee adenoviral vectors for the prevention of HIV-1 and HCV coinfection. *Front. Immunol.* 9:3175. doi: 10.3389/fimmu.2018.03175

Haynes, B. F., Gilbert, P. B., McElrath, M. J., Zolla-Pazner, S., Tomaras, G. D., Alam, S. M., et al. (2012). Immune-correlates analysis of an HIV-1 vaccine efficacy trial. *N. Engl. J. Med.* 366, 1275–1286. doi: 10.1056/NEJMoa1113425

Hayton, E. J., Rose, A., Ibrahimsa, U., Del Sorbo, M., Capone, S., Crook, A., et al. (2014). Safety and tolerability of conserved region vaccines vectored by plasmid DNA, simian adenovirus and modified vaccinia virus ankara administered to human immunodeficiency virus type 1-uninfected adults in a randomized, single-blind phase I trial. *PLoS ONE* 9:e101591. doi: 10.1371/journal.pone.0101591

Holz, L. E., Prier, J. E., Freestone, D., Steiner, T. M., English, K., Johnson, D. N., et al. (2018). CD8(+) T cell activation leads to constitutive formation of liver tissue-resident memory T cells that seed a large and flexible niche in the liver. *Cell Rep.* 25, 68–79 e4. doi: 10.1016/j.celrep.2018.08.094

Houghton, M. (2011). Prospects for prophylactic and therapeutic vaccines against the hepatitis C viruses. *Immunol. Rev.* 239, 99–108. doi: 10.1111/j.1600-065X.2010.00977.x

Ishizuka, A. S., Lyke, K. E., DeZure, A., Berry, A. A., Richie, T. L., Mendoza, F. H., et al. (2016). Protection against malaria at 1 year and immune correlates following PfSPZ vaccination. *Nat. Med.* 22, 614–623. doi: 10.1038/nm.4110

Jechlinger, W. (2006). Optimization and delivery of plasmid DNA for vaccination. *Expert Rev. Vaccines* 5, 803–825. doi: 10.1586/14760584.5.6.803

Jin, X., Morgan, C., Yu, X., DeRosa, S., Tomaras, G. D., Montefiori, D. C., et al. (2015). Multiple factors affect immunogenicity of DNA plasmid HIV vaccines in human clinical trials. *Vaccine* 33, 2347–2353. doi: 10.1016/j.vaccine.2015.03.036

Jorritsma, S. H. T., Gowans, E. J., Grubor-Bauk, B., and Wijesundara, D. K. (2016). Delivery methods to increase cellular uptake and immunogenicity of DNA vaccines. *Vaccine* 34, 5488–5494. doi: 10.1016/j.vaccine.2016.09.062

Joseph, S. B., Swanstrom, R., Kashuba, A. D., and Cohen, M. S. (2015). Bottlenecks in HIV-1 transmission: insights from the study of founder viruses. *Nat. Rev. Microbiol.* 13, 414–425. doi: 10.1038/nrmicro3471

Kawakami, S., Fumoto, S., Nishikawa, M., Yamashita, F., and Hashida, M. (2000). *In vivo* gene delivery to the liver using novel galactosylated cationic liposomes. *Pharm. Res.* 17, 306–313. doi: 10.1023/A:1007501122611

Kibuuka, H., Kimutai, R., Maboko, L., Sawe, F., Schunk, M. S., Kroidl, A., et al. (2010). A Phase I/II study of a multiclade HIV-1 DNA plasmid prime and recombinant adenovirus-type 5 boost vaccine in HIV uninfected East Africans (RV 172). *J. Infect. Dis.* 201, 600–607. doi: 10.1086/650299

Kim, T. J., Jin, H. T., Hur, S. Y., Yang, H. G., Seo, Y. B., Hong, S. R., et al. (2014). Clearance of persistent HPV infection and cervical lesion by therapeutic DNA vaccine in CIN3 patients. *Nat. Commun.* 5:5317. doi: 10.1038/ncomms6317

Larocca, R. A., Abbink, P., Peron, J. P., Zanotto, P. M., Iampietro, M. J., Badamchi-Zadeh, A., et al. (2016). Vaccine protection against Zika virus from Brazil. *Nature* 536, 474–478. doi: 10.1038/nature18952

Latimer, B., Toporovski, R., Yan, J., Pankhong, P., Morrow, M. P., Khan, A. S., et al. (2014). Strong HCV NS3/4a, NS4b, NS5a, NS5b-specific cellular immune responses induced in *Rhesus macaques* by a novel HCV genotype 1a/1b consensus DNA vaccine. *Hum. Vaccin. Immunother.* 10, 2357–2365. doi: 10.4161/hv.29590

Liu, F., Lei, J., Vollmer, R., and Huang, L. (2004). Mechanism of liver gene transfer by mechanical massage. *Mol. Ther.* 9, 452–7. doi: 10.1016/j.ymthe.2003.12.003

MacGregor, R. R., Boyer, J. D., Ugen, K. E., Lacy, K. E., Gluckman, S. J., Bagarazzi, M. L., et al. (1998). First human trial of a DNA-based vaccine for treatment of human immunodeficiency virus type 1 infection: safety and host response. *J. Infect. Dis.* 178, 92–100.

MacGregor, R. R., Ginsberg, R., Ugen, K. E., Baine, Y., Kang, C. U., Tu, X. M., et al. (2002). T-cell responses induced in normal volunteers immunized with a DNA-based vaccine containing HIV-1 env and rev. *AIDS* 16, 2137–2143. doi: 10.1097/00002030-200211080-00005

MacKay, L. K., Stock, A. T., Ma, J. Z., Jones, C. M., Kent, S. J., Mueller, S. N., et al. (2012). Long-lived epithelial immunity by tissue-resident memory T (TRM) cells in the absence of persisting local antigen presentation. *Proc. Natl. Acad. Sci. U.S.A.* 109, 7037–7042. doi: 10.1073/pnas.1202288109

Masopust, D., Choo, D., Vezys, V., Wherry, E. J., Duraiswamy, J., Akondy, R., et al. (2010). Dynamic T cell migration program provides resident memory within intestinal epithelium. *J. Exp. Med.* 207, 553–564. doi: 10.1084/jem.20090858

Masopust, D., Vezys, V., Marzo, A. L., and Lefrançois, L. (2001). Preferential localization of effector memory cells in nonlymphoid tissue. *Science* 291, 2413–2417. doi: 10.1126/science.1058867

McMichael, A. J., and Koff, W. C. (2014). Vaccines that stimulate T cell immunity to HIV-1: the next step. *Nat. Immunol.* 15, 319–322. doi: 10.1038/ni.2844

Moyo, N., Borthwick, N. J., Wee, E. G., Capucci, S., Crook, A., Dorrell, L., et al. (2017). Long-term follow up of human T-cell responses to conserved HIV-1 regions elicited by DNA/simian adenovirus/MVA vaccine regimens. *PLoS ONE* 12:e0181382. doi: 10.1371/journal.pone.0181382

Mueller, S. N., and Mackay, L. K. (2016). Tissue-resident memory T cells: local specialists in immune defence. *Nat. Rev. Immunol.* 16, 79–89. doi: 10.1038/nri.2015.3

Muruganandah, V., Sathkumara, H. D., Navarro, S., and Kupz, A. (2018). A systematic review: the role of resident memory T cells in infectious diseases and their relevance for vaccine development. *Front. Immunol.* 9:1574. doi: 10.3389/fimmu.2018.01574

Nagata, T., Uchijima, M., Yoshida, A., Kawashima, M., and Koide, Y. (1999). Codon optimization effect on translational efficiency of DNA vaccine in mammalian cells: analysis of plasmid DNA encoding a CTL epitope derived from microorganisms. *Biochem. Biophys. Res. Commun.* 261, 445–451. doi: 10.1006/bbrc.1999.1050

Nganou-Makamdop, K., Ploemen, I., Behet, M., Van Gemert, G. J., Hermsen, C., Roestenberg, M., et al. (2012). Reduced *Plasmodium berghei* sporozoite liver load associates with low protective efficacy after intradermal immunization. *Parasite Immunol.* 34, 562–569. doi: 10.1111/pim.12000.x

Okuda, K., Xin, K. O., Tsuji, T., Bukawa, H., Tanaka, S., Koff, W. C., et al. (1997). DNA vaccination followed by macromolecular multicomponent peptide vaccination against HIV-1 induces strong antigen-specific immunity. *Vaccine* 15, 1049–1056.

Olsen, T. M., Stone, B. C., Chuenchob, V., and Murphy, S. C. (2018). Prime-and-Trap malaria vaccination to generate protective CD8(+) liver-resident memory T cells. *J. Immunol.* 201, 1984–1993. doi: 10.4049/jimmunol.1800740

Osburn, W. O., Snider, A. E., Wells, B. L., Latanich, R., Bailey, J. R., Thomas, D. L., et al. (2014). Clearance of hepatitis C infection is associated with the early appearance of broad neutralizing antibody responses. *Hepatology* 59, 2140–2151. doi: 10.1002/hep.27013

Pallett, L. J., Davies, J., Colbeck, E. J., Robertson, F., Hansi, N., Easom, N. J. W., et al. (2017). IL-2(high) tissue-resident T cells in the human liver: sentinels for hepatotropic infection. *J. Exp. Med.* 214, 1567–1580. doi: 10.1084/jem.20162115

Park, S. L., Zaid, A., Hor, J. L., Christo, S. N., Prier, J. E., Davies, B., et al. (2018). Local proliferation maintains a stable pool of tissue-resident memory T cells after antiviral recall responses. *Nat. Immunol.* 19, 183–191. doi: 10.1038/s41590-017-0027-5

Parsons, M. S., Lloyd, S. B., Lee, W. S., Kristensen, A. B., Amarasena, T., Center, R. J., et al. (2017). Partial efficacy of a broadly neutralizing antibody against cell-associated SHIV infection. *Sci. Transl. Med.* 9:eaaf1483. doi: 10.1126/scitranslmed.aaf1483

Platt, L., Easterbrook, P., Gower, E., McDonald, B., Sabin, K., McGowan, C., et al. (2016). Prevalence and burden of HCV co-infection in people living with HIV: a global systematic review and meta-analysis. *Lancet Infect. Dis.* 16, 797–808. doi: 10.1016/S1473-3099(15)00485-5

Pontesilli, O., Klein, M. R., Kerkhof-Garde, S. R., Pakker, N. G., de Wolf, F., Schuitemaker, H., et al. (1998). Longitudinal analysis of human immunodeficiency virus type 1-specific cytotoxic T lymphocyte responses: a predominant gag-specific response is associated with nonprogressive infection. *J. Infect. Dis.* 178, 1008–1018.

Rolland, M., Nickle, D. C., and Mullins, J. I. (2007). HIV-1 group M conserved elements vaccine. *PLoS Pathog.* 3:e157. doi: 10.1371/journal.ppat.0030157

Rosato, P. C., Beura, L. K., and Masopust, D. (2017). Tissue resident memory T cells and viral immunity. *Curr. Opin. Virol.* 22, 44–50. doi: 10.1016/j.coviro.2016.11.011

Sacks-Davis, R., Grebely, J., Dore, G. J., Osburn, W., Cox, A. L., Rice, T. M., et al. (2015). Hepatitis C virus reinfection and spontaneous clearance of reinfection–the InC3 study. *J. Infect. Dis.* 212, 1407–1419. doi: 10.1093/infdis/jiv220

Saez-Cirion, A., Lacabaratz, C., Lambotte, O., Versmisse, P., Urrutia, A., Boufassa, F., et al. (2007). HIV controllers exhibit potent CD8 T cell capacity to suppress HIV infection ex vivo and peculiar cytotoxic T lymphocyte activation phenotype. *Proc. Natl. Acad. Sci. U.S.A.* 104, 6776–6781. doi: 10.1073/pnas.0611244104

Sato, T., Thorlacius, H., Johnston, B., Staton, T. L., Xiang, W., Littman, D. R., et al. (2005). Role for CXCR6 in recruitment of activated CD8+ lymphocytes to inflamed liver. *J. Immunol.* 174, 277–283.doi: 10.4049/jimmunol.174.1.277

Schenkel, J. M., Fraser, K. A., Vezys, V., and Masopust, D. (2013). Sensing and alarm function of resident memory CD8(+) T cells. *Nat. Immunol.* 14, 509–513. doi: 10.1038/ni.2568

Shin, H., and Iwasaki, A. (2012). A vaccine strategy that protects against genital herpes by establishing local memory T cells. *Nature* 491, 463–467. doi: 10.1038/nature11522

Shin, S. Y. (2016). Recent update in HIV vaccine development. *Clin. Exp. Vaccine Res.* 5, 6–11. doi: 10.7774/cevr.2016.5.1.6

Smyk-Pearson, S., Tester, I. A., Klarquist, J., Palmer, B. E., Pawlotsky, J. M., Golden-Mason, L., et al. (2008). Spontaneous recovery in acute human hepatitis C virus infection: functional T-cell thresholds and relative importance of CD4 help. *J. Virol.* 82, 1827–1837. doi: 10.1128/jvi.01581-07

Stone, J., Martin, N. K., Hickman, M., Hellard, M., Scott, N., McBryde, E., et al. (2016). The potential impact of a hepatitis C vaccine for people who inject drugs: is a vaccine needed in the age of direct-acting antivirals? *PLoS ONE* 11:e0156213. doi: 10.1371/journal.pone.0156213

Sun, Y., Peng, S., Qiu, J., Miao, J., Yang, B., Jeang, J., et al. (2015). Intravaginal HPV DNA vaccination with electroporation induces local CD8+ T-cell immune responses and antitumor effects against cervicovaginal tumors. *Gene Ther.* 22, 528–535. doi: 10.1038/gt.2015.17

Swadling, L., Capone, S., Antrobus, R. D., Brown, A., Richardson, R., Newell, E. W., et al. (2014). A human vaccine strategy based on chimpanzee adenoviral and MVA vectors that primes, boosts, and sustains functional HCV-specific T cell memory. *Sci. Transl. Med.* 6:261ra153. doi: 10.1126/scitranslmed.3009185

Tavel, J. A., Martin, J. E., Kelly, G. G., Enama, M. E., Shen, J. M., Gomez, P. L., et al. (2007). Safety and immunogenicity of a Gag-Pol candidate HIV-1 DNA vaccine administered by a needle-free device in HIV-1–seronegative subjects. *J. AIDS* 44, 601–605. doi: 10.1097/QAI.0b013e3180417cb6

Tay, S. S., Wong, Y. C., McDonald, D. M., Wood, N. A., Roediger, B., Sierro, F., et al. (2014). Antigen expression level threshold tunes the fate of CD8 T cells during primary hepatic immune responses. *Proc. Natl. Acad. Sci. U.S.A.* 111, E2540–E2549. doi: 10.1073/pnas.1406674111

Tebas, P., Roberts, C. C., Muthumani, K., Reuschel, E. L., Kudchodkar, S. B., Zaidi, F. I., et al. (2017). Safety and immunogenicity of an anti-Zika virus DNA vaccine - preliminary report. *N. Engl. J. Med.* doi: 10.1056/NEJMoa1708120

Tomusange, K., Wijesundara, D., Gummow, J., Garrod, T., Li, Y., Gray, L., et al. (2016). A HIV-Tat/C4-binding protein chimera encoded by a DNA vaccine is highly immunogenic and contains acute EcoHIV infection in mice. *Sci. Rep.* 6:29131. doi: 10.1038/srep29131

Trimble, C. L., Morrow, M. P., Kraynyak, K. A., Shen, X., Dallas, M., Yan, J., et al. (2015). Safety, efficacy, and immunogenicity of VGX-3100, a therapeutic synthetic DNA vaccine targeting human papillomavirus 16 and 18 E6 and E7 proteins for cervical intraepithelial neoplasia 2/3: a randomised, double-blind, placebo-controlled phase 2b trial. *Lancet* 386, 2078–2088. doi: 10.1016/S0140-6736(15)00239-1

Tse, S. W., Radtke, A. J., Espinosa, D. A., Cockburn, I. A., and Zavala, F. (2014). The chemokine receptor CXCR6 is required for the maintenance of liver memory CD8(+) T cells specific for infectious pathogens. *J. Infect. Dis.* 210, 1508–16. doi: 10.1093/infdis/jiu281

UNAIDS (2018). *Miles to Go- Closing Gaps, Breaking Barriers, Righting Injustices*. Global AIDS update 2018.

Vanniasinkam, T., Reddy, S. T., and Ertl, H. C. J. (2006). DNA immunization using a non-viral promoter. *Virology* 344, 412–420. doi: 10.1016/j.virol.2005.08.040

Vujkovic-Cvijin, I., Dunham, R. M., Iwai, S., Maher, M. C., Albright, R. G., Broadhurst, M. J., et al. (2013). Dysbiosis of the gut microbiota is associated with HIV disease progression and tryptophan catabolism. *Sci. Transl. Med.* 5:193ra91. doi: 10.1126/scitranslmed.3006438

Wang, H. B., Mo, Q. H., and Yang, Z. (2015). HIV vaccine research: the challenge and the way forward. *J. Immunol. Res.* 2015:503978. doi: 10.1155/2015/503978

Weiland, O., Ahlen, G., Diepolder, H., Jung, M. C., Levander, S., Fons, M., et al. (2013). Therapeutic DNA vaccination using *in vivo* electroporation followed by standard of care therapy in patients with genotype 1 chronic hepatitis C. *Mol. Ther.* 21, 1796–1805. doi: 10.1038/mt.2013.119

WHO (2017). *Global Hepatitis Report, 2017*. Geneva: World Health Organisation. Available online at: https://www.who.int/hepatitis/publications/global-hepatitis-report2017/en/. (accessed November 06, 2018).

Wijesundara, D. K., Gummow, J., Li, Y., Yu, W., Quah, B. J., Ranasinghe, C., et al. (2018). Induction of genotype cross-reactive, hepatitis C virus-specific, cell-mediated immunity in DNA-vaccinated mice. *J. Virol.* 92:e02133-17. doi: 10.1128/jvi.02133-17

Yu, W., Grubor-Bauk, B., Gargett, T., Garrod, T., and Gowans, E. J. (2014). A novel challenge model to evaluate the efficacy of hepatitis C virus vaccines in mice. *Vaccine* 32, 3409–3416. doi: 10.1016/j.vaccine.2014.04.014

Zhang, X. (2016). Direct anti-HCV agents. *Acta Pharm. Sin. B* 6, 26–31. doi: 10.1016/j.apsb.2015.09.008

Conflict of Interest Statement: 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 Cellular and Infection Microbiology

REVIEW
published: 17 April 2019
doi: 10.3389/fcimb.2019.00107

The Future of Flu: A Review of the Human Challenge Model and Systems Biology for Advancement of Influenza Vaccinology

Amy Caryn Sherman, Aneesh Mehta, Neal W. Dickert, Evan J. Anderson and Nadine Rouphael*

Department of Medicine, Division of Infectious Diseases, Emory University, Atlanta, GA, United States

Objectives: Novel approaches to advance the field of vaccinology must be investigated, and are particularly of importance for influenza in order to produce a more effective vaccine. A systematic review of human challenge studies for influenza was performed, with the goal of assessing safety and ethics and determining how these studies have led to therapeutic and vaccine development. A systematic review of systems biology approaches for the study of influenza was also performed, with a focus on how this technology has been utilized for influenza vaccine development.

Methods: The PubMed database was searched for influenza human challenge studies, and for systems biology studies that have addressed both influenza infection and immunological effects of vaccination.

Results: Influenza human challenge studies have led to important advancements in therapeutics and influenza immunization, and can be performed safely and ethically if certain criteria are met. Many studies have investigated the use of systems biology for evaluating immune response to influenza vaccine, and several promising molecular signatures may help advance our understanding of pathogenesis and be used as targets for influenza interventions. Combining these methodologies has the potential to lead to significant advances in the field of influenza vaccinology and therapeutics.

Conclusions: Human challenge studies and systems biology approaches are important tools that should be used in concert to advance our understanding of influenza infection and provide targets for novel therapeutics and immunizations.

Keywords: human challenge model, influenza vaccination, systems biology, universal influenza vaccination, bioethics, transcriptomics, metabolomics

OPEN ACCESS

Edited by:
Yanmin Wan,
Fudan University, China

Reviewed by:
Francesco Santoro,
University of Siena, Italy
Kevin Alby,
University of North Carolina at Chapel Hill, United States

****Correspondence:***
Amy Caryn Sherman
amy.sherman@emory.edu

Specialty section:
This article was submitted to Clinical Microbiology, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *31 January 2019*
Accepted: *28 March 2019*
Published: *17 April 2019*

Citation:
Sherman AC, Mehta A, Dickert NW, Anderson EJ and Rouphael N (2019) The Future of Flu: A Review of the Human Challenge Model and Systems Biology for Advancement of Influenza Vaccinology. Front. Cell. Infect. Microbiol. 9:107. doi: 10.3389/fcimb.2019.00107

INTRODUCTION

Although influenza virus was recognized as an important pathogen over a century ago, influenza continues to cause a significant burden of disease. In the United States alone, it's estimated that in the 2017–2018 season there were 959,000 hospitalizations related to influenza illness, and 79,400 deaths (CDC, 2018). Worldwide, WHO estimates that annual influenza epidemics cause 3–5 million cases of severe disease, with 290,000–650,000 of these severe cases resulting in death (Influenza (Seasonal), 2018). Although annual influenza immunizations are recommended and

antivirals are available, both have several limitations. The efficacy of the seasonal influenza vaccine is compromised by several factors: antigenic changes over time (requiring a strain specific match each year), slow manufacturing processing, vaccine strain egg-adapted changes, short duration of protection, lack of cross-reactivity, and poor immunogenicity in certain populations (e.g., the elderly) (Goodwin et al., 2006; Soema et al., 2015; Raymond et al., 2016). Antiviral agents such as neuraminidase inhibitors are most effective if administered early in the disease course, and even then have only a modest impact upon the duration of clinical symptoms (McNicholl and McNicholl, 2001). Furthermore, data are inconclusive regarding the ability of neuraminidase inhibitors to reduce the risk of complications, such as hospitalizations or progression to pneumonia (Doll et al., 2017).

Novel platforms to understand influenza immunology are essential in order to address the burden of influenza disease and develop a more effective influenza vaccine that does not rely on annual updates. Combining old modalities—human challenge studies—with new technology—systems biology—has the potential to lead to exciting discoveries that can achieve this goal. There are many reasons why human challenge studies are essential for scientific progress, especially for the influenza field. Human challenge models have several benefits over traditional models such as animal and epidemiological models. Although mice and ferrets have been used in influenza challenges, animal models do not directly translate to humans in regards to their baseline immunity and subsequent immunological responses. Epidemiological or field studies have also been applied to study influenza vaccine efficacy, such as Petrie et al who followed a cohort of over 1,000 individuals from 2014 to 2015 to assess vaccine effectiveness (Petrie et al., 2017). However, these studies require a large sample size and often require numerous sampling points and several years to acquire sufficient data, with many conflicting and cofounding variables. In contrast, the human challenge model is efficient (relatively few participants are required to power a study), immunological responses of humans can be studied directly, and the exact timing of infection is known so that specific time points and measurements are precisely determined.

Human challenge studies for influenza are particularly attractive, with current national emphasis on development of a universal influenza vaccine. As outlined by NIAID's strategic plan, a universal flu vaccine would be at least 75% effective, maintain protection for at least 1 year, protect against group I (e.g., H1, H5) and II (e.g., H3, H7) influenza A virus strains, and be effective for all age groups (Erbelding et al., 2018). The strategic plan also elucidated how a human challenge model could offer unique benefits to better understand the concept of imprinting, determine correlates of protection against influenza, and evaluate different universal influenza vaccine candidates.

In this review, we will examine influenza human challenge studies that have been conducted and their safety, as well as review the ethical considerations in designing a challenge model. We will also review how the use of systems biology techniques in the context of human influenza challenge studies has great potential to advance our current understanding of the host response to acute influenza infection, and ultimately aid in the development of a universal influenza vaccine. The PubMed database was used to search for relevant clinical trials related to these subjects. We propose that successful integration of the right model (the human challenge study) in combination with systems biology approaches will help to better understand the immunological mechanisms of influenza infection and effects of vaccination, which will ultimately aid in the development of an improved vaccine (and perhaps even a universal influenza vaccine), and novel influenza therapeutics.

History and Safety of Influenza Challenge Models

In medical research, there is a long and complex history of human challenge studies, in which healthy participants are intentionally infected in order to study the natural history of a disease or to test experimental therapeutic and preventative measures. Perhaps the most famous challenge study in infectious disease was Edward Jenner's use of cowpox in 1796. Although he is not the first to use intentional infection to protect against disease—historical records show that similar practices were likely occurring in Africa, India and China (Gross and Sepkowitz, 1998) long before the eighteenth century—he was responsible for publishing and popularizing the idea. Jenner inoculated the 8-year-old boy James Phipps with cowpox derived from a lesion on the hand of a dairymaid, Sarah Nelms (Riedel, 2005). This successful challenge lead to the creation of the first vaccine, with widespread use in Europe by the year 1800, and eventual eradication of smallpox.

The first well described influenza challenge study was published by Smorodintseff et al. in 1937. The authors infected 72 volunteers via inhalation of a human influenza virus, that had been maintained through the passage of ferrets and mice (Smorodintseff et al., 1937). They discovered that only a small percent (∼20%) developed disease that was mild in intensity. The model was deemed safe and was utilized for several decades thereafter to understand immune responses to influenza and test preventative and therapeutic measures.

However, in the early 2000s, influenza human challenge studies came to a halt. This was a direct result of an adverse outcome associated with a human challenge study that was investigating the use of peramivir as a prophylactic agent (Ison et al., 2005). The subject was a 21-year-old, previously healthy individual with no prior cardiac history. Following the infection with mild influenza B infection and receipt of peramivir, he had asymptomatic ECG changes (described as new T wave inversions of leads II, aVF, and v4-6) on day 4 of the study. Repeat ECG at day 15 had returned to baseline, and he had no cardiac symptoms or enzyme elevation of CPK. He then traveled to Indonesia for 2 weeks, and became ill with an URI. When he returned to the US, he had an echocardiography performed 51 days after challenge since he had had initial ECG changes earlier. He had new reduced ejection fraction, with left ventricular enlargement and remained asymptomatic. Extensive work-up was unrevealing for infectious etiologies. Over the next 5 months, repeat echocardiograms showed gradual improvement and return to normal ejection fraction.

Despite the subject's return to baseline health, and despite the lack of direct causality linking myocarditis to the influenza challenge stock, no further influenza challenge studies were conducted in the US for nearly a decade later. Internationally, as well, only a few challenge studies for influenza were conducted in this time period. After the H1N1 pandemic, Memoli et al. at the NIH re-visited the human challenge model with their validation of a A/H1N1 challenge strain (Memoli et al., 2015). His group challenged 46 healthy participants with a virus that was rescued using reverse genetics (A/CA/04/2009) , with the goal to determine the dose needed to produce mild to moderate influenza infection in at least 60% of the participants who had baseline HAI titers of $\leq$1:40. The experimental influenza virus was delivered intranasally, and the participants were kept in isolation for at least 9 days following the challenge. The participants' symptoms were monitored and their immune response (serum cytokines, HAI and neuraminidase inhibition titers) documented at specific pre- and post-challenge time points. Discharge from isolation occurred only after the participant had two negative nasal washes on consecutive days. All of the participants demonstrated clinical symptoms of infection (as intended), and 70% of participants had both viral shedding and symptoms. At the dose of 10^7 $TCID_{50}$, 85% of the participants who received that dose had a $\geq$4-fold rise in HAI titer by week 8. No significant adverse effects or complications of the influenza infection occurred. Carrat et al. also performed a large and thorough review of 56 different influenza challenge studies, and confirmed that infection from a challenge stock induced only mild disease, with one third of participants having a fever and one fifth of participants developing lower respiratory symptoms (Carrat et al., 2008). This extensive review, in addition to further studies by Memoli's team (Memoli et al., 2016; Hunsberger and Memoli, 2017; Han et al., 2018), demonstrated that influenza challenges can be implemented safely and used in influenza research.

Ethical Considerations for Human Challenge Studies

Human challenge studies raise specific ethical concerns because they place research subjects at risk with no potential for direct benefit. The main considerations that must be weighed include the level of risk to the participants, the overall social value of the study, the absence of good alternative study designs, and informed consent.

In human subject research, the risk-to-benefit ratio is usually favorable to the subject, before the risk-to-benefit ratio is weighed for the broader society (Pollard et al., 2012). For example, an individual with a rare and life-threatening disease may choose to participate in a trial that tests a novel therapy, because they personally may benefit from a cure for an otherwise untreatable condition. However, for the human challenge model, the participant is not directly gaining anything for their health, and the risk-to-benefit ratio leans toward the side of risk. Therefore, an acceptable challenge model must have risks that are reasonable to the participants, because the challenge model can be justified only by the benefits to society and not to the individuals. To mitigate risk to the participants, certain criteria should be evaluated. The study hypothesis must only be answerable by challenging human subjects; if the question and endpoint of the study could be determined by animal models or *in vitro* techniques, the human challenge model should not be used. Infected subjects should also have therapy available (in the case of influenza, antivirals such as oseltamivir or peramivir) in the event that they develop severe influenza infection during the experimental challenge. Participants should receive compensation for their time and effort; however, the more controversial topic is how much participants should receive. Is there a certain amount that is too much, and thus coercive? And likewise, is there an amount that is too little based on the risk inherent in the challenge study? Several authors, such as Miller and Grady, have offered a framework to evaluate the ethical considerations of an infection-inducing challenge study (Miller and Grady, 2001). To maintain ethical integrity, other specific considerations must be addressed in designing an influenza human challenge study, as outlined in **Table 1**.

If these criteria and considerations are carried out in a thoughtful manner, influenza challenge studies can be implemented safely and ethically.

A limitation of the human challenge model is the requirement to have healthy volunteers with no significant comorbidities. As stated above, healthy volunteers must be recruited in order to be ethically sound, in order to reduce the overall risk and complications to the individual. However, this limits our understanding of influenza pathogenesis and vaccine performance in high-risk populations who have the greatest likelihood of severe influenza complications, such as the very young and elderly, immunocompromised, and those with comorbidities. Another inherent limitation of this model is the goal to produce only mild to moderate influenza disease, not severe disease. Again, the efficacy of new therapeutics would be most important in the population suffering severe disease, which cannot be tested in this model for ethical reasons.

TABLE 1 | Ethical considerations in the design of an influenza challenge model.

Selection of the appropriate strain and dose of influenza challenge stock to achieve mild to moderate influenza illness.
Strict inclusion and exclusion criteria to ensure healthy volunteers with minimal comorbidities.
Full review of the proposed study by a third-party ethics committee.
Selection of appropriate clinical and microbiological endpoints to minimize risk to participants.
Transparent informed consent and fair compensation for participants.
Facilities and trained staff that can ensure close and careful monitoring of infected participants.
Proof of decreased infectivity (e.g., undetectable virus by molecular testing) upon discharge to eliminate possibility of transmission to general public.
Adequate clinical follow-up and evaluation for adverse events or sequelae of influenza infection.

**Loosely adapted from Darton et al. (2015) and Miller and Grady (2001).*

Advancing Influenza Therapeutics With the Influenza Human Challenge Model

The influenza challenge model has proven useful in advancing the development of several current antivirals to the market, while also useful in terminating the clinical development of others. Two large randomized controlled trials were conducted in 1997 by Hayden et al. which investigated the use of oseltamivir, an oral neuraminidase inhibitor, for both prophylaxis and treatment (Hayden et al., 1999). Healthy participants ($N = 117$) were inoculated with influenza A/Texas/36/91 (H1N1), and given oseltamivir (at two different doses) or placebo. The results showed that prophylaxis and early treatment with oseltamivir significantly reduced symptoms and had anti-viral effects. This trial is the basis for the standard of care practiced today—oseltamvir continues to be recommended for influenza prophylaxis and treatment by clinical treatment guidelines (Uyeki et al., 2019). Peramivir, another neuraminidase inhibitor agent, was also tested using a similar design. Four randomized, double-blind, placebo-controlled trials were conducted, with 288 healthy volunteers inoculated with either A/Texas/36/91 (H1N1) or B/Yamagata/16/88 (Barroso et al., 2005). At the time of the study, oseltamivir was the only oral neuraminidase inhibitor available. Therefore, the authors sought to determine the tolerability and antiviral efficacy of oral peramivir for treatment and prophylaxis of influenza A and B, using the experimental influenza virus. The results showed that an oral dose of 200 mg twice daily or 400 mg once a day was effective for influenza A, and that prophylaxis with peramivir did not significantly reduce viral shedding. The authors also found relatively low blood peramivir concentrations, and recommended pursuing further study with parental dosing. In both the oseltamivir and peramivir studies, the challenge model was essential for understanding the exact timing of infection in order to test the efficacy of the new agents and identify the appropriate dosing schedule.

Testing of therapies that did not work were equally important in advancing the field. For example, a topical interferon inducer was tested in subjects experimentally infected with an influenza A H3N2 strain (Douglas et al., 1975). They found no difference in frequency or severity of illness, or in quantitative viral shedding between the placebo group and the group who received the interferon inducer. Another study investigated a new antiviral (agent ICI 130,685) that was similar to amantadine for both prophylactic and therapeutic use (Al-Nakib et al., 1986). The authors found that that the higher dosage of 200 mg/day of the new agent did have protective efficacy as compared to placebo when used for prophylaxis; however, the number of side effects in this group were double the side effects reported for the placebo group. For the therapeutic group, the new agent did show a reduced mean daily clinical score and decreased virus in the nasal washings as compared to the placebo arm. Yet, these reductions were not statistically significant for viral concentration until day 3, and for symptom score until day 4. Therefore, this product did not forward toward licensure since the risks (increased side effects) were greater than the benefits (only minor reductions in viral shedding and late symptom improvement).

Advancing Vaccine Development With the Influenza Challenge Model

Human challenge studies have also been critical for the development of influenza vaccines. The first influenza vaccines were developed and distributed in the US in 1938, and provided to soldiers in World War II (Hannoun, 2013). Thomas Francis, who was a leader in the field of influenza and the author of "Doctrine of Original Antigenic Sin," published a small trial in 1940 in which he inoculated active influenza viral cultures into the nares of 11 human subjects (Francis, 1940). He reported that none of the subjects experienced significant signs or symptoms of infection, and one individual showed a rise of influenza antibodies. He proposed that this technique may have potential for vaccine development. Another study in 1942–1943 challenged individuals with active influenza virus who had received an allantoic fluid vaccine at least 4 months before (Henle et al., 1943). Although it was a small study, the investigators were able to determine the efficacy of the vaccine. Of their controls, ten of twenty-eight individuals developed clinical influenza after inhalation of the isolated and active virus. In contrast, only one of forty-four individuals became ill with influenza after receiving the vaccination. Other early investigations had similar goals, and tested vaccine efficacy for both influenza A and B (Francis and Magill, 1937; Francis et al., 1945; Henle et al., 1946). In 1971, Couch et al. investigated the use of a recombinant influenza A vaccine (X-31 influenza A_2, Hong Kong variant) in comparison to the standard vaccine (Couch et al., 1971). Two groups of healthy volunteers were given either the recombinant or the standard vaccine, and then challenged with the live virus strain (Hong Kong variant) used in the vaccine a month later. They measured neuraminidase-inhibiting antibody in the serum, neutralizing antibody in nasal secretions, influenza virus in the nasal secretions, and degree of clinical illness. The results demonstrated that the two vaccines were equally effective. Treanor et al. later challenged healthy adults with wild-type influenza strains to compare the efficacy of a trivalent, live, cold-adapted influenza vaccine (CAIV-T) against the trivalent inactivated influenza vaccine (TIV) (Treanor et al., 1999). After challenging the immunized individuals, they measured influenza illness (defined as respiratory symptoms with wild-type influenza virus isolated from nasal passages or >4-fold increase of HAI antibody from baseline). The efficacy of the immunizations was calculated as 85% for CAIV-T and 71% for TIV, although the difference was not statistically significant.

IMMUNOLOGY AND APPLYING SYSTEMS BIOLOGY TO INFLUENZA VACCINOLOGY

Pathogenesis and Immunology of Influenza Infection

Several studies have used experimental influenza inoculation of humans to investigate the pathogenesis and immune mechanisms associated with infection. Many sought to better understand viral infectivity by route of transmission (e.g., aerosolization), or determine if influenza could be transmitted across different

host species (Kasel et al., 1965). For example, one study sought to determine if equine influenza virus could produce clinical symptoms if given to a human (Alford et al., 1966).

Others investigated various human immune responses following experimental infection. The mechanism of systemic and local antibody responses to infection was examined in a study conducted by Murphy et al. Their volunteers were children, aged 1.5 to 4.5 years old, and were inoculated intranasally with either A/Alaska/7/77 (H3N2) or A/Hong Kong/123/77 (H1N1) viruses (developed as candidate vaccine viruses) (Murphy et al., 1982). They found that there was a correlation between the IgA HA antibodies recovered from the nasal passageways and the serum IgG response. Thus, they proposed that intranasal inoculation stimulates both systemic and local immune responses and could be used for immunization purposes. Brown et al. sought to further explore the distinction between serum and secretory IgA antibodies in response to infection. By challenging 13 human volunteers with intranasal A/Peking/2/79(H3N2) wild-type virus, they measured serum and nasal IgA antibodies and their subclasses pre- and post- inoculation with the influenza virus (Brown et al., 1985). They found that IgA1 accounted for most of the increase in IgA anti-HA levels after infection, and determined that the origin of serum IgA antibodies to HA were from the mucosa.

Other investigations examined cell-mediated immune responses, challenging healthy volunteers with different strains of influenza A and measuring serum antibody, viral shedding, and other peripheral blood parameters (such as white blood cell counts), as well as local and systemic cytokine responses. In 1977, Dolin et al administered influenza A virus to 19 volunteers in order to assess cell-mediated immune responses up to 4 weeks after the challenge (Dolin et al., 1977). Eight of the nineteen volunteers had clinical symptoms and 4-fold increases of serum antibody, and lymphopenia was described in this cohort. The authors found that depression of lymphocytes and decreased functionality of the lymphocytes were present even at 4 weeks post-challenge. Another study by Hayden et al challenged volunteers with a H1N1 influenza strain and examined the relationship between clinical symptoms and cytokine responses (Hayden et al., 1998). They collected nasal lavage fluids, plasma and serum levels from the participants and analyzed various cytokines (IL-1 β, IL-2, IL-6, IL-8, IFN-α, TGF-β, and TNF-α) over time. The authors described an association between nasal fluid IFN-α, IL-6, and TNF-α levels and fever on day 2 post-challenge, and IFN-α and IL-6 levels with lower respiratory symptoms on days 5 and 6. They found that IL-6 levels were associated with total, systemic, and upper respiratory symptom scores on days 2 and 4. They concluded that IL-6 is likely the main factor involved in causing fever in influenza (not TNF-α, which is usually described as contributing to fever symptoms in other infections) based on the larger magnitude of IL-6 response early in the course of infection, and IFN-α is responsible for early systemic and local symptoms experienced in influenza infection. The authors hoped that their description of the cytokine response to influenza could be utilized to either (a) develop therapeutic agents that would target specific cytokine responses, or (b) use cytokine levels to more accurately measure the impact of an antiviral intervention. An interesting study by Gentile et al. utilized the influenza human challenge model to control for inflammatory responses seen in patients with allergic rhinitis, which they hypothesized would cause more severe disease (Gentile et al., 2001). Gentile et al enrolled 27 participants who had a history of allergic or nonallergic rhinitis, and then inoculated the participants with an influenza A H1N1 strain and measured anti-IgE-induced leukocyte histamine release, plasma histamine levels, and serum IgG, IgA, IgM, and IgE. The results showed no significant enhancement of systemic immune or inflammatory responses after inoculation in the allergic rhinitis group. The authors speculated that perhaps no difference was observed because the experimental infection was so mild in nature.

Systems Biology and Influenza Immunization

The field of systems biology applied to vaccines uses mathematical modeling, networking, and other measurements to describe and predict the human immune response to vaccines. In addition, systems biology approaches have the potential to better explain the host-viral interaction and elucidate specific immunological pathways and mechanisms in regards to both influenza natural infection and influenza vaccination. Using systems biology methodologies may be especially useful in the context of influenza human challenge studies.

Nakaya et al. have done extensive work describing specific molecular signatures in individuals who have received seasonal influenza vaccines (Nakaya et al., 2011). They compared immune responses in individuals who had received either the trivalent inactivated vaccine (TIV) or live attenuated influenza vaccine (LAIV). They identified molecular signatures that correlated with B cell responses at day 7 and 28 after immunization, and demonstrated how these could be used to predict an individual's later immune response to the vaccine. Several specific genes were identified that corresponded with HAI response, many of which were regulated by XBP-1 (transcription factor). XBP-1 has been shown to be necessary for the differentiation of plasma cells and the unfolded protein response (Iwakoshi et al., 2003). Another important feature of an effective vaccine is its ability to produce and maintain a long-term durable antibody response to provide long-lasting protection. In a separate study, Nakaya et al. investigated antibody responses after influenza vaccination at day 28 vs. 180, with emphasis on identifying molecular pathways associated with either a persistent or waning antibody response with time (Nakaya et al., 2015). One potentially important pathway associated with persistent antibody response involves P-selectin (SELP), which affects mobility of leukocytes to the vaccine administration site from the peripheral blood stream. The authors were also able to identify transcriptional responses to vaccination, with particular interest in micro RNA expression of miR-424. MiR-424 is a regulator of the interferon response that occurs post vaccination. Another contribution was the finding of specific signatures associated with different age groups. The authors demonstrated that in the elderly population, modules

TABLE 2 | Using systems biology to study immune responses to influenza vaccination.

Study title (first author)	Year	Key molecular correlate(s) identified	Contribution
Systems analysis of immunity to influenza vaccination across multiple years and in diverse populations reveals shared molecular signatures (Nakaya et al., 2015)	2015	**P-Selectin (SELP)**: pathway associated with persistent antibody response. **miR-424:** regulator of the interferon response post vaccination	Identified pathways involved in a more long-term, durable response to influenza immunization. Identified interferon response and inflammatory markers that differ according to age. Persistent inflammatory state in elderly may explain immunosenescence.
Systems biology of immunity to MF59-adjuvanted vs. nonadjuvanted trivalent seasonal influenza vaccines in early childhood (Nakaya et al., 2016)	2016	**Module 75**: "antiviral interferon signature." **Module 165**: "enriched in activated dendritic cells"	Identified modules that were correlated with strong HAI response post-vaccination with adjuvant.
Systems biology of vaccination for seasonal influenza in humans (Nakaya et al., 2011)	2011	**XBP-1:** corresponds with HAI response	Identified molecular signatures that correlated with B cell response to vaccinations, and showed how these can be used to predict vaccine response.
Global analyses of human immune variation reveal baseline predictors of post-vaccination responses (Tsang et al., 2014)	2014	PBMC subpopulation frequencies (baseline)	Described baseline characteristics that can be used to predict serologic response to influenza vaccines.
Apoptosis and other immune biomarkers predict influenza vaccine responsiveness (Furman et al., 2013)	2013	**APO module:** involved in apoptosis	Described a positive association between two gene modules involved with apoptosis and vaccine response to influenza.
Differences in antibody responses between trivalent inactivated influenza vaccine and live attenuated influenza vaccine correlate with the kinetics and magnitude of interferon signaling in children (Cao et al., 2014)	2014	**IFN-related modules:** M1.2, M3.4, M5.12, encode for IFN-inducible proteins. Overexpressed day 1 post-vaccination for the TIV group only, and more prominent in younger children.	Identified transcriptional patterns post-vaccination demonstrating that vaccines induced expression of interferon-related genes, which also was associated with antibody production.
Early patterns of gene expression correlate with the humoral immune response to influenza vaccination in humans (Bucasas et al., 2011)	2011	**494-gene signature** (mediates interferon response): positive correlation with antibody response to vaccination	Described a signature that corresponded to antibody response to the trivalent influenza vaccine.
Integrative genomic analysis of the human immune response to influenza vaccination (Franco et al., 2013)	2013	20 genes identified with correlation between transcriptional and antibody responses to vaccination, which influence the immune response to vaccine: **TAP2, SNX29, FGD2, NAPSA, NAPSB, GM2A C1orf85, JUP, FBLN5, CHST13, DIP2a, PAM, D4S234E, C3AR1, HERC2, LST1, LRRC37A4, OAS1, RPL14, DYNLT1**	Identified potential predictive biomarkers that can describe vaccine response. Many of the genes described are involved in membrane trafficking, antigen processing and antigen presentation.

associated with antiviral and IFN-related genes were impaired in the early innate response as compared to the younger population. In the elderly population, there was an enhanced NK-cell related expression and higher proportions of monocytes at baseline and post vaccination. It is hypothesized that perhaps these persistent inflammatory responses seen in the elderly may actually be having a negative effect by inhibiting appropriate vaccine immune responses, and perhaps explains the underlying mechanism of immunosenescence.

Several other studies have examined patterns of gene expression in response to influenza vaccination. The results of key experiments are summarized in **Table 2**.

Systems biology approaches have also been used to study human influenza infection, mostly in human cell culture, in order to better define pathogenesis, virulence factors, and immune responses. For example, transcriptomic responses to a human tissue culture cell line infected with various strains of influenza were examined by Josset et al. (2014). By analyzing transcriptomic responses to Influenza A Virus (IAV), the authors described that the avian H7N9 had further adapted to the human host. They also were able to test various therapeutics *in vitro*, and demonstrated antiviral responses associated with gene expression alterations. Other authors have studied proteomics in macrophages and monocytes of the lung after IAV infection. These studies showed interferon and TNF-alpha expression in response to infection, as well as pathways leading to secretions of specific proteins and antiviral cytokines (Lee et al., 2009; Lietzén et al., 2011; Cypryk et al., 2017). Ultimately, these findings demonstrate that macrophages could be used as a biomarker to determine the severity of influenza infection.

Applying Systems Biology to the Human Influenza Challenge Model

To continue to advance our understanding of influenza, the techniques of systems biology should be furthered applied to the influenza challenge model. Very few studies have been published that use both techniques in concert. An influenza human challenge study was conducted by Woods in

collaboration with Retroscreen Virology (London, UK), with pre-vaccination and post-vaccination RNA extracted from volunteers to examine gene expression, with the purpose of exploring new diagnostic options (Woods et al., 2013). Twenty-four healthy volunteers were inoculated with A/Brisbane/59/2007 (H1N1), and 17 were inoculated with A/Wisconsin/67/2005 (H3N2). The peripheral blood transcriptome was then analyzed at several time points over the course of 7 days. The results showed that peripheral blood gene expression after infection had a distinct signature that was specific to either H1N1 or H3N2 infection. Importantly, these genomic signatures were able to identify infected individuals before they manifested symptoms or had only mild, non-specific symptoms. This early recognition could lead to earlier administration of antivirals and have a more significant impact on lessening the severity of influenza illness. Sobel Leonard et al. used the same 17 participants inoculated with H3N2 from Woods' trial to evaluate evolution of the influenza virus at the time of transmission (Sobel Leonard et al., 2016). Using deep sequencing techniques, the nasal washings of the participants inoculated were compared with the viral stock and the reference strain (A/Wisconsin/67/2005) used to create the stock virus. They found that in acute infection in the human host, the influenza virus populations can undergo rapid viral evolution and changes, which largely occurs during a transmission bottleneck effect that is highly selective. Sobel Leonard et al. were able to further characterize these findings by describing viral evolution within the host and identifying genetic selection factors (Sobel Leonard et al., 2017).

A recent study by Jochems et al investigated the role of influenza infection leading to secondary bacterial pneumonia, using a "double" experimental human challenge model and systems biology approaches (Jochems et al., 2018). Subjects were first inoculated with human type 6B *Streptococcus pneumoniae* (Spn) to simulate carriage, which is an important pre-requisite of clinical pneumococcal pneumonia. The authors then gave the participants live attenuated influenza virus (LAIV) to simulate influenza infection. Using systems biology approaches of nasal secretions, they were able to identify specific immune mediators that control Spn carriage, and determine how influenza infection can affect these pathways. The results showed that LAIV increased the carriage density of Spn by impairing degranulation of nasal neutrophils and decreasing the recruitment of monocytes, which are two mechanisms essential for bacterial clearance. Although the study has limitations—only one pneumococcal serotype (6B) was used, and the influenza challenge was with LAIV, not wild-type influenza—the findings are important and highlight how the innate immune system is involved in Spn carriage and clearance, and how pre-existing viral infections can negatively affect immune-mediated control of infection. This study also underscores the power of applying systems biology approaches to the human challenge model. The granular details and elucidation of immune mechanisms were only able to be determined by integrating these two techniques.

CONCLUSIONS

There is an urgent need to utilize novel platforms that can lead to the development of more effective vaccines and therapeutics for influenza, which continues to cause a significant burden of disease. Human challenge models have been successfully used for centuries. With advancing technologies and new methods to investigate the host-pathogen interaction, human challenge studies will be essential for progress, and can be performed in a safe and ethical manner. Furthermore, systems biology (e.g., transcriptomics, metabolomics, proteomics, lipidomics, etc.) allows for fundamental changes and patterns of the human immune system to be dissected. Harmonizing these two modalities is very promising; future studies should address using systems biology in a human challenge model to identify important gaps in our knowledge of influenza pathogenesis, and identify essential pathways involved in producing effective immune responses to vaccination. The ultimate goal would be to use these methods in concert to discover novel therapeutics, and potentially even lead to the development of a universal influenza vaccine.

AUTHOR CONTRIBUTIONS

AS and NR drafted the article. AS contributed to the collection and assembly of data, and the analysis and interpretation of the data. ND, AM, EA, and NR critically revised the article. ND contributed to the revision of the article and provided intellectual content for the ethics section.

FUNDING

This work was supported by the NIH NIAID (Vaccinology T32, Award No. T32AI074492).

REFERENCES

Alford, R. H., Kasel, J. A., Gerone, P. J., and Knight, V. (1966). *Human Influenza Resulting from Aerosol Inhalation*. Available online at: https://www.researchgate.net/publication/17267143_Human_Influenza_Resulting_from_Aerosol_Inhalation (accessed December 17, 2018).

Al-Nakib, W., Higgins, P. G., Willman, J., Tyrrell, D. A., Swallow, D. L., Hurst, B. C., et al. (1986). Prevention and treatment of experimental influenza A virus infection in volunteers with a new antiviral ICI 130,685. *J. Antimicrob. Chemother.* 18, 119–129. doi: 10.1093/jac/18.1.119

Barroso, L., Treanor, J., Gubareva, L., and Hayden, F. G. (2005). Efficacy and tolerability of the oral neuraminidase inhibitor peramivir in experimental human influenza: randomized, controlled trials for prophylaxis and treatment. *Antivir. Ther.* 10, 901–910.

Brown, T. A., Murphy, B. R., Radl, J., Haaijman, J. J., and Mestecky, J. (1985). Subclass distribution and molecular form of immunoglobulin A hemagglutinin antibodies in sera and nasal secretions after experimental secondary infection with influenza A virus in humans. *J. Clin. Microbiol.* 22, 259–264.

Bucasas, K. L., Franco, L. M., Shaw, C. A., Bray, M. S., Wells, J. M., Niño, D., et al. (2011). Early patterns of gene expression correlate with the humoral immune response to influenza vaccination in humans. *J. Infect. Dis.* 203, 921–929. doi: 10.1093/infdis/jiq156

Cao, R. G., Suarez, N. M., Obermoser, G., Lopez, S. M., Flano, E., Mertz, S. E., et al. (2014). Differences in antibody responses between trivalent inactivated

influenza vaccine and live attenuated influenza vaccine correlate with the kinetics and magnitude of interferon signaling in children. *J. Infect. Dis.* 210, 224–233. doi: 10.1093/infdis/jiu079

Carrat, F., Vergu, E., Ferguson, N. M., Lemaitre, M., Cauchemez, S., Leach, S., et al. (2008). Time lines of infection and disease in human influenza: a review of volunteer challenge studies. *Am. J. Epidemiol.* 167, 775–785. doi: 10.1093/aje/kwm375

CDC (2018). *Estimated Influenza Illnesses, Medical Visits, Hospitalizations, and Deaths in the United States — 2017–2018 Influenza Season | CDC*. Available online at: https://www.cdc.gov/flu/about/burden/estimates.htm (accessed November 15, 2018).

Couch, R. B., Gordon Douglas, R., Fedson, D. S., and Kasel, J. A. (1971). Correlated studies of a recombinant influenza-virus vaccine. III. Protection against experimental influenza in man. *J. Infect. Dis.* 124, 473–480. doi: 10.1093/infdis/124.5.473

Cypryk, W., Lorey, M., Puustinen, A., Nyman, T. A., and Matikainen, S. (2017). Proteomic and bioinformatic characterization of extracellular vesicles released from human macrophages upon influenza A virus infection. *J. Proteome Res.* 16, 217–227. doi: 10.1021/acs.jproteome.6b00596

Darton, T. C., Blohmke, C. J., Moorthy, V. S., Altmann, D. M., Hayden, F. G., Clutterbuck, E. A., et al. (2015). Design, recruitment, and microbiological considerations in human challenge studies. *Lancet Infect. Dis.* 15, 840–851. doi: 10.1016/S1473-3099(15)00068-7

Dolin, R., Richman, D. D., Murphy, B. R., and Fauci, A. S. (1977). Cell-mediated immune responses in humans after induced infection with influenza A virus. *J. Infect. Dis.* 135, 714–719. doi: 10.1093/infdis/135.5.714

Doll, M. K., Winters, N., Boikos, C., Kraicer-Melamed, H., Gore, G., and Quach, C. (2017). Safety and effectiveness of neuraminidase inhibitors for influenza treatment, prophylaxis, and outbreak control: a systematic review of systematic reviews and/or meta-analyses. *J. Antimicrob. Chemother.* 72, 2990–3007. doi: 10.1093/jac/dkx271

Douglas, R. G., Betts, R. F., Simons, R. L., Hogan, P. W., and Roth, F. K. (1975). Evaluation of a topical interferon inducer in experimental influenza infection in volunteers. *Antimicrob. Agents Chemother.* 8, 684–687. doi: 10.1128/AAC.8.6.684

Erbelding, E. J., Post, D. J., Stemmy, E. J., Roberts, P. C., Augustine, A. D., Ferguson, S., et al. (2018). A Universal influenza vaccine: the strategic plan for the National Institute of Allergy and Infectious Diseases. *J. Infect. Dis.* 218, 347–354. doi: 10.1093/infdis/jiy103

Francis, T. (1940). Intranasal inoculation of human individuals with the virus of epidemic influenza. *Proc. Soc. Exp. Biol. Med.* 43, 337–339. doi: 10.3181/00379727-43-11188P

Francis, T., and Magill, T. P. (1937). The antibody response of human subjects vaccinated with the virus of human influenza. *J. Exp. Med.* 65, 251–259. doi: 10.1084/jem.65.2.251

Francis, T., Salk, J. E., Pearson, H., and Brown, P. N. (1945). Protective effect of vaccination against induced influenza A 1. *J. Clin. Invest.* 24, 536–546. doi: 10.1172/JCI101633

Franco, L. M., Bucasas, K. L., Wells, J. M., Niño, D., Wang, X., Zapata, G. E., et al. (2013). Integrative genomic analysis of the human immune response to influenza vaccination. *ELife* 2:e00299. doi: 10.7554/eLife.00299

Furman, D., Jojic, V., Kidd, B., Shen-Orr, S., Price, J., Jarrell, J., et al. (2013). Apoptosis and other immune biomarkers predict influenza vaccine responsiveness. *Mol. Syst. Biol.* 9:659. doi: 10.1038/msb.2013.15

Gentile, D. A., Doyle, W. J., Fireman, P., and Skoner, D. P. (2001). Effect of experimental influenza A infection on systemic immune and inflammatory parameters in allergic and nonallergic adult subjects. *Ann. Allergy Asthma Immunol.* 87, 496–500. doi: 10.1016/S1081-1206(10)62263-6

Goodwin, K., Viboud, C., and Simonsen, L. (2006). Antibody response to influenza vaccination in the elderly: a quantitative review. *Vaccine* 24, 1159–1169. doi: 10.1016/j.vaccine.2005.08.105

Gross, C. P., and Sepkowitz, K. A. (1998). The Myth of the medical breakthrough: smallpox, vaccination, and Jenner reconsidered. *Int. J. Infect. Dis.* 3, 54–60. doi: 10.1016/S1201-9712(98)90096-0

Han, A., Poon, J. L., Powers, J. H., Leidy, N. K., Yu, R., and Memoli, M. J. (2018). Using the Influenza Patient-Reported Outcome (FLU-PRO) diary to evaluate symptoms of influenza viral infection in a healthy human challenge model. *BMC Infect. Dis.* 18:353. doi: 10.1186/s12879-018-3220-8

Hannoun, C. (2013). The evolving history of influenza viruses and influenza vaccines. *Expert Rev. Vacc.* 12, 1085–1094. doi: 10.1586/14760584.2013.824709

Hayden, F. G., Fritz, R., Lobo, M. C., Alvord, W., Strober, W., and Straus, S. E. (1998). Local and systemic cytokine responses during experimental human influenza A virus infection. Relation to symptom formation and host defense. *J. Clin. Invest.* 101, 643–649. doi: 10.1172/JCI1355

Hayden, F. G., Treanor, J. J., Fritz, R. S., Lobo, M., Betts, R. F., Miller, M., et al. (1999). Use of the oral neuraminidase inhibitor oseltamivir in experimental human influenza: randomized controlled trials for prevention and treatment. *JAMA* 282, 1240–1246. doi: 10.1001/jama.282.13.1240

Henle, G., Henle, W., Stokes, J Jr. (1943). Demonstration of the efficacy of vaccination against influenza type A by experimental infection of human beings. *J. Immunol.* 46, 163–175.

Henle, W., Henle, G., Stokes, J., and Maris, E. P. (1946). Experimental exposure of human subjects to viruses of influenza. *J. Immunol.* 52, 145–165.

Hunsberger, S., and Memoli, M. J. (2017). Efficacy analysis in healthy-volunteer influenza challenge trials: intention to treat. *Antimicrob. Agents Chemother.* 62, 1–2. doi: 10.1128/AAC.02018-17

Influenza (Seasonal) (2018). *"Influenza (Seasonal)."* Available online at: https://www.who.int/news-room/fact-sheets/detail/influenza-(seasonal) (accessed December 20, 2018).

Ison, M. G., Campbell, V., Rembold, C., Dent, J., and Hayden, F. G. (2005). Cardiac findings during uncomplicated acute influenza in ambulatory adults. *Clin. Infect. Dis.* 40, 415–422. doi: 10.1086/427282

Iwakoshi, N. N., Lee, A. H., Vallabhajosyula, P., Otipoby, K. L., Rajewsky, K., and Glimcher, L. H. (2003). Plasma cell differentiation and the unfolded protein response intersect at the transcription factor XBP-1. *Nat. Immunol.* 4, 321–329. doi: 10.1038/ni907

Jochems, S. P., Marcon, F., Carniel, B. F., Holloway, M., Mitsi, E., Smith, E., et al. (2018). Inflammation induced by influenza virus impairs human innate immune control of pneumococcus. *Nat. Immunol.* 19, 1299–1308. doi: 10.1038/s41590-018-0231-y

Josset, L., Zeng, H., Kelly, S. M., Tumpey, T. M., and Katze, M. G. (2014). Transcriptomic characterization of the novel Avian-origin influenza A (H7N9) virus: specific host response and responses intermediate between Avian (H5N1 and H7N7) and human (H3N2) viruses and implications for treatment options. *MBio* 5:e01102-13. doi: 10.1128/mBio.01102-13

Kasel, J. A., Alford, R. H., Knight, V., Waddell, G. H., and Sigel, M. M. (1965). Experimental infection of human volunteers with equine influenza virus. *Nature* 206, 41–43. doi: 10.1038/206041a0

Lee, S. M., Gardy, J. L., Cheung, C. Y., Cheung, T. K., Hui, K. P., Ip, N. Y., et al. (2009). Systems-level comparison of host-responses elicited by Avian H5N1 and seasonal H1N1 influenza viruses in primary human macrophages. *PLoS ONE* 4:e8072. doi: 10.1371/journal.pone.0008072

Lietzén, N., Ohman, T., Rintahaka, J., Julkunen, I., Aittokallio, T., Matikainen, S., et al. (2011). Quantitative subcellular proteome and secretome profiling of influenza A virus-infected human primary macrophages. *PLOS Pathog.* 7:e1001340. doi: 10.1371/journal.ppat.1001340

McNicholl, I. R., and McNicholl, J. J. (2001). Neuraminidase Inhibitors: Zanamivir and Oseltamivir. *Ann. Pharmacother.* 35, 57–70. doi: 10.1345/aph.10118

Memoli, M. J., Czajkowski, L.,Reed, S., Athota, R., Bristol, T., Proudfoot, K., et al. (2015). Validation of the wild-type influenza a human challenge model H1N1pdMIST: an A(H1N1)Pdm09 dose-finding investigational new drug study. *Clin. Infect. Dis.* 60, 693–702. doi: 10.1093/cid/ciu924

Memoli, M. J., Shaw, P. A., Han, A., Czajkowski, L., Reed, S., Athota, R., et al. (2016). Evaluation of antihemagglutinin and antineuraminidase antibodies as correlates of protection in an influenza A/H1N1 virus healthy human challenge model. *MBio* 7:e00417-16. doi: 10.1128/mBio.00417-16

Miller, F. G., and Grady, C. (2001). The ethical challenge of infection-inducing challenge experiments. *Clin. Infect. Dis.* 33, 1028–1033. doi: 10.1086/322664

Murphy, B. R., Nelson, D. L., Wright, P. F., Tierney, E. L., Phelan, M. A., and Chanock, R. M. (1982). Secretory and systemic immunological response in children infected with live attenuated influenza a virus vaccines. *Infect. Immun.* 36, 1102–1108.

Nakaya, H. I., Clutterbuck, E., Kazmin, D., Wang, L., Cortese, M., Bosinger, S. E., et al. (2016). Systems biology of immunity to MF59-adjuvanted versus

nonadjuvanted trivalent seasonal influenza vaccines in early childhood. *Proc. Natl. Acad. Sci. U.S.A.* 113, 1853–1858. doi: 10.1073/pnas.1519690113

Nakaya, H. I., Hagan, T., Duraisingham, S. S., Lee, E. K., Kwissa, M., Rouphael, N., et al. (2015). Systems analysis of immunity to influenza vaccination across multiple years and in diverse populations reveals shared molecular signatures. *Immunity* 43, 1186–1198. doi: 10.1016/j.immuni.2015.11.012

Nakaya, H. I., Wrammert, J., Lee, E. K., Racioppi, L., Marie-Kunze, S., Haining, W. N., et al. (2011). Systems biology of vaccination for seasonal influenza in humans. *Nat. Immunol.* 12, 786–795. doi: 10.1038/ni.2067

Petrie, J. G., Malosh, R. E., Cheng, C. K., Ohmit, S. E., Martin, E. T., Johnson, E., et al. (2017). The household influenza vaccine effectiveness study: lack of antibody response and protection following receipt of 2014-2015 influenza vaccine. *Clin. Infect. Dis.* 65, 1644–1651. doi: 10.1093/cid/cix608

Pollard, A. J., Savulescu, J., Oxford, J., Hill, A. V., Levine, M. M., Lewis, D. J., et al. (2012). Human microbial challenge: the ultimate animal model. *Lancet Infect. Dis.* 12, 903–905. doi: 10.1016/S1473-3099(12)70292-X

Raymond, D. D., Stewart, S. M., Lee, J., Ferdman, J., Bajic, G., Do, K. T., et al. (2016). Influenza immunization elicits antibodies specific for an egg-adapted vaccine strain. *Nat. Med.* 22, 1465–1469. doi: 10.1038/nm.4223

Riedel, S. (2005). Edward Jenner and the History of Smallpox and Vaccination. *Proceedings (Bayl. Univ. Med. Cent.)* 18, 21–25. doi: 10.1080/08998280.2005.11928028

Smorodintseff, A. A., Tushinsky, M. D., Drobyshevskaya, A. I., Korovin, A. A., and Osetroff, A. I. (1937). Investigation on volunteers infected with the influenza virus. *Am. J. Med. Sci.* 194, 159–170. doi: 10.1097/00000441-193708000-00002

Sobel Leonard, A., McClain, M. T., Smith, G. J., Wentworth, D. E., Halpin, R. A., Lin, X., et al. (2016). Deep sequencing of influenza A virus from a human challenge study reveals a selective bottleneck and only limited intrahost genetic diversification. *J. Virol.* 90, 11247–11258. doi: 10.1128/JVI.01657-16

Sobel Leonard, A., McClain, M. T., Smith, G. J., Wentworth, D. E., Halpin, R. A., Lin, X., et al. (2017). The effective rate of influenza reassortment is limited during human infection. *PLoS Pathog.* 13:e1006203. doi: 10.1371/journal.ppat.1006203

Soema, P. C., Kompier, R., Amorij, J. P., and Kersten, G. F. (2015). Current and next generation influenza vaccines: formulation and production strategies. *Eur. J. Pharmac. Biopharmac.* 94, 251–263. doi: 10.1016/j.ejpb.2015.05.023

Treanor, J. J., Kotloff, K., Betts, R. F., Belshe, R., Newman, F., Lacuzio, D., et al. (1999). Evaluation of trivalent, live, cold-adapted (CAIV-T) and inactivated (TIV) influenza vaccines in prevention of virus infection and illness following challenge of adults with wild-type influenza A (H1N1), A (H3N2), and B viruses. *Vaccine* 18, 899–906. doi: 10.1016/S0264-410X(99)00334-5

Tsang, J. S., Schwartzberg, P. L., Kotliarov, Y., Biancotto, A., Zhi, X., Germain, R. N., et al. (2014). Global analyses of human immune variation reveal baseline predictors of post-vaccination responses. *Cell* 157, 499–513. doi: 10.1016/j.cell.2014.03.031

Uyeki, T. M., Bernstein, H. H., Bradley, J. S., Englund, J. A., File, T. M., Fry, A. M., et al. (2019). Clinical practice guidelines by the infectious diseases society of America: 2018 update on diagnosis, treatment, chemoprophylaxis, and institutional outbreak management of seasonal influenza. *Clin. Infect. Dis.* 68, e1–e47. doi: 10.1093/cid/ciy874

Woods, C. W., McClain, M. T., Chen, M., Zaas, A. K., Nicholson, B. P., Varkey, J., et al. (2013). A host transcriptional signature for presymptomatic detection of infection in humans exposed to influenza H1N1 or H3N2. *PLoS ONE* 8:e52198. doi: 10.1371/journal.pone.0052198

Conflict of Interest Statement: 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 Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 26 April 2019
doi: 10.3389/fcimb.2019.00125

A Pertussis Outer Membrane Vesicle-Based Vaccine Induces Lung-Resident Memory CD4 T Cells and Protection Against *Bordetella pertussis,* Including Pertactin Deficient Strains

María Eugenia Zurita[1], *Mieszko M. Wilk*[2], *Francisco Carriquiriborde*[1], *Erika Bartel*[1], *Griselda Moreno*[3], *Alicja Misiak*[2], *Kingston H. G. Mills*[2] *and Daniela Hozbor*[1]*

[1] *Laboratorio VacSal, Facultad de Ciencias Exactas, Instituto de Biotecnología y Biología Molecular (IBBM), CCT-CONICET La Plata, Universidad Nacional de La Plata, La Plata, Argentina,* [2] *School of Biochemistry and Immunology, Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin, Ireland,* [3] *Facultad de Ciencias Exactas, Instituto de Estudios Inmunológicos y Fisiopatológicos (IIFP), CCT-CONICET La Plata, Universidad Nacional de La Plata, La Plata, Argentina*

OPEN ACCESS

Edited by:
Alberto Moreno,
Emory University School of Medicine, United States

Reviewed by:
Riti Sharan,
Texas Biomedical Research Institute, United States
Gideon Kersten,
Intravacc, Netherlands

***Correspondence:**
Daniela Hozbor
hozbor.daniela@gmail.com;
hozbor@biol.unlp.edu.ar

Specialty section:
This article was submitted to Clinical Microbiology, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *08 December 2018*
Accepted: *09 April 2019*
Published: *26 April 2019*

Citation:
Zurita ME, Wilk MM, Carriquiriborde F, Bartel E, Moreno G, Misiak A, Mills KHG and Hozbor D (2019) A Pertussis Outer Membrane Vesicle-Based Vaccine Induces Lung-Resident Memory CD4 T Cells and Protection Against Bordetella pertussis, Including Pertactin Deficient Strains. Front. Cell. Infect. Microbiol. 9:125. doi: 10.3389/fcimb.2019.00125

Pertussis is a respiratory infectious disease that has been resurged during the last decades. The change from the traditional multi-antigen whole-cell pertussis (wP) vaccines to acellular pertussis (aP) vaccines that consist of a few antigens formulated with alum, appears to be a key factor in the resurgence of pertussis in many countries. Though current aP vaccines have helped to reduce the morbidity and mortality associated with pertussis, they do not provide durable immunity or adequate protection against the disease caused by the current circulating strains of *Bordetella pertussis,* which have evolved in the face of the selection pressure induced by the vaccines. Based on the hypothesis that a new vaccine containing multiple antigens could overcome deficiencies in the current aP vaccines, we have designed and characterized a vaccine candidate based on outer membrane vesicle (OMVs). Here we show that the OMVs vaccine, but not an aP vaccine, protected mice against lung infection with a circulating pertactin (PRN)-deficient isolate. Using isogenic bacteria that in principle only differ in PRN expression, we found that deficiency in PRN appears to be largely responsible for the failure of the aP vaccine to protect against this circulating clinical isolates. Regarding the durability of induced immunity, we have already reported that the OMV vaccine is able to induce long-lasting immune responses that effectively prevent infection with *B. pertussis*. Consistent with this, here we found that CD4 T cells with a tissue-resident memory (T_{RM}) cell phenotype ($CD44^{+}CD62L^{low}CD69^{+}$ and/or $CD103^{+}$) accumulated in the lungs of mice 14 days after immunization with 2 doses of the OMVs vaccine. CD4 T_{RM} cells, which have previously been shown to play a critical role sustained protective immunity against *B. pertussis,* were also detected in mice immunized with wP vaccine, but not in the animals immunized with a commercial aP vaccine.

The CD4 T_{RM} cells secreted IFN-γ and IL-17 and were significantly expanded through local proliferation following respiratory challenge of mice with *B. pertussis*. Our findings that the OMVs vaccine induce respiratory CD4 T_{RM} cells may explain the ability of this vaccine to induce long-term protection and is therefore an ideal candidate for a third generation vaccine against *B. pertussis*.

Keywords: *Bordetella pertussis*, pertussis, outer membrane vesicles, T_{RM} cells, pertactin deficient strains, protection

INTRODUCTION

Although vaccination is the most cost-effective strategy to prevent life threatening infectious diseases, these diseases continue to be the main cause of global morbidity and mortality (Plotkin, 2005). Vaccine preventable diseases (e.g., measles, tetanus, diphtheria, polio, etc.) are responsible for ∼25% of the 10 million deaths that occur annually among children under 5 years of age. In addition, around 25% of adult deaths (15–59 years) are still attributed to infectious diseases. Therefore, there is an urgent need to improve some traditional vaccines of the Expanded Program on Immunization and to design new vaccines for emerging, re-emerging or resurgent pathogens (WHO, 2010a,b). One of the traditional vaccines that needs to be improved is that designed to prevent the severe respiratory disease whooping cough or pertussis. During the last decades the incidence of this disease has increased in adults, adolescents, as well as in children despite good vaccine coverage (Berti et al., 2014; Clark, 2014). Individuals at the extreme ends of life may be the most vulnerable to severe *Bordetella pertussis* (the etiologic agent of the disease) infection, though hospitalization can be necessary across all age groups (Mbayei et al., 2018).

The incidence of pertussis was drastically reduced following introduction of global immunization with the whole cell pertussis (wP) vaccine, consisting in a suspension of the causative agent *B. pertussis* killed by heat and chemically detoxified (Cherry, 1984). The wP vaccine was introduced in the 1940 and 1950s and it is still in use, in developing countries for the pediatric population. However safety concerns with wP vaccines (Desauziers et al., 2004; Klein, 2014) and its acceptance diminished in different countries (Romanus et al., 1987; Klein, 2014). This lead to development of acellular pertussis (aP) vaccines containing purified antigenic protein components of *B. pertussis* (2, 3, or 5 immunogens) (Sato and Sato, 1985; Edwards and Karzon, 1990). The aP vaccines have a better safety profile and gradually replaced wP vaccine in many industrialized countries (Zhang et al., 2012).

During the last two decades the epidemiology of pertussis has changed (Clark, 2014; Tan et al., 2015), with major outbreaks in many developing countries but also in developed countries (Hozbor et al., 2009; Clark, 2012), even in those with high rates of vaccination (He and Mertsola, 2008; Anon, 2010; Clark, 2014; Mbayei et al., 2018). There have been a number of explanations for the resurgence of pertussis, including waning of immunity induced by vaccines, in particular aP vaccines (Koepke et al., 2014; McGirr and Fisman, 2015), pathogen adaptation to escape vaccine induced immunity (Mäkelä P. H., 2000; King et al., 2001; Mooi et al., 2001; He et al., 2003; David et al., 2004; Gzyl et al., 2004; Bottero et al., 2007; Bowden et al., 2016), and the failure of pertussis vaccines, in particular aP vaccines, to prevent infection and spread of *B. pertussis*.

Regarding pathogen evolution, the first reports were related to polymorphism in genes coding for proteins included in the vaccine [pertactin (PRN) and pertussis toxin (PTx) among others] and later in the pertussis toxin promoter (*ptx*P) (Advani et al., 2011; Kallonen et al., 2012). Recently, there has been an increase in *B. pertussis* isolates that do not produce some of the vaccine antigens (Bodilis and Guiso, 2013; Hegerle and Guiso, 2014; Lam et al., 2014). In particular in US, Canada and Australia it was reported that PRN-deficient isolates [PRN(-)] increased substantially in the last years (Lam et al., 2014; Pawloski et al., 2014; Tsang et al., 2014). These isolates are expected to be resistant to the phagocytosis mediated by anti-pertactin antibodies (Hellwig et al., 2003). It has been proposed that the loss of this vaccine antigen probably provides a selective advantage for bacterial survival in populations vaccinated with aP vaccines. Commercial aP vaccines containing PTx, PRN, and filamentous hemagglutinin (FHA) are not as effective as expected in controlling the infection caused by the recent circulating bacteria that do not express PRN (Hegerle et al., 2014). Moreover, recently it was demonstrated in a mixed infection mouse model that PRN(-) *B. pertussis* colonizes the respiratory tract of aP immunized mice more effectively than the PRN(+) strain, out-competing the PRN(+) strain (Safarchi et al., 2015).

Regarding waning immunity, it is well known that while wP vaccines induce potent Th1 and Th17 responses, the current aP vaccines are inefficient at promoting Th1 responses, but do induce potent antibody and Th2-polarized responses and weak Th17 responses (Ross et al., 2013; Brummelman et al., 2015). Furthermore, immunization with wP vaccines appear to be more effective than current aP vaccines at inducing immunological memory and in conferring long-term protection against pertussis (Brummelman et al., 2015). Recent data has demonstrated that wP but not aP vaccines induced CD4 T memory cells that reside in the lungs (Allen et al., 2018; Borkner et al., 2018). These respiratory tissue-resident memory CD4 T cells that express $CD44^{+}CD62L^{low}CD69^{+}$ confer long-term protective immunity against *B. pertussis*.

Possible solutions to improved control of pertussis disease, in the short term, include the return to the use of the wP vaccine in the primary dose and/or add more boosters. However, adding many boosters implies having both, resources to sustain the vaccination schedule and pharmaceuticals capable

of responding to the demands of all countries that require them. Regarding those countries that switched to aP, it seems difficult to reintroduce the same wP vaccine since the acceptability of this vaccine by the population had been lost (Plotkin, 2014).

We have designed a new multi-antigen aP vaccine formulation that shares the beneficial properties of current aP vaccines in terms of biosafety and those of wP vaccines in terms of immunogenicity and protective capacity (International patent granted in the USA and in process in other countries, Application Number: PCT/IB2014/060143) (Roberts et al., 2008; Asensio et al., 2011; Gaillard et al., 2014). This new acellular formulation has been obtained from membrane components of *B. pertussis* (outer membrane vesicles, OMVs) in which antigens are presented in their native conformation, with membrane-associated PAMPs acting as immunostimulatory molecules, such as in the commercial wP vaccines. We have reported that the OMVs-based vaccine was capable of inducing a more robust immune response than current aP vaccines with a Th1/Th17 and Th2 cellular profile (Bottero et al., 2016), that confers long lasting protection against *B. pertussis* (Gaillard et al., 2014).

In this study we have evaluated whether our OMVs vaccine is capable of overcoming the deficiencies of commercial vaccines in both controlling infections caused by PRN(-) isolate/strain and inducing memory immunity. We found that our OMVs-based formulation has a higher protective capacity against the PRN(-) bacteria than that induced with a commercial aP vaccine. We found that CD4 T cells with a tissue-resident memory (T_{RM}) cell phenotype ($CD44^{+}CD62L^{low}CD69^{+}$ and/or $CD103^{+}$) accumulated in the lungs of mice after the second OMVs vaccine immunization. CD4 T_{RM} cells were also detected in mice immunized with wP vaccine, but not in the animals immunized with a commercial aP vaccine. The CD4 T_{RM} cell population was significantly expanded through local proliferation following respiratory challenge of mice with *B. pertussis*. These CD4 T_{RM} cells secreted IFN-γ and IL-17 that have previously been shown to play a critical role in adaptive immunity against *B. pertussis* infection. Our findings suggest that the OMVs-vaccine is an ideal candidate for the development of a third generation pertussis vaccine.

MATERIALS AND METHODS

Animals

C57BL/6 (8-week-old) mice were obtained from Harlan Laboratories U.K. or the Comparative Medicine Unit, Trinity College Dublin and housed in a specific pathogen–free facility. All animal experiments performed in Dublin were conducted in accordance with the recommendations and guidelines and under licenses approved by the Health Products Regulatory Authority of Ireland in accordance with protocols approved by the Trinity College Dublin Animal Research Ethics Committee. Animal experiments using female BALB/c mice with 3–4 weeks of age, obtained from the Instituto Biológico Argentino (Biol. SAIC, Argentina) were also performed in Argentina. The studies have been approved by Ethical Committee for Animal Experiments of the Faculty of Science at La Plata National University (Argentina, approval number 004-06-15 and 003-06-15).

Bacterial Strains and Growth Conditions

Bordetella pertussis Tohama phase I strain, its isogenic mutant strain defective in PRN (Roberts et al., 1991), and PRN-deficient clinical isolate (Bp935) were used throughout this study. Bp935, which was kindly provided by CDC (Atlanta) is a representative PRN(-) isolate obtained from the 2012 Washington (US) outbreak (Pawloski et al., 2014). PRN deficiency in this isolate was caused by IS481 insertion into *prn* gene, and its MLVA-MLST type is the most prevalent among the isolates from that outbreak (*ptx*P3, *ptx*A1, *prn*2). Bacteria were grown on Bordet-Gengou agar (BGA, Difco) supplemented with 10% defibrinated sheep blood at 36.5°C for 72 h and plated again on the same medium for 24 h before each infection.

Isolation and Characterization of Outer Membrane Vesicles (OMVs)

OMVs were produced and characterized as previously described (Hozbor et al., 1999; Asensio et al., 2011). Briefly, culture samples from the decelerating growth phase were centrifuged and the bacterial pellet obtained was resuspended in 20 mM Tris–HCl, 2 mM EDTA pH 8.5. The suspension was sonicated (ultrasonic bath) in cool water for 20 min. After two centrifugations at 10,000 × g for 20 min at 4°C, the supernatant was pelleted at 100,000 × g for 2 h at 4°C. This pellet was re-suspended in Tris buffer (20 mM pH 7.6). The samples obtained were negatively stained for electron microscope examination. Protein content was estimated by the Bradford method using bovine serum albumin as standard. The presence of the main immunogenic proteins in the OMVs was corroborated by immunoblot assays using specific antibodies as we previously described (Roberts et al., 2008).

Formulation of OMV-Based Vaccine

The characterized OMVs that range in size from ∼50 to 200 nanometers in diameter were used to formulate the vaccine with tetanus (5–7 Lf/ dose with a power ≥2 UIA/ml serum) and diphtheria (1–3 Lf / dose with an output of 0.1 UIA/ml serum) toxoids as we previously described (Gaillard et al., 2014). The LPS concentration determined by GC-MS ranged from 0.275 to 0.352 μg per dose of OMV based vaccine. The safety of this vaccine was evaluated by a mouse weight-gain test (WHO, 2007) and the murine and human whole-blood IL-6–release assays (Stoddard et al., 2010; Bottero et al., 2016). To perform the experiments described below we verified that the OMV based vaccine prepared by us fulfilled the WHO criteria for safety in the weight-gain test. The safety of OMV-based vaccines was also confirmed by human whole-blood assays.

Immunization of Mice

Groups of 4–5 female C57BL/6 or BALB/c mice (discriminated in the legends to the figures) were immunized with OMVs-based vaccine formulated as previously described (3 μg total protein per dose formulated with alum as adjuvant) (Asensio et al., 2011), 1:50 or 1:10 human dose of aP vaccine BOOSTRIX® [GlaxoSmithKline, with composition per human dose: pertussis toxoid (8 μg), pertactin (2.5 μg), FHA (8 μg), tetanus toxoid (20 IU), diphtheria toxoid (2 IU), and alum as adjuvant], or

1:40 human dose of wP vaccine (National Institute of Biological Standards and Control, South Mimms, UK; NIBSC batch 41S) in 200 μl PBS via intraperitoneal (i.p.) injection using a two-dose schedule. The i.p route, although not translatable to humans, was chosen as it allowed us to compare our results with previous studies on the immunogenicity and protective OMVs-based pertussis vaccines that used this route of immunization (Gaillard et al., 2014; Bottero et al., 2016). Two weeks after the second immunization, mice were sacrificed, and the immune response was evaluated in lungs and spleen. For protection assays mice were challenged with *B. pertussis* by exposure to an aerosol of 5×10^8 bacteria per ml or by intranasal inoculation (sublethal dose 10^7-10^8 CFU 40 μl^{-1}) as is described below. In both case, mice were killed 1 and/or 2 weeks after challenge.

B. pertussis Respiratory Challenge

For protection assays, mice were challenged with *B. pertussis* by exposure to an aerosol of 5×10^8 bacteria per ml or by intranasal inoculation (sublethal dose 10^7-10^8 CFU 40 μl^{-1}). For aerosol challenge, *B. pertussis* bacteria were grown from a frozen stock on Bordet Gengou plates containing glycerol and horse blood (Cruinn) at 36.5°C. After 3 d of culture, the bacteria were collected in supplemented Stainer–Scholte medium and cultured overnight at 36.5°C in a shaking incubator at 220 rpm. Bacteria were centrifuged and resuspended in 1% casein solution, and the OD was measured at 600 nm. *B. pertussis* infection of C57BL/6 mice was performed by aerosol challenge (BP338 strain; 1 × 108 CFU/ml) administered using a nebulizer (PARI TurboBOY SX) over 10 min (Zhang et al., 2012). The course of infection was followed by performing CFU counts on lung homogenates at intervals post-infection (p.i.), as described (Zhang et al., 2012). For intranasal challenge, mice were infected with a sublethal dose (10^7-10^8 CFU 40 μl^{-1}) of *B. pertussis* clinical isolate/strain. Bacterial counts were performed 7 days after the challenge as described previously (Asensio et al., 2011; Gaillard et al., 2014).

Intravascular Staining for Discriminating Circulating Cells From Lung-Retained Cells

To discriminate blood-borne circulating cells from lung-localized cells, we used a well-described approach in which anti-mouse PE-CD45 Ab (eBioscience) was administered i.v. to mice 10 min before they were euthanized and lungs were harvested (Gzyl et al., 2004).

Isolation and FACS Analysis of Cells From Lung or Spleen Tissue

Lung and nasal tissue mononuclear cell suspensions were prepared by mechanical (chopping with a scalpel) followed by enzymatic disruption of tissue for 1 h at 37°C with Collagenase D (1 mg/ml; Sigma-Aldrich) and DNAse I (20 U/ml; Sigma-Aldrich). Next, lungs or spleens were passed through a 40-mm cell strainer to a obtain single-cell suspension, followed by RBC lysis. The cells were incubated with CD16/CD32 FcgRIII (1:100) to block IgG Fc receptors. Cells were incubated with LIVE/DEAD Aqua (Invitrogen), followed by surface staining with fluorochrome-conjugated anti-mouse Abs for various markers. To detect cytokines, cells were stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) in the presence of brefeldin A (5 mg/ml) for 4 h at 37°C. The following surface Abs were used: CD45R-PE, CD3-BV421, CD44-BV605 (Biolegend), CD62L-PE-CF594 (BD), CD103-BV786, CD4-APC-eF780, CD69-FITC (eBioscience). For detection of intracellular cytokines, cells were fixed in 2% PFA and permeabilized with 0.5% saponin (Sigma-Aldrich, Ireland), followed by staining with IL-17A–PerCP-Cy5.5 and IFN-γ-BV650 (eBiosciences). Fluorescence minus one or non-specific isotype Abs were used as controls. Flow cytometric analysis was performed on an LSR Fortessa, and data were acquired using Diva software (BD Biosciences). The results were analyzed using FlowJo software (TreeStar).

Ag-Specific IL-17, IL-5, and IFN-γ Production by Spleen Cells

Spleens from untreated and immunized mice were passed through a 40-mm cell strainer to obtain a single-cell suspension. Spleen cells were cultured with sonicated *B. pertussis* (sBp; 5 μ/ml), or medium only. After 72 h of incubation, IFN-γ, IL-5, and IL-17A concentrations were quantified in supernatants by ELISA. sBp was obtained by sonication of the bacterial suspension in PBS (10^{10} CFU/ml) on ice using ultrasonic homogenizer (10 × 15 s. pulse; Sonopuls Bandelin). The sonicated bacterial suspension was then centrifuged at 10,000 rpm × 15 min. Supernatant was collected and protein concentration was determined as described above.

Statistical Analysis

For the analysis of CFU counts in animal lungs, before applying the statistical methods described below we evaluated the normality of the data by using Shapiro-Wilk test (http://scistatcalc.blogspot.com.ar/2013/10/shapiro-wilk-test-calculator.html). After verifying that our CFUs data follows a normal distribution, we statistically analyzed them by using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test (GraphPadPrism®). Differences were considered to be significant when $p < 0.05$. For fluorescence values analysis, we used Mann-Whitney statistical analysis ($p < 0.05$). All statistical analysis of data was performed using GraphPadPrism® version 6.00 for Windows, GraphPad® Software.

RESULTS

OMVs Vaccine Protects Against PRN(-) *B. pertussis* Clinical Isolate

We compared the protective capacity of the OMVs vaccine with that induced with a commercially available aP vaccine against infection with *B. pertussis* Tohama phase I strain [*ptx*P1 variant and *prn*1 allele, PRN(+)] or PRN(-) clinical isolate of *B. pertussis* Bp935. The OMVs vaccine and the commercial aP vaccine conferred protective immunity against the *B. pertussis* Tohama phase I strain. The bacterial counts in the lungs were significantly lower in immunized compared with non-immunized mice (**Figure 1A**). In contrast, the protective capacity of OMVs vaccine against PRN(-) isolate was significantly higher than that of the

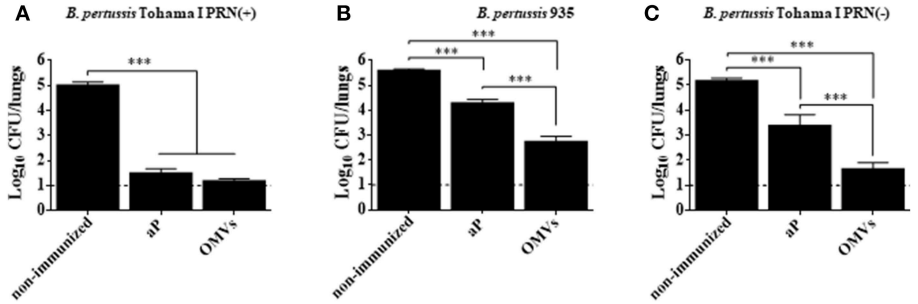


FIGURE 1 | Protection against *B. pertussis* PRN(-) isolate induced with an OMVs vaccine in a mouse model. BALB/c mice were immunized (i.p.) twice, 2 weeks apart. Mice were challenged with sublethal doses (5×10^7 CFUs/40 μl) of *B. pertussis* Tohama I PRN(+) **(A)**, *B. pertussis* 935 PRN(-) **(B)** or *B. pertussis* Tohama I PRN(-) **(C)** 2 weeks after the second immunization with aP or OMVs vaccine. Three independent experiments were performed for each strain/isolate. Results from one representative experiment are shown. Results depicted are means of 5 mice per group at 7 days post-challenge. The dashed line indicates the lower limit of detection. The number of bacteria recovered from mouse lungs is expressed as the log 10 means ± SEM (error bars) of colony forming units (CFU) per lung. Data obtained were analyzed statistically by using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test (GraphPadPrism). In **(A,B,C)** ***Significant differences with $p < 0.001$.

commercial aP vaccine (**Figure 1B**). Mice immunized with the OMVs had 3 Log_{10} reduction in colonies 7 days after challenge with *B. pertussis* PRN(-) compared with the non-immunized mice, whereas the CFU counts in the lungs of mice immunized with the commercial aP vaccine was only reduced by 1 log_{10} (**Figure 1B**). Since there may be other unidentified features other than the absence of PRN in the clinical isolate, we also evaluated the efficacy of the studied acellular vaccines against *B. pertussis* Tohama phase I isogenic bacteria that only differ in PRN expression. We found that immunization with the OMVs vaccine significantly ($p < 0.001$) reduced the bacterial counts in the lung to near the detection limit of the assay after challenge with the PRN-defective *B. pertussis* Tohama mutant (**Figure 1C**). Importantly, the protective capacity of commercial aP vaccine was impaired (at least in 1 log_{10}, $p < 0.001$) against PRN defective mutant strain (**Figure 1C**) compared with that observed again the wild type strain (**Figure 1A**). The findings demonstrate that the current aP vaccine has impaired capacity to protect against mutant strain or certain circulating strains of *B. pertussis* that lack PRN.

CD4 T_{RM} Cell Accumulate in the Lungs of Mice Immunized With the OMVs Vaccine

We used the murine model to evaluate whether the OMVs-based candidate pertussis vaccine induced systemic antigen-specific immune responses and CD4 T cells with a T_{RM} cell phenotype in lungs. The Mills group had already demonstrated that natural infection or immunization with an wP vaccine primed respiratory T_{RM} cells, whereas an aP vaccine did not (Wilk et al., 2017; Allen et al., 2018). Therefore, we performed experiments comparing the responses with that induced with the OMV-based vaccine with aP or wP vaccines, with the wP vaccines acting as positive control. First, we used the murine respiratory challenge model to confirm that the immunization with the different vaccines tested in this study were capable of inducing protection against infection (**Figure 2**). We found that two parenteral immunizations with the OMVs vaccine was highly effective at preventing lung infection; the CFU counts were at or close to background 7 and 14 days post-challenge (**Figure 2**). The protection induced with the OMVs vaccine was similar to that generated with an wP vaccine (**Figure 2** upper panel) and was significantly better than induced with the aP vaccine (**Figure 2**, lower panel).

To examine antigen-specific immune responses to *B. pertussis,* spleen cells from immunized mice 14 days after the second dose were stimulated with sonicated *B. pertussis* (sBp), cytokines were quantified in the supernatants by ELISA. The results revealed that spleen cells from mice immunized with the OMVs vaccine produced significantly higher concentrations of IFN-γ and IL-17 than spleen cells from mice immunized with the aP vaccine (**Figure 3**). *Bordetella pertussis*-specific IFN-γ and IL-17 was also detected in spleen cells from mice immunized with the wP vaccines. However, antigen-specific IL-17 production was significantly stronger in mice immunized with the OMVs vaccine. In contrast, the aP vaccine predominantly induced antigen-specific IL-5. These data demonstrate that OMVs and wP vaccine induce a mixed Th1/Th17 response while the current aP vaccines induce Th2-polarized responses.

We next assessed T_{RM} cell in the lungs (the gating strategy is shown in **Figure S1**). In order to discriminate blood borne circulating leukocytes from lung-retained leukocytes, we used a validated approach (Gzyl et al., 2004) in which a fluorescently labeled anti-CD45 antibody (Ab) was administered i.v. to mice 10 min prior to sacrifice. Circulating cells become labeled with the Ab ($CD45^+$), whereas the Ab cannot penetrate the tissue to stain the lung resident cells and therefore remain unstained ($CD45^-$). We found that the OMVs vaccine and the wP vaccine (used as a positive control) induced cytokine-secreting respiratory CD4 T_{RM} cells. There were significant higher number of lung-resident CD4 T cells that expressed the T_{RM} cell markers CD69 and/or CD103 between in the lungs of mice immunized with OMVs compared with aP immunized or non-immunized mice ($14.02 \times 10^4 \pm 2.09 \times 10^4$ T_{RM} for OMVs, $14.99 \times 10^4 \pm 4.70 \times 10^4$ T_{RM} for wP, $4.44 \times 10^4 \pm 0.76 \times 10^4$ T_{RM} for aP, $1.59 \times 10^4 \pm$

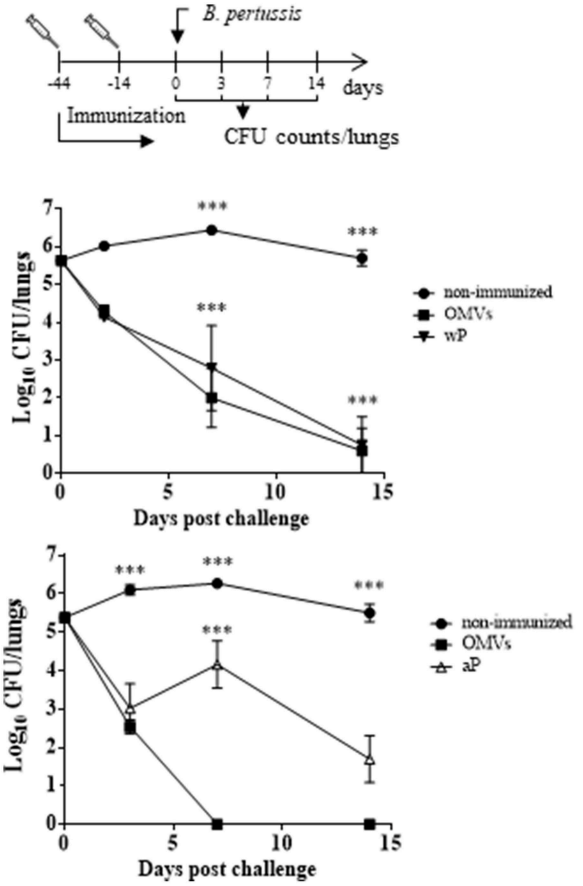


FIGURE 2 | Protection of mice against *B. pertussis* infection induced with the OMVs vaccine compared with wP and aP vaccines. C57BL/6 mice were immunized (i.p.) twice, 4 weeks apart, with either wP, aP, or OMVs vaccines or were non-immunized. Mice were challenge 2 weeks after the second immunization by aerosol with *B. pertussis* BP338 strain (1×10^8 CFU/ml) administered using a nebulizer (PARI TurboBOY SX) over 10 min. Results are shown as the number of bacteria recovered from the mouse lungs at different time points, expressed as the $\log_{10}$ of the means ± SEM (error bars) of the CFUs per lung, plotted on the *ordinate* for the samples from the lungs of the experimental groups indicated on the *abscissa.* The experiment involved three biological replicates with the results from a representative one being presented. The dotted horizontal line marks the lower limit of detection. ***$p < 0.001$ (ANOVA followed by the Bonferroni *post-hoc* test).

0.26×10^4 T_{RM} for PBS; **Figure 4**). To evaluate the functionality of the induced T_{RM} cell we used intracellular cytokine staining for IFN-**γ** or IL-17. The results showed that the immunization with the wP vaccine or the OMVs vaccine induced IFN-γ or IL-17-secreting CD4 T_{RM} cells in the lungs. Although the numbers were highest in mice immunized with the wP vaccine, the number of cytokine-secreting CD4 T_{RM} cells significantly ($p < 0.05$) higher in the lungs of mice immunized with the OMVs vaccine when compared with the aP vaccine (**Figure 5**). Furthermore, CD4 T cells with a tissue-resident memory phenotype expanded significantly (5-fold) after *B. pertussis* challenge (**Figure 6**) in mice vaccinated with the wP or OMVs-based vaccine, but not in mice immunized with the aP vaccine (**Figure 6**). These findings demonstrate that immunization of mice with the OMVs vaccine, like the wP vaccine but not the aP vaccine, is capable of generating T_{RM} cells that expand in the lung after *B. pertussis* challenge.

DISCUSSION

In this study we demonstrated that the OMVs vaccine, like the wP vaccine, promoted induction of protective immunity against *B. pertussis* lung infection in mice and that while both vaccines were effective at inducing *B. pertussis*-specific INF-γ (marker of Th1 cells) and IL-17 (marker of Th17 cells) by spleen cells, the aP vaccine mainly induced IL-5 (marker of Th2 cells) (Ryan et al., 1998; Bottero et al., 2016). Though antibodies are known to be involved in protection against pertussis, Th1 and Th17 responses are also crucial for mediating adaptive immunity and clearance of *B. pertussis* from the respiratory tract (Mills et al., 1993; Ryan et al., 1997).

Consistent with previous studies showing that OMV-based vaccine was capable of conferring both long-lasting immunity and protection against pertussis (Gaillard et al., 2014), here we demonstrated that OMV-based vaccine was also effective at inducing respiratory INF-γ- and IL-17-secreting T_{RM} cells. T_{RM} cells were also expanded in the lungs of mice immunized with the wP vaccine, not in mice immunized with a commercial aP vaccine. The failure of aP vaccine to induce T_{RM} cells, even using the i.p. route of vaccination, which induces the strongest immune response in mice, may explain the waning immunity reported in populations immunized with this vaccine (Klein et al., 2013; McGirr and Fisman, 2015). The growing evidences that pertussis affects all age groups suggests that pertussis vaccines, particularly aP vaccines, do not provide long-lasting immunity. The induction of respiratory T_{RM} cells in immunized mice with the OMVs vaccine is an important finding since this memory CD4 T cell population not only provide much more immediate protection than the anti-pathogen responses conferred by naïve T-cells, they also could provide more effective immune protection to the host as demonstrated in other models of infection (Teijaro et al., 2011; Glennie et al., 2017). CD4 T_{RM} cells have been shown to play a crucial role in protective immunity against re-infection with *B. pertussis* (Wilk et al., 2017) and following vaccination with aP vaccine formulated with a novel adjuvant, comprising agonists for TLR2 and intracellular receptor stimulator of interferon genes (STING) (Allen et al., 2018). Our experiments with the OMV focused on lung T_{RM} cells, but we do not rule out the possibility that OMV induces T_{RM} cells in upper respiratory tract or in the gut. Thus, the immunization with the OMVs as well as wP vaccine induces long-term memory T cells that could spread to different parts of the body and a fraction of these cells would constitute the respiratory TRM cell population. We found that the $CD69^+CD4^+$ T_{RM} cells induced in the lungs by immunization with OMVs or wP vaccines secreted INF-γ and IL-17. Moreover, these $CD69^+CD4^+$ T_{RM} cells were expanded in the lungs after *B. pertussis* challenge in mice immunized with OMVs or wP vaccine, but not in mice immunized with a commercial aP vaccine.

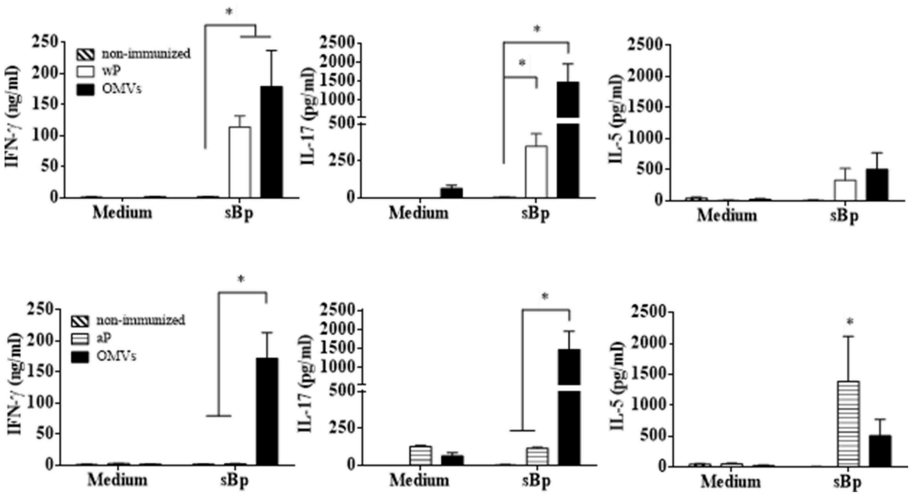


FIGURE 3 | Cytokine production by spleen cells from immunized mice. C57BL/6 mice were immunized as described in **Figure 2**. Fourteen days after the last immunization, mice were sacrificed and their spleen cells stimulated with sonicated *B. pertussis* (sBp) or medium alone (negative control). After 72 h of culture, the concentrations of IFN-γ, IL-17, and IL-5 were determined in the culture supernatant by ELISA. The results are expressed as mean values (± SEM) of three biological replicates with each having 4 mice per group. Significant differences were analyzed for each cytokine between non-immunized and immunized mice. *$p < 0.05$, ANOVA followed by the Bonferroni *post-hoc* test.

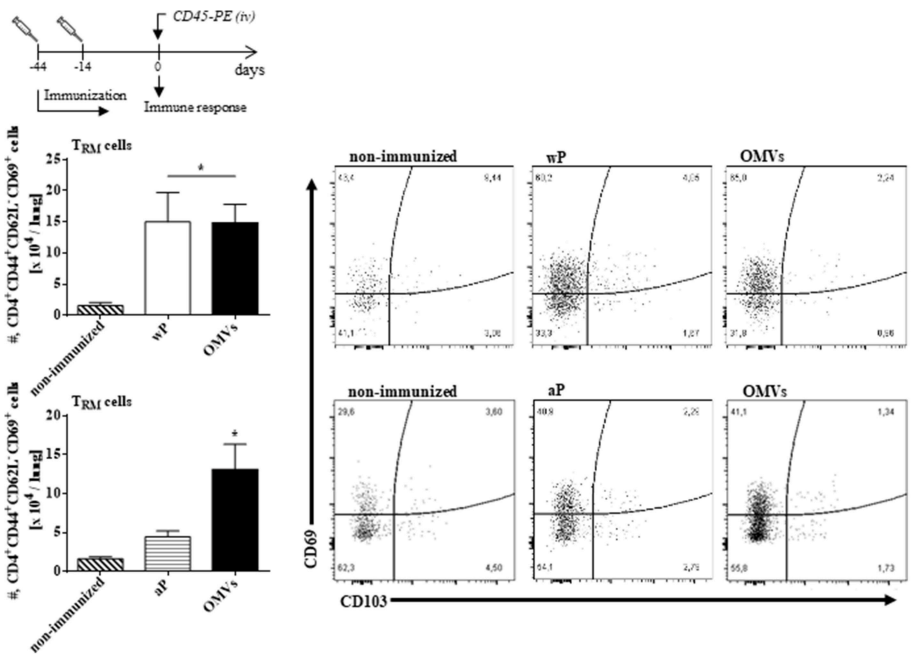


FIGURE 4 | OMVs and wP vaccines induce CD4 T_{RM} cells in lungs. C57BL/6 mice were immunized as described in **Figure 2**. Fourteen days post-immunization, mice were i.v. injected with anti-CD45-PE antibody 10 min before euthanasia. Lung tissue was taken from all mice and the T cell response was analyzed by flow cytometry. Only tissue-resident cells (CD45-PE negative) were included in the analysis. Absolute counts of $CD4^+$ T_{RM} (CD45–, CD44+, $CD62L^-$, CD69+, CD103+/–, CD4+). Data are mean ± SEM ($n = 4$ mice) for $CD4^+$ T_{RM} cells from lungs. *$p < 0.05$, two-way ANOVA with Bonferroni multiple-comparison test. Representative flow cytometry plots are shown on the right.

The long-lived IL-17-producing Th17 memory T cells have also been detected in the respiratory mucosa of *B. pertussis*-infected baboons (Warfel and Merkel, 2013). The studies in this non-human primate model showed that Th17 cells persist long after pertussis infection and suggest that these cells play an important role in adaptive immunity to *B. pertussis* (Warfel and Merkel, 2013). Our findings suggest that, in contrast to the aP vaccine, immunization with the OMVs vaccine is an effective

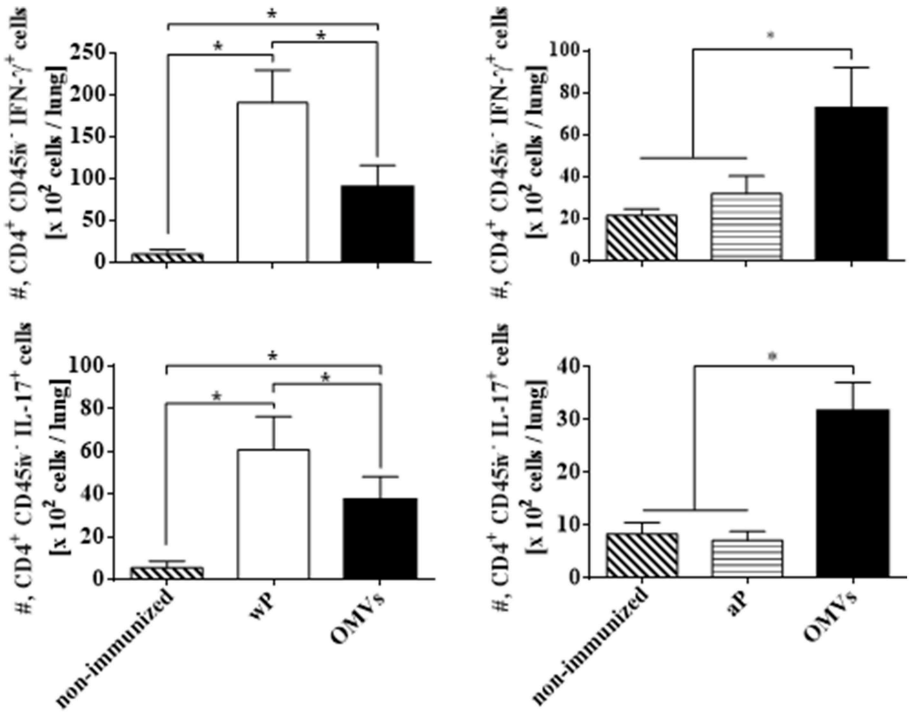


FIGURE 5 | IFN-γ and IL-17 production by CD4 T_{RM} cells in lungs of immunized mice on the day of challenge. C57BL/6 mice were immunized as described in **Figure 2** and CD4 T_{RM} were quantified in the lungs 2 weeks after the 2nd immunization (the day before challenge) by i.v. labeling with anti-CD45 as described in **Figure 3**. Intracellular cytokine staining was performed to quantify IFN-γ and IL-17-secreting T_{RM} cells. Only tissue-resident cells (CD45-PE negative) were included in the analysis. Results are absolute number of IFN-γ-producing $CD4^+$ $CD45^-$ T cells and IL-17-producing $CD4^+$ $CD45^-$ T cells in the lung. The bars indicate the mean ± SEM. *$p < 0.05$, two-way ANOVA with Bonferroni multiple-comparison test.

approach for inducing lung INF-γ and IL-17-secreting T_{RM} cells (present study) and for conferring long lasting protection against *B. pertussis* colonization (Gaillard et al., 2014). Although the used i.p. route of immunization is not translatable to humans, several previous studies, including our own had used this route as useful and highly reproducible route of systemic immunization as proof-of-principle in mice. However, we have reported that intranasal immunization with an aP vaccine formulated with a novel adjuvant is much more effective than the i.p. route for the generation of *B. pertussis*-specific T_{RM} cells (Allen et al., 2018). Furthermore, studies with *B. pertussis* OMV have demonstrated that pulmonary immunization is more effective than the s.c. route of immunization for induction of $CD103^+$ T_{RM} cells (Raeven et al., 2018). Therefore, a "prime and pull" strategy of systemic priming followed by nasal boosting might be an interesting approach for induction of systemic and local memory immune response against *B. pertussis* (Shin and Iwasaki, 2012).

The OMVs derived from *B. pertussis* represent an attractive aP vaccine candidate (Roberts et al., 2008; Asensio et al., 2011; Ormazabal et al., 2014; Hozbor, 2016) not only because of its safety and ability to induce protective Th1, Th17 cells (Mills et al., 1993; Ryan et al., 1997) and T_{RM} cells, but because it contains a greater number of immunogens in conformations close to those found in pathogen, when compared with the current aP vaccines (Hozbor, 2016). This broad immunogenic composition is an important characteristic of our vaccine candidate since is expected to exert a lower selection pressure on the circulating bacterial population than that exerted by the commercial aP vaccines consisting of only a few antigens. The prevalence of bacteria that do not express vaccine antigens in regions that only use aP vaccine provides indirect evidence of selection pressure being exerting by the aP on the circulating bacterial population (Bodilis and Guiso, 2013; Hegerle and Guiso, 2014; Lam et al., 2014). In particular in United States, Canada and Australia it was reported that PRN(-) strains have increased substantially in recent years (Lam et al., 2014; Pawloski et al., 2014; Tsang et al., 2014). The polymorphism in PRN described first and the spread of PRN-deficient isolates later, have elicited deep concerns in the healthcare systems since it was hypothesized that these changes might represent a selective advantage of the bacteria against immunity induced by the aP vaccines. In particular PRN-deficient clinical isolates may have an advantage in an aP-vaccine primed immunity (Martin et al., 2015). It has been reported that PRN-deficient clinical isolates are able to overcome the anti-PRN mediated inhibition of macrophage cytotoxicity *in vitro*. Moreover, a recent study revealed that recent PRN-deficient *B. pertussis* clinical isolates harboring *ptx*P3 variant and *prn*2 allele remain at higher CFUs/lung and are capable of sustaining infection longer than isolates still producing this adhesin, in mice immunized with a 3-component aP vaccine

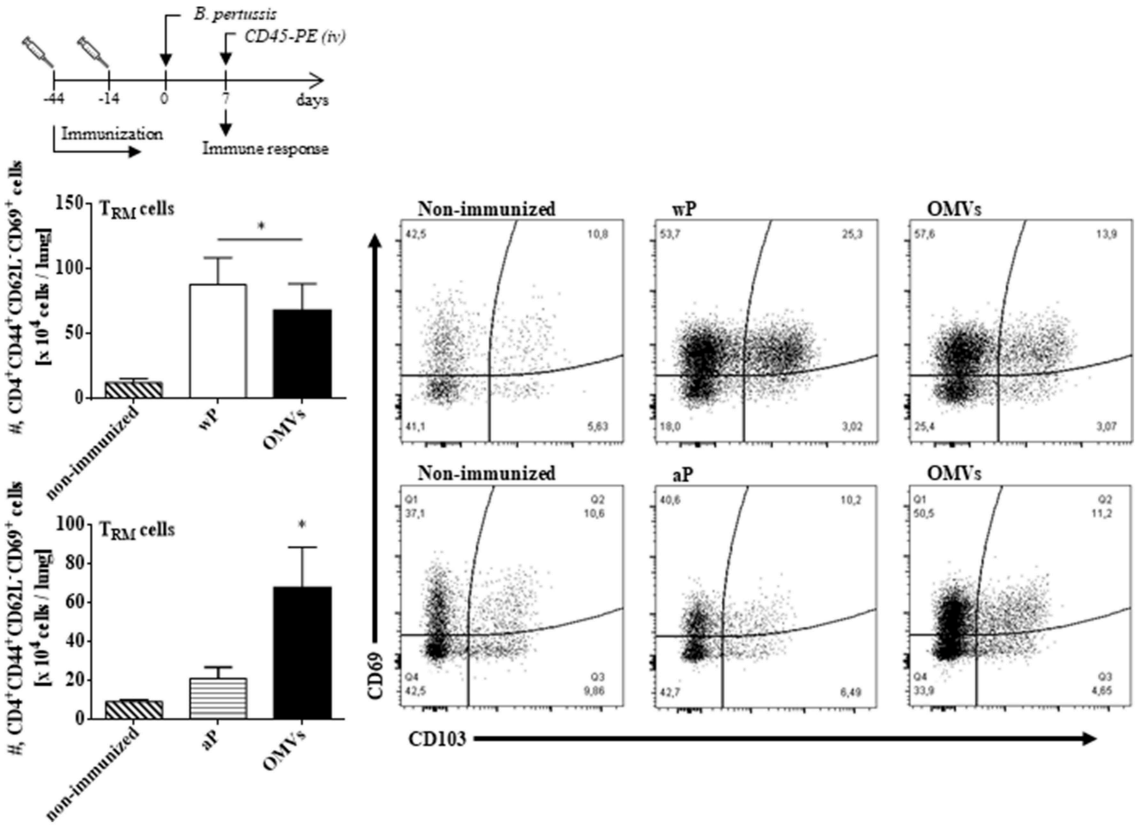


FIGURE 6 | CD4 T_{RM} cells in lungs of immunized mice after challenge with *B. pertussis*. C57BL/6 mice were immunized as described in **Figure 2** and CD4 T_{RM} were quantified in the lungs of immunized mice 7 days post-*B. pertussis* challenge as described in **Figure 4**. Absolute counts of $CD4^+$ T_{RM} (CD45–, CD44+, $CD62L^-$, CD69+, CD103+/–, CD4+) are represented. Data are mean ± SEM ($n = 4$ mice) for $CD4^+$ T_{RM} cells from lungs. *$p < 0.05$, two-way ANOVA with Bonferroni multiple-comparison test. Representative flow cytometry plots are shown on the right.

mice (Hegerle et al., 2014). The authors of such study speculated that these particular isolates might thus be capable of infecting immunized individuals at an earlier stage of waning immunity post-aP vaccine immunization or post-infection, presenting an advantage when compared to isolates producing PRN. These findings (Hegerle et al., 2014) are consistent with those on the higher fitness of PRN negative strain in the immunized mice recently reported by Safarchi et al. (2015).

Consistent with previous reports (Hegerle et al., 2014; Safarchi et al., 2015), we found that immunization with commercial aP vaccine does not protect against PRN(-) isolate as effectively as against *B. pertussis* Tohama strain (PRN+). However, the PRN(-) strain used in this study was a clinical isolate that is not isogenic to *B. pertussis* Tohama strain (PRN+) and contains polymorphisms at other loci that may affect the fitness of these bacteria. Therefore, we also examined protection against a PRN defective mutant derived from *B. pertussis* Tohama strain. Consistent with previous results (Roberts et al., 1991), we showed that in addition to PRN, other virulence factors and key antigens are equally expressed in parental and derived strain. We found that the commercial aP vaccine exhibits lower level of protection against the PRN(-) strain when compared with the parental PRN(+) positive strain. These results clearly showed the impact of the absence of PRN expression in the effectiveness of aP vaccine against *B. pertussis* when comparisons are made on strains that contain the same genetic background.

The results obtained here clearly showed that the OMVs vaccine is more effective than a current commercial aP vaccine against PRN(-) strains. Therefore the OMV formulation appears as an attractive vaccine candidate that could replace the current aP without causing concern on the reactogenicity associated with wP vaccines because of the proven safety of the OMVs vaccines (Bottero et al., 2016). Since major limitations of the current aP are their strong selection pressure exerted on the circulating bacterial population and their failure to induce sustained protective immunity, the OMV-based vaccine, that contains high number of antigens and that induces INF-γ and IL17-secreting T_{RM} cells, has the potential to replace the current aP vaccine.

ETHICS STATEMENT

This study was conducted in accordance with the recommendations and guidelines and under licenses approved

by the Health Products Regulatory Authority of Ireland and Argentina. The protocol was approved by the Trinity College Dublin Animal Research Ethics Committee and the Ethical Committee for Animal Experiments of the Faculty of Science at La Plata National University (Argentina, approval number 004-06-15 and 003-06-15).

AUTHOR CONTRIBUTIONS

DH and KM planned the study, interpreted data, and wrote the manuscript. MZ and MW planned the study, interpreted data, and edited the figures and manuscript. FC, EB, GM, and AM performed experiments and laboratory analyses. All authors approved the final manuscript.

FUNDING

This study was funded by a grant from the ANCPyT (PICT 2014-3617), CONICET and FCE-UNLP (Argentina) grants to DH and by a Science Foundation Ireland grant (16/IA/4468) to KM. DH, GM, and MZ are members of the Scientific Career of CONICET. FC and EB are fellows from CONICET. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2019.00125/full#supplementary-material

REFERENCES

Advani, A., Gustafsson, L., Ahren, C., Mooi, F. R., and Hallander, H. O. (2011). Appearance of Fim3 and ptxP3-*Bordetella pertussis* strains, in two regions of Sweden with different vaccination programs. *Vaccine* 29, 3438–3442. doi: 10.1016/j.vaccine.2011.02.070

Allen, A. C., Wilk, M. M., Misiak, A., Borkner, L., Murphy, D., and Mills, K. H. G. (2018). Sustained protective immunity against *Bordetella pertussis* nasal colonization by intranasal immunization with a vaccine-adjuvant combination that induces IL-17-secreting TRM cells. *Mucosal Immunol.* 11, 1763–1776. doi: 10.1038/s41385-018-0080-x

Anon (2010). Pertussis vaccines: WHO position paper. *Wkly. Epidemiol. Rec.* 85, 385–400.

Asensio, C. J., Gaillard, M. E., Moreno, G., Bottero, D., Zurita, E., Rumbo, M., et al. (2011). Outer membrane vesicles obtained from *Bordetella pertussis* Tohama expressing the lipid A deacylase PagL as a novel acellular vaccine candidate. *Vaccine* 29, 1649–1656. doi: 10.1016/j.vaccine.2010.12.068

Berti, E., Chiappini, E., Orlandini, E., Galli, L., and de Martino, M. (2014). Pertussis is still common in a highly vaccinated infant population. *Acta Paediatr*. 103, 846–849. doi: 10.1111/apa.12655

Bodilis, H., and Guiso, N. (2013). Virulence of pertactin-negative *Bordetella pertussis* isolates from infants, France. *Emerg. Infect. Dis.* 19, 471–474. doi: 10.3201/eid1903.121475

Borkner, L., Misiak, A., Wilk, M. M., and Mills, K. H. G. (2018). Azithromycin clears *Bordetella pertussis* infection in mice but also modulates innate and adaptive immune responses and T cell memory. *Front. Immunol.* 9:1764. doi: 10.3389/fimmu.2018.01764

Bottero, D., Gaillard, M. E., Fingermann, M., Weltman, G., Fernandez, J., Sisti, F., et al. (2007). Pulsed-field gel electrophoresis, pertactin, pertussis toxin S1 subunit polymorphisms, and surfaceome analysis of vaccine and clinical *Bordetella pertussis* strains. *Clin. Vaccine Immunol.* 14, 1490–1498. doi: 10.1128/CVI.00177-07

Bottero, D., Gaillard, M. E., Zurita, E., Moreno, G., Martinez, D. S., Bartel, E., et al. (2016). Characterization of the immune response induced by pertussis OMVs-based vaccine. *Vaccine* 34, 3303–3309. doi: 10.1016/j.vaccine.2016.04.079

Bowden, K. E., Weigand, M. R., Peng, Y., Cassiday, P. K., Sammons, S., Knipe, K., et al. (2016). Genome structural diversity among 31 *Bordetella pertussis* Isolates from two recent U.S. whooping cough statewide epidemics. *mSphere* 1:e00036-16. doi: 10.1128/mSphere.00036-16

Brummelman, J., Wilk, M. M., Han, W. G., van Els, C. A., and Mills, K. H. (2015). Roads to the development of improved pertussis vaccines paved by immunology. *Pathog. Dis.* 73:ftv067. doi: 10.1093/femspd/ftv067

Cherry, J. D. (1984). The epidemiology of pertussis and pertussis immunization in the United Kingdom and the United States: a comparative study. *Curr. Probl. Pediatr.* 14, 1–78. doi: 10.1016/0045-9380(84)90016-1

Clark, T. A. (2012). Responding to pertussis. *J. Pediatr.* 161, 980–982. doi: 10.1016/j.jpeds.2012.07.014

Clark, T. A. (2014). Changing pertussis epidemiology: everything old is new again. *J. Infect. Dis.* 209, 978–981. doi: 10.1093/infdis/jiu001

David, S., van Furth, R., and Mooi, F. R. (2004). Efficacies of whole cell and acellular pertussis vaccines against Bordetella parapertussis in a mouse model. *Vaccine* 22, 1892–1898. doi: 10.1016/j.vaccine.2003.11.005

Desauziers, E., Hessel, L., Decker, M. D., Caro, J. J., and Liese, J. G. (2004). Systematic review of the effects of pertussis vaccines in children. *Vaccine* 22, 2681–4; author reply 2685. doi: 10.1016/j.vaccine.2004.03.004

Edwards, K. M., and Karzon, D. T. (1990). Pertussis vaccines. *Pediatr. Clin. North Am.* 37, 549–566. doi: 10.1016/S0031-3955(16)36904-8

Gaillard, M. E., Bottero, D., Errea, A., Ormazabal, M., Zurita, M. E., Moreno, G., et al. (2014). Acellular pertussis vaccine based on outer membrane vesicles capable of conferring both long-lasting immunity and protection against different strain genotypes. *Vaccine* 32, 931–937. doi: 10.1016/j.vaccine.2013.12.048

Glennie, N. D., Volk, S. W., and Scott, P. (2017). Skin-resident CD4+ T cells protect against Leishmania major by recruiting and activating inflammatory monocytes. *PLoS Pathog.* 13:e1006349. doi: 10.1371/journal.ppat.1006349

Gzyl, A., Augustynowicz, E., Gniadek, G., Rabczenko, D., Dulny, G., and Slusarczyk, J. (2004). Sequence variation in pertussis S1 subunit toxin and pertussis genes in *Bordetella pertussis* strains used for the whole-cell pertussis vaccine produced in Poland since 1960, efficiency of the DTwP vaccine-induced immunity against currently circulating *B. pertussis* isolates. *Vaccine* 22, 2122–2128. doi: 10.1016/j.vaccine.2003.12.006

He, Q., Makinen, J., Berbers, G., Mooi, F. R., Viljanen, M. K., Arvilommi, H., et al. (2003). *Bordetella pertussis* protein pertactin induces type-specific antibodies: one possible explanation for the emergence of antigenic variants? *J. Infect. Dis.* 187, 1200–1205. doi: 10.1086/368412

He, Q., and Mertsola, J. (2008). Factors contributing to pertussis resurgence. *Future Microbiol.* 3, 329–339. doi: 10.2217/17460913.3.3.329

Hegerle, N., Dore, G., and Guiso, N. (2014). Pertactin deficient *Bordetella pertussis* present a better fitness in mice immunized with an acellular pertussis vaccine. *Vaccine* 32, 6597–6600. doi: 10.1016/j.vaccine.2014.09.068

Hegerle, N., and Guiso, N. (2014). *Bordetella pertussis* and pertactin-deficient clinical isolates: lessons for pertussis vaccines. *Expert Rev. Vaccines* 13, 1135–1146. doi: 10.1586/14760584.2014.932254

Hellwig, S. M., Rodriguez, M. E., Berbers, G. A., van de Winkel, J. G., and Mooi, F. R. (2003). Crucial role of antibodies to pertactin in *Bordetella pertussis* immunity. *J. Infect. Dis.* 188, 738–742. doi: 10.1086/377283

Hozbor, D., Mooi, F., Flores, D., Weltman, G., Bottero, D., Fossati, S., et al. (2009). Pertussis epidemiology in Argentina: trends over 2004-2007. *J. Infect.* 59, 225–231. doi: 10.1016/j.jinf.2009.07.014

Hozbor, D., Rodriguez, M. E., Fernandez, J., Lagares, A., Guiso, N., and Yantorno, O. (1999). Release of outer membrane vesicles from *Bordetella pertussis*. *Curr. Microbiol.* 38, 273–278. doi: 10.1007/PL00006801

Hozbor, D. F. (2016). Outer membrane vesicles: an attractive candidate for pertussis vaccines. *Expert Rev. Vaccines* 16, 193–196. doi: 10.1080/14760584.2017.1276832

Kallonen, T., Mertsola, J., Mooi, F. R., and He, Q. (2012). Rapid detection of the recently emerged *Bordetella pertussis* strains with the ptxP3 pertussis toxin promoter allele by real-time PCR. *Clin. Microbiol. Infect.* 18, E377–E379. doi: 10.1111/j.1469-0691.2012.04000.

King, A. J., Berbers, G., van Oirschot, H. F., Hoogerhout, P., Knipping, K., and Mooi, F. R. (2001). Role of the polymorphic region 1 of the *Bordetella pertussis* protein pertactin in immunity. *Microbiology* 147(Pt 11), 2885–2895. doi: 10.1099/00221287-147-11-2885

Klein, N. P. (2014). Licensed pertussis vaccines in the United States. History and current state. *Hum. Vaccin. Immunother.* 10, 2684–2690. doi: 10.4161/hv.29576

Klein, N. P., Bartlett, J., Fireman, B., Rowhani-Rahbar, A., and Baxter, R. (2013). Comparative effectiveness of acellular versus whole-cell pertussis vaccines in teenagers. *Pediatrics* 131, e1716–e1722. doi: 10.1542/peds.2012-3836

Koepke, R., Eickhoff, J. C., Ayele, R. A., Petit, A. B., Schauer, S. L., Hopfensperger, D. J., et al. (2014). Estimating the effectiveness of Tdap vaccine for preventing pertussis: evidence of rapidly waning immunity and differences in effectiveness by Tdap brand. *J. Infect. Dis.* 210, 942–953 doi: 10.1093/infdis/jiu322

Lam, C., Octavia, S., Ricafort, L., Sintchenko, V., Gilbert, G. L., Wood, N., et al. (2014). Rapid increase in Pertactin-deficient *Bordetella pertussis* isolates, Australia. *Emerg. Infect. Dis.* 20, 626–633. doi: 10.3201/eid2004.131478

Mäkelä P. H. (2000). Vaccines, coming of age after 200 years. *FEMS Microbiol. Rev.* 24, 9–20. doi: 10.1111/j.1574-6976.2000.tb00530.x

Martin, S. W., Pawloski, L., Williams, M., Weening, K., DeBolt, C., Qin, X., et al. (2015). Pertactin-negative *Bordetella pertussis* strains: evidence for a possible selective advantage. *Clin. Infect. Dis.* 60, 223–227. doi: 10.1093/cid/ciu788

Mbayei, S. A., Faulkner, A., Miner, C., Edge, K., Cruz, V., Pena, S. A., et al. (2018). Severe Pertussis Infections in the United States, 2011-2015. *Clin. Infect. Dis.* ciy889. doi: 10.1093/cid/ciy889

McGirr, A., and Fisman, D. N. (2015). Duration of Pertussis immunity after DTaP immunization: a meta-analysis. *Pediatrics* 135, 331–343. doi: 10.1542/peds.2014-1729

Mills, K. H., Barnard, A., Watkins, J., and Redhead, K. (1993). Cell-mediated immunity to *Bordetella pertussis*: role of Th1 cells in bacterial clearance in a murine respiratory infection model. *Infect. Immun.* 61, 399–410.

Mooi, F. R., van Loo, I. H., and King, A. J. (2001). Adaptation of *Bordetella pertussis* to vaccination: a cause for its reemergence? *Emerg Infect Dis.* 7(3 Suppl), 526–528. doi: 10.3201/eid0707.017708

Ormazabal, M., Bartel, E., Gaillard, M. E., Bottero, D., Errea, A., Zurita, M. E., et al. (2014). Characterization of the key antigenic components of pertussis vaccine based on outer membrane vesicles. *Vaccine* 32, 6084–6090. doi: 10.1016/j.vaccine.2014.08.084

Pawloski, L. C., Queenan, A. M., Cassiday, P. K., Lynch, A. S., Harrison, M. J., Shang, W., et al. (2014). Prevalence and molecular characterization of pertactin-deficient *Bordetella pertussis* in the United States. *Clin. Vaccine Immunol.* 21, 119–125. doi: 10.1128/CVI.00717-13

Plotkin, S. A. (2005). Six revolutions in vaccinology. *Pediatr. Infect. Dis. J.* 24, 1–9. doi: 10.1097/01.inf.0000148933.08301.02

Plotkin, S. A. (2014). The pertussis problem. *Clin. Infect. Dis.* 58, 830–833. doi: 10.1093/cid/cit934

Raeven, R. H. M., Brummelman, J., Pennings, J. L. A., van der Maas, L., Helm, K., Tilstra, W., et al. (2018). Molecular and cellular signatures underlying superior immunity against *Bordetella pertussis* upon pulmonary vaccination. *Mucosal Immunol.* 11:1009. doi: 10.1038/mi.2017.110

Roberts, M., Fairweather, N. F., Leininger, E., Pickard, D., Hewlett, E. L., Robinson, A., et al. (1991). Construction and characterization of *Bordetella pertussis* mutants lacking the vir-regulated P.69 outer membrane protein. *Mol. Microbiol.* 5, 1393–1404. doi: 10.1111/j.1365-2958.1991.tb00786.x

Roberts, R., Moreno, G., Bottero, D., Gaillard, M. E., Fingermann, M., Graieb, A., et al. (2008). Outer membrane vesicles as acellular vaccine against pertussis. *Vaccine* 26, 4639–4646. doi: 10.1016/j.vaccine.2008.07.004

Romanus, V., Jonsell, R., and Bergquist, S. O. (1987). Pertussis in Sweden after the cessation of general immunization in 1979. *Pediatr. Infect. Dis. J.* 6, 364–371. doi: 10.1097/00006454-198704000-00005

Ross, P. J., Sutton, C. E., Higgins, S., Allen, A. C., Walsh, K., Misiak, A., et al. (2013). Relative contribution of Th1 and Th17 cells in adaptive immunity to *Bordetella pertussis*: towards the rational design of an improved acellular pertussis vaccine. *PLoS Pathog.* 9:e1003264. doi: 10.1371/journal.ppat.1003264

Ryan, M., Murphy, G., Gothefors, L., Nilsson, L., Storsaeter, J., and Mills, K. H. (1997). *Bordetella pertussis* respiratory infection in children is associated with preferential activation of type 1 T helper cells. *J. Infect. Dis.* 175, 1246–1250. doi: 10.1086/593682

Ryan, M., Murphy, G., Ryan, E., Nilsson, L., Shackley, F., Gothefors, L., et al. (1998). Distinct T-cell subtypes induced with whole cell and acellular pertussis vaccines in children. *Immunology* 93, 1–10. doi: 10.1046/j.1365-2567.1998.00401.x

Safarchi, A., Octavia, S., Luu, L. D., Tay, C. Y., Sintchenko, V., Wood, N., et al. (2015). Pertactin negative *Bordetella pertussis* demonstrates higher fitness under vaccine selection pressure in a mixed infection model. *Vaccine* 33, 6277–6281. doi: 10.1016/j.vaccine.2015.09.064

Sato, H., and Sato, Y. (1985). Protective antigens of *Bordetella pertussis* mouse-protection test against intracerebral and aerosol challenge of *B. pertussis*. *Dev. Biol. Stand.* 61, 461–467.

Shin, H., and Iwasaki, A. (2012). A vaccine strategy that protects against genital herpes by establishing local memory T cells. *Nature* 491, 463–467. doi: 10.1038/nature11522

Stoddard, M. B., Pinto, V., Keiser, P. B., and Zollinger, W. (2010). Evaluation of a whole-blood cytokine release assay for use in measuring endotoxin activity of group B Neisseria meningitidis vaccines made from lipid A acylation mutants. *Clin. Vaccine Immunol.* 17, 98–107. doi: 10.1128/CVI.00342-09

Tan, T., Dalby, T., Forsyth, K., Halperin, S. A., Heininger, U., Hozbor, D., et al. (2015). Pertussis across the globe: recent epidemiologic trends from 2000-2013. *Pediatr. Infect. Dis. J.* 34:e222–32. doi: 10.1097/INF.0000000000000795

Teijaro, J. R., Turner, D., Pham, Q., Wherry, E. J., Lefrancois, L., and Farber, D. L. (2011). Cutting edge: tissue-retentive lung memory CD4 T cells mediate optimal protection to respiratory virus infection. *J. Immunol.* 187, 5510–5514. doi: 10.4049/jimmunol.1102243

Tsang, R. S., Shuel, M., Jamieson, F. B., Drews, S., Hoang, L., Horsman, G., et al. (2014). Pertactin-negative *Bordetella pertussis* strains in Canada: characterization of a dozen isolates based on a survey of 224 samples collected in different parts of the country over the last 20 years. *Int. J. Infect. Dis.* 28, 65–69. doi: 10.1016/j.ijid.2014.08.002

Warfel, J. M., and Merkel, T. J. (2013). *Bordetella pertussis* infection induces a mucosal IL-17 response and long-lived Th17 and Th1 immune memory cells in nonhuman primates. *Mucosal Immunol.* 6, 787–796. doi: 10.1038/mi.2012.117

WHO (2007). *Annex 6. Recommendations for Whole-Cell Pertussis Vaccine.* WHO Technical Report Series No 941, 2007.

WHO (2010a). *The Initiative for Vaccine Research, Strategic Plan 2010–2020.* Geneva: World Health Organization. Available online at: http://www.who.int/iris/handle/10665/70254

WHO (2010b). *The Initiative for Vaccine Research. Report 2008–2009.* Available online at: https://apps.who.int/iris/bitstream/handle/10665/70443/WHO_IVB_10.06_eng.pdf?sequence=1

Wilk, M. M., Misiak, A., McManus, R. M., Allen, A. C., Lynch, M. A., and Mills, K. H. G. (2017). Lung CD4 tissue-resident memory T cells mediate adaptive immunity induced by previous infection of mice with *Bordetella pertussis*. *J. Immunol.* 199, 233–243. doi: 10.4049/jimmunol.1602051

Zhang, L., Prietsch, S. O., Axelsson, I., and Halperin, S. A. (2012). Acellular vaccines for preventing whooping cough in children. *Cochrane Database Syst. Rev.* 3:CD001478. doi: 10.1002/14651858.CD001478.pub5

Conflict of Interest Statement: 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 Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 01 May 2019
doi: 10.3389/fcimb.2019.00135

A Multi-Stage *Plasmodium vivax* Malaria Vaccine Candidate Able to Induce Long-Lived Antibody Responses Against Blood Stage Parasites and Robust Transmission-Blocking Activity

Jessica N. McCaffery[1†], *Jairo A. Fonseca*[1,2†‡], *Balwan Singh*[1], *Monica Cabrera-Mora*[1], *Caitlin Bohannon*[1], *Joshy Jacob*[1,3], *Myriam Arévalo-Herrera*[4] *and Alberto Moreno*[1,2*]

[1] *Emory Vaccine Center, Yerkes National Primate Research Center, Emory University, Atlanta, GA, United States,* [2] *Division of Infectious Diseases, Department of Medicine, Emory University, Atlanta, GA, United States,* [3] *Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA, United States,* [4] *Caucaseco Scientific Research Center, Malaria Vaccine and Drug Development Center, Cali, Colombia*

Malaria control and interventions including long-lasting insecticide-treated nets, indoor residual spraying, and intermittent preventative treatment in pregnancy have resulted in a significant reduction in the number of *Plasmodium falciparum* cases. Considerable efforts have been devoted to *P. falciparum* vaccines development with much less to *P. vivax*. Transmission-blocking vaccines, which can elicit antibodies targeting *Plasmodium* antigens expressed during sexual stage development and interrupt transmission, offer an alternative strategy to achieve malaria control. The post-fertilization antigen P25 mediates several functions essential to ookinete survival but is poorly immunogenic in humans. Previous clinical trials targeting this antigen have suggested that conjugation to a carrier protein could improve the immunogenicity of P25. Here we report the production, and characterization of a vaccine candidate composed of a chimeric *P. vivax* Merozoite Surface Protein 1 (cPvMSP1) genetically fused to *P. vivax* P25 (Pvs25) designed to enhance $CD4^+$ T cell responses and its assessment in a murine model. We demonstrate that antibodies elicited by immunization with this chimeric protein recognize both the erythrocytic and sexual stages and are able to block the transmission of *P. vivax* field isolates in direct membrane-feeding assays. These findings provide support for the continued development of multi-stage transmission blocking vaccines targeting the life-cycle stage responsible for clinical disease and the sexual-stage development accountable for disease transmission simultaneously.

Keywords: malaria, *Plasmodium vivax*, multi-stage, chimeric vaccine, transmission-blocking, P25

OPEN ACCESS

Edited by:
Ricardo Ataide,
Burnet Institute, Australia

Reviewed by:
Rhea Jessica Longley,
Walter and Eliza Hall Institute of Medical Research, Australia
Takafumi Tsuboi,
Ehime University, Japan

****Correspondence:***
Alberto Moreno
alberto.moreno@emory.edu

†These authors have contributed equally to this work

‡Present Address:
Jairo A. Fonseca,
Division of Leukemia and Lymphoma, Department of Pediatrics, Emory University School of Medicine, Atlanta, GA, United States

Specialty section:
This article was submitted to Clinical Microbiology, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *30 January 2019*
Accepted: *15 April 2019*
Published: *01 May 2019*

Citation:
McCaffery JN, Fonseca JA, Singh B, Cabrera-Mora M, Bohannon C, Jacob J, Arévalo-Herrera M and Moreno A (2019) A Multi-Stage Plasmodium vivax Malaria Vaccine Candidate Able to Induce Long-Lived Antibody Responses Against Blood Stage Parasites and Robust Transmission-Blocking Activity. Front. Cell. Infect. Microbiol. 9:135. doi: 10.3389/fcimb.2019.00135

INTRODUCTION

Malaria remains one of the most serious threats to global health. In 2017, there were an estimated 219 million malaria cases resulting in 435,000 deaths worldwide (World Health Organization, 2018). Of the five *Plasmodium* species that cause malaria in humans, *P. vivax* is the most widely distributed with ∼2.8 billion people at risk of infection (Guerra et al., 2010). Its wide geographical range is mainly due to the ability of *P. vivax* to develop within the *Anopheles* mosquito vector at lower temperatures, allowing for its survival at higher altitudes and temperate climates (World Health Organization, 2014). Furthermore, *P. vivax* has the ability to produce hypnozoites, dormant liver-stage parasites present in *P. vivax* but not in *P. falciparum* (Krotoski et al., 1982), causing relapse infections weeks to months after the initial infection. Effective malaria control programs, therefore, require comprehensive measures that involve targeting both *Plasmodium* species (Battle et al., 2012; Gething et al., 2012).

Current malaria control efforts have mainly been focused on the use of vector-based interventions, including long-lasting insecticide-treated nets (LLIN), indoor residual spraying (IRS), and preventative therapies. Preventive therapies include intermittent preventative treatment in pregnancy (IPTp) with sulfadoxine-pyrimethamine and seasonal malaria chemoprevention (SMC) in children aged 3–59 months living in areas of high seasonal malaria transmission (World Health Organization, 2018). While these interventions have resulted in a significant reduction in *P. falciparum* cases (Mendis et al., 2009), *P. falciparum* vector-based interventions are less efficacious against *P. vivax* (Bockarie and Dagoro, 2006). Anomalous climate patterns, as well as the emergence of mosquito resistance to insecticides (Corbel et al., 2007; Dondorp et al., 2009) and parasite resistance to antimalarial treatments (Thomas et al., 2016; Haldar et al., 2018; World Health Organization, 2018), pose additional challenges to the prevention and treatment of malaria despite improved malaria control coverage.

Due to the numerous challenges faced by traditional malaria control methods, the development of novel intervention tools is essential. One potential strategy is the use of transmission-blocking vaccines as they are considered one of the best alternatives to achieve malaria control. Since the life cycle of *Plasmodium* requires the female *Anopheles* mosquito to ingest gametocytes during a blood meal from an infected human host to reach the mosquito midgut and begin the next stage of development outside the human red blood cells, this transition could be interrupted by anti-parasite antibodies present in the blood meal (Saxena et al., 2007).

There are two kinds of transmission blocking antigens that can be targeted by vaccines: pre-fertilization and post-fertilization antigens. Pre-fertilization antigens are expressed by gametocytes and gametes; antibodies against these antigens can block the formation of zygotes by binding to the gametes (Sauerwein and Bousema, 2015). Post-fertilization antigens are expressed by zygotes and ookinetes, antibodies that recognize these forms prevent the mosquito midgut invasion (Saxena et al., 2007; Sauerwein and Bousema, 2015). Under natural conditions, the human host is not exposed to post-fertilization antigens. However, transmission-blocking vaccination can be used to elicit antibodies targeting post-fertilization antigens that the mosquito will be exposed to during the blood meal.

Of the post-fertilization antigens described to date, the P25 protein present on the surface of ookinetes and oocysts, first described by Tsuboi et al. (1998), is one of the best characterized (Blagborough et al., 2016). P25 mediates several functions including promoting the clustering of the ookinetes and allowing them to survive the midgut proteolytic environment (Gass and Yeates, 1979). P25 also mediates the attachment and invasion of the mosquito midgut by damaging the midgut epithelium (Han et al., 2000; Zieler and Dvorak, 2000; Vlachou et al., 2004), and binding to laminin and collagen IV in the basal membrane which serves as the starting signal for the ookinete to oocyst development (Vlachou et al., 2001; Arrighi and Hurd, 2002).

While previous phase I clinical trials using the *P. vivax* P25 protein (Pvs25) have demonstrated that humans can produce antibodies against this antigen, an ideal formulation has not been reported. The first clinical trial of a protein-based Pvs25 vaccine candidate formulated with alum as an adjuvant showed poor immunogenicity and no transmission blocking effect (Malkin et al., 2005). A subsequent clinical trial using a protein-based Pvs25 formulated with Montanide ISA 51 as an adjuvant, showed that low doses of the formulation were able to induce transmission blocking immunity, but higher doses were associated with systemic adverse events (Wu et al., 2008). However, pre-clinical and clinical studies aimed at improving the suboptimal immunogenicity observed by immunization with the *Plasmodium* P25 proteins, Pfs25, and Pvs25 have suggested that the addition of a carrier protein could potentially enhance the immunogenicity of this protein (Qian et al., 2007; Parzych et al., 2017; Radtke et al., 2017).

A vaccine targeting only a transmission-blocking antigen faces challenges in maintaining an antibody response to parasite antigens to which there would be no bosting effect by natural exposure. Furthermore, this type of vaccine would not provide the human host with protection against infection and would likely have low compliance especially if multiple vaccinations are required. We hypothesize that the development of a bifunctional *P. vivax* vaccine able to target both a blood stage antigen and a sexual stage antigen could provide protection against infection to the vaccinated individual as well as reduce transmission. A multi-stage transmission-blocking vaccine is particularly relevant for *P. vivax* given the fact that most relapses are asymptomatic (Van den Eede et al., 2011). These individuals are less likely to receive treatment to clear the infection, resulting in longer periods where the parasite can be transmitted to mosquitoes. In addition to targeting a reservoir of malaria transmission, a *P. vivax* bifunctional blood stage and transmission blocking vaccine may also improve vaccine uptake due to its potential to provide clinical immunity, as well as a reduction in transmission.

Our group has previously defined several $CD4^+$ T cell epitopes within the erythrocytic stage antigen Merozoite Surface Protein 1 (MSP1) of *P. vivax*. These epitopes contain features that define them as promiscuous T cell epitopes (i.e., able to bind

a broad range of MHC class II alleles) (Caro-Aguilar et al., 2002). Synthetic peptides representing these *P. vivax* MSP1 T cell epitopes are recognized by lymphocytes from individuals naturally infected with *P. vivax* (Caro-Aguilar et al., 2002). We have designed and expressed a chimeric *P. vivax* MSP1 (cPvMSP1) by genetically linking five of these promiscuous T cell epitopes arrayed in tandem conformation to an extended version of the carboxyl-terminal 19kDa fragment of the *P. vivax* MSP1 Merozoite Surface Protein 1 ($PvMSP1_{19}$) (Fonseca et al., 2016). We have shown that immunization with cPvMSP1 induced significantly increased cellular and humoral immune responses in the murine model when compared to the native protein (Fonseca et al., 2016). Here we report the design, production, and characterization of a chimeric bifunctional protein composed of the previously described cPvMSP1 (Fonseca et al., 2016), now genetically fused to recombinant Pvs25 (cPvMSP1-Pvs25). We hypothesize that cPvMSP1 will serve both as a carrier protein that can improve Pvs25 immunogenicity while also inducing robust anti-blood stage protective immune responses. Here we assessed the cellular and humoral immunogenicity of cPvMSP1-Pvs25 in mice and its ability to induce long-lived plasma cells, as well as the ability of antibodies elicited by vaccination with cPvMSP1-Pvs25 to reduce transmission when tested in functional assays.

METHODS

Ethics Statements

This study including human samples was carried out in accordance with the recommendations of the ICH/GCP guidelines, Comité de Etica para Investigación con Humanos, Centro Internacional de Vacunas (CECIV, Cali, Colombia), and the protocol approved by the CECIV. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

All animal protocols that include experimental animal procedures using mice and NHP were carried out in accordance with the US Animal Welfare Act and approved by the Emory University's Institutional Animal Care and Use Committee and followed accordingly.

Design and Biochemical Characterization of the *P. vivax* Chimeric Pvs25-MSP1 Protein

The 861 bp synthetic gene encoding the chimeric *P. vivax* merozoite surface protein 1 protein (cPvMSP1) used for these studies has been previously described (Fonseca et al., 2016). A 546 bp synthetic gene encoding Pvs25 (codon optimized for expression in *E. coli*) was produced by Geneart (Regensburg, Germany). The sequence for the synthetic gene was derived from the Salvador I strain (XP_001608460; A23 to L195), which does not include its signal peptide and the GPI anchor. A sequence encoding the peptide MAVD was added upstream of the amino-terminal A23 for protein expression. The synthetic gene was subcloned into a pET24d(+) vector. For the production of the synthetic gene encoding the bifunctional chimeric protein, the chimeric PvMSP1 plasmid construct was digested with XhoI and the Pvs25 plasmid amplified by PCR using XhoI/NcoI specific primers. The fragments were annealed and then ligated with T4 DNA ligase. The proper configurations of the *Pvs25* and *cPvMSP1-Pvs25* genes were verified by enzyme restriction analysis and the sequence confirmed using an automatic sequencer. The recombinant pET plasmids were transformed into BL21 (DE3) cells with kanamycin selection. The sequence of the recombinant bifunctional erythrocytic stage-transmission blocking chimeric protein, designated cPvMSP1-Pvs25 (**Figure 1A**), includes: (i) MAVD amino terminus to reduce degradation during synthesis in *E. coli* and to provide a start signal; (ii) Five promiscuous T cell epitopes derived from *P. vivax* MSP1 capable of binding to a broad range of MHC class II alleles, arranged in tandem interspaced with GPGPG spacers: PvT4 (N_{78}-L_{97}), PvT6 (F_{118}-H_{137}), PvT8 (L_{158}-D_{177}), PvT19 (L_{378}-S_{397}), and PvT53 (S_{1058}-N_{1077}); (iii) An extended version of the *P. vivax* $MSP1_{19}$ kD protein fragment, which includes two promiscuous T cell epitopes derived from $MSP1_{33}$ protein fragment; (iv) A $(NANP)_6$ tag from the original chimeric PvMSP1 derived from the *P. falciparum* circumsporozoite protein included for biochemical characterization of antigenic integrity of the chimeric protein and to provide an optional affinity purification tag; and (v) The Pvs25 sequence derived from the *P. vivax* Salvador I strain, without the signal peptide and the GPI anchor, but including the MAVD sequence derived from the plasmid Pvs25.

Protein expression was induced with 1 mM IPTG, and the soluble Pvs25 was purified with a Ni-NTA affinity column. cPvMSP1-Pvs25 was expressed in inclusion bodies and refolded as previously described (Singh et al., 2001) using 4M concentration of urea in the refolding solution. After refolding, the protein was purified using gel filtration chromatography. The integrity of the proteins was analyzed by western blot using the anti-Pvs25 monoclonal antibody (mAb) N1-1H10 (MRA-471, BEI Resources), an anti-His tag mAb, or the mAb 2A10 that recognize the $(NANP)_6$ carboxyl terminal tag of the cPvMSP1-Pvs25 (**Figure 1** and **Supplementary Figure 1**). Additionally, endotoxin levels of the purified protein product were determined using the E-Toxate *Limulus* amebocyte lysate kit (Sigma), according to the manufacturer's instructions, and were determined to range between 25 and 42 EU/mg of protein.

Synthetic Peptides

A library of 61 15-mer synthetic peptides overlapped by 11 residues and spanning the complete cPvMSP1-Pvs25 chimeric protein sequence was commercially synthesized by the multiple solid-phase technique (Sigma-Aldrich). Peptide pools were used to characterize cellular reactivity, with the cPvMSP1 peptide pool 1 representing the sequence of the cognate T cell epitopes included in our chimeric construct and the cPvMSP1 pool 2 representing the complete amino acid sequence of the $MSP1_{19}$ kD protein fragment. Pvs25 pool 1 and pool 2 represent the amino acid sequence of Pvs25 (**Table 1**).

Mice Immunizations

Groups of 10 female CB6F1/J ($H\text{-}2^{d/b}$) mice, 6–8 weeks of age, were purchased from The Jackson Laboratory. The animals were immunized subcutaneously on days 0, 20, and 40, in

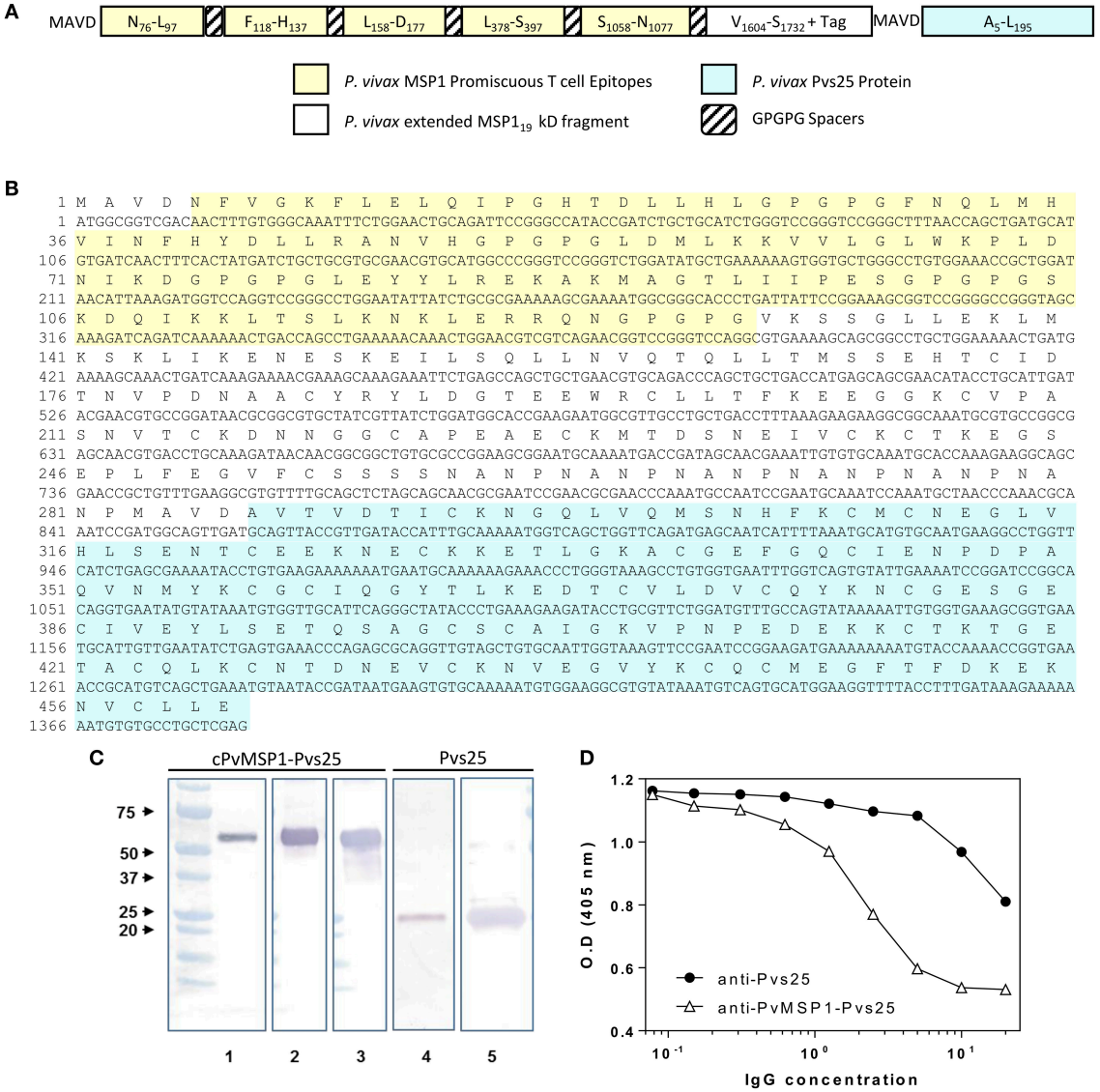


FIGURE 1 | cPvMSP1-Pvs25 protein structure, sequence, and characterization. **(A)** cPvMSP1-Pvs25 structure. This protein includes five promiscuous T cell epitopes of cPvMSP1 (yellow), each separated by a GPGPG spacer (diagonal lines). The promiscuous T cell epitopes are linked to a fragment derived from *P. vivax* $MSP1_{33}$ and the entire *P. vivax* $MSP1_{19}$ kD fragment (white), and the *P. vivax* Pvs25 protein (blue), with each separated by GPGPG spacers (diagonal lines). **(B)** cPvMSP1-Pvs25 amino acid sequence is shown in single letter code. The carboxyl-terminal $(H)_6$ tag provided by the vector was not included in the sequence. The yellow shaded area shows the region of the chimeric protein that contains the promiscuous T cell epitopes. The blue shaded area shows the *P. vivax* Pvs25 protein. **(C)** Western blot analysis of the purified cPvMSP1-Pvs25 (lanes 1–3) and the purified Pvs25 proteins (lanes 4 and 5). Each protein was run as separate PAGE gels using 1.0 μg total of purified protein under reducing conditions, and blots were stained with individual antibodies. Full uncut blots are shown in **Supplementary Figure 1**. Samples were incubated with following antibodies: Lane 1 and 4, the monoclonal antibody N1-1H10 which targets Pvs25; lane 2 and 5, an anti-His-Tag monoclonal antibody targeting the C terminal tags of the cPvMSP1-Pvs25 and Pvs25 proteins; lane 3, the monoclonal antibody 2A10 which targets the cPvMSP1 C terminal tag. The molecular weight markers (BioRad) are indicated. **(D)** Polyclonal anti-cPvMSP1-Pvs25 and anti-Pvs25 elicited in rabbits compete for binding with the transmission blocking monoclonal antibody N1-1H10. Fixed amounts of N1-1H10 (1 μg) were tested with 2-fold dilutions of purified rabbit IgG using Pvs25 as antigen. O.D. values (y-axis) are shown for anti-Pvs25 in closed circles and anti-cPvMSP1-Pvs25 in open triangles using polyclonal antibodies ranging from 0.078 ng/ml to 20 μg/ml (x-axis). Data are presented as geometric mean values.

the base of the tail and the interscapular area, using 20 μg of either the cPvMSP1-Pvs25 or Pvs25 proteins emulsified in the adjuvant Montanide ISA 51 VG (Seppic). As a control, a group of mice received PBS emulsified in the same adjuvant. A summary of the immunization regimens and groups can be found in **Table 2**. All animal protocols were approved by the Emory University's Institutional Animal Care and Use Committee and followed accordingly. Rabbits were immunized four times either with cPvMSP1-Pvs25 or Pvs25 at twenty days intervals, and sera were obtained prior to the first immunization and after each immunization. Rabbit immunization and sera collections were performed by Convance Inc.

TABLE 1 | Chimeric PvMSP1 and Pvs25 peptide pools.

Peptide pool	Sequence	Peptide pool	Sequence
cPvMSP1 Pool 1	MANFVGKFLELQIPG	Pvs25 Pool 1	MAVDAVTVDTICKNG
	VGKFLELQIPGHTDL		AVTVDTICKNGQLVQ
	LELQIPGHTDLLHLG		DTICKNGQLVQMSNH
	IPGHTDLLHLGPGPG		KNGQLVQMSNHFKCM
	TDLLHLGPGPGFNQL		LVQMSNHFKCMCNEG
	HLGPGPGFNQLMHVI		SNHFKCMCNEGLVHL
	GPGFNQLMHVINFHY		KCMCNEGLVHLSENT
	NQLMHVINFHYDLLR		NEGLVHLSENTCEEK
	HVINFHYDLLRANVH		VHLSENTCEEKNECK
	FHYDLLRANVHGPGP		ENTCEEKNECKKETL
	LLRANVHGPGPGLDM		EEKNECKKETLGKAC
	NVHGPGPGLDMLKKV		ECKKETLGKACGEFG
	PGPGLDMLKKVVLGL		ETLGKACGEFGQCIE
	LDMLKKVVLGLWKPL		KACGEFGQCIENPDP
	KKVVLGLWKPLDNIK		EFGQCIENPDPAQVN
	LGLWKPLDNIKDGPG		CIENPDPAQVNMYKC
	KPLDNIKDGPGPGLE		PDPAQVNMYKCGCIQ
	NIKDGPGPGLEYYLR		QVNMYKCGCIQGYTL
	GPGPGLEYYLREKAK		YKCGCIQGYTLKEDT
	GLEYYLREKAKMAGT		CIQGYTLKEDTCVLD
	YLREKAKMAGTLIIP		
	KAKMAGTLIIPESGP		
	AGTLIIPESGPGPGS		
	IIPESGPGPGSKDQI		
	SGPGPGSKDQIKKLT		
	PGSKDQIKKLTSLKN		
	DQIKKLTSLKNKLER		
	KLTSLKNKLERRQNG		
	LKNKLERRQNGPGPG		
	LERRQNGPGPGVKSS		
cPvMSP1 Pool 2	QNGPGPGVKSSGLLE	Pvs25 Pool 2	YTLKEDTCVLDVCQY
	GPGVKSSGLLEKLMK		EDTCVLDVCQYKNCG
	KSSGLLEKLMKSKLI		VLDVCQYKNCGESGE
	LLEKLMKSKLIKENE		CQYKNCGESGECIVE
	LMKSKLIKENESKEI		NCGESGECIVEYLSE
	KLIKENESKEILSQL		SGECIVEYLSETQSA
	ENESKEILSQLLNVQ		IVEYLSETQSAGCSC
	KEILSQLLNVQTQLL		LSETQSAGCSCAIGK
	SQLLNVQTQLLTMSS		QSAGCSCAIGKVPNP
	NVQTQLLTMSSEHTC		CSCAIGKVPNPEDEK
	QLLTMSSEHTCIDTN		IGKVPNPEDEKKCTK
	MSSEHTCIDTNVPDN		PNPEDEKKCTKTGET
	HTCIDTNVPDNAACY		DEKKCTKTGETACQL
	DTNVPDNAACYRYLD		CTKTGETACQLKCNT
	PDNAACYRYLDGTEE		GETACQLKCNTDNEV
	ACYRYLDGTEEWRCL		CQLKCNTDNEVCKNV
	YLDGTEEWRCLLTFK		CNTDNEVCKNVEGVY
	TEEWRCLLTFKEEGG		NEVCKNVEGVYKCQC
	RCLLTFKEEGGKCVP		KNVEGVYKCQCMEGF
	TFKEEGGKCVPASNV		GVYKCQCMEGFTFDK
	EGGKCVPASNVTCKD		CQCMEGFTFDKEKNV
	CVPASNVTCKDNNGG		EGFTFDKEKNVCLLE
	SNVTCKDNNGGCAPE		
	CKDNNGGCAPEAECK		
	NGGCAPEAECKMTDS		
	APEAECKMTDSNEIV		
	ECKMTDSNEIVCKCT		
	TDSNEIVCKCTKEGS		
	EIVCKCTKEGSEPLF		
	KCTKEGSEPLFEGVF		
	EGSEPLFEGVFCSSS		

Peptide pools used for stimulation of T cells for assessment of cytokine production after the second immunization. cPvMSP1 Pool 1 represents MSP1 T cell epitopes present in cPvMSP1. Pool 2 represents the extended MSP1$_{19}$ kD protein fragment. Pvs25 pools 1 and 2 cover the full sequence of the Pvs25 protein.

ELISA Assays

The procedures for the assessment of IgG antibody titers, subclasses, and avidity have been previously described (Fonseca et al., 2016). Antibody titers elicited by immunization of mice were determined by ELISA using Immulon 4HBX plates (Thermo Scientific) coated with 1 μg/ml of cPvMSP1-Pvs25, Pvs25, or PvMSP1$_{19}$ diluted in carbonate buffer as described (Singh et al., 2010).

Briefly, plates were allowed to incubate overnight with 100 μl of the 1 μg/ml protein solution. The solution was removed, and plates were washed 3 times with wash buffer consisting of PBS 1X with 0.05% Tween 20. 200 μl of blocking solution, BSA (KPL) diluted 1:10 in distilled water, was added to each well and plates were incubated again for 2 h at 37°C. Blocking solution was removed without washing. Sera were diluted in a dilution solution composed of 1:20 BSA (KPL) in distilled water at a starting dilution of 1:320. Cutoffs for positive titers were set at the highest dilution of sera where the O.D. was greater than that of the mean plus three standard deviations above the optical densities obtained using pre-immune sera**.** Following a 1-h incubation with 100 μl of the diluted mouse sera at 37°C, the plates were washed five times with wash buffer before the addition of 100 μl of peroxidase labeled goat anti-mouse IgG antibody (KPL) at 1:1,000 in dilution solution. Plates were again incubated for 1 h at 37°C before washing five times with wash buffer. ABTS solution (KPL) was used as a substrate following a 1-h incubation. Optical densities were determined using a VERSAmax ELISA reader (Molecular Device Corporation) with a 405 nm filter. Results are presented as the reciprocal of the end-point dilution.

IgG1 and IgG2a subclass profiles of vaccine-induced antibodies were also determined. ELISA assays were performed as described for the determination of antibody titers, except that after incubation with sera the plates were washed and incubated with biotinylated rat anti-mouse mAbs IgG1 or IgG2a, (BD Pharmingen) for 2 h. After washing, the bound antibodies were detected using horseradish peroxidase (HRP)-streptavidin (KPL) and the SureBlue^{TM} TMB Microwell Peroxidase Substrate (KPL). The peroxidase reaction was stopped with the TMB Stop Solution (KPL). Optical densities were determined using a VERSAmax ELISA reader (Molecular Device Corporation) with a 450 nm filter.

The avidity indices of the antibodies were assessed by ammonium thiocyanate elution-based ELISA using sera samples obtained at day 60, corresponding to 20 days after the final immunization, and day 730, (2 years after the first immunization). The avidity ELISA was conducted similarly to the total IgG titer ELISA, with slight modifications. Briefly, serial dilutions of the sera were assayed in the absence and presence of 1M NH_4SCN (Sigma Aldrich) in PBS. The plates were incubated for 15 min at room temperature before washing and proceeding with the assay as described above. The avidity index was calculated as the ratio between the antilog of the absorbance curves obtained with (x_1) and without (x_2) NH4SCN, as previously described (Perciani et al., 2007).

For ELISA competition assays, purified polyclonal anti-cPvMSP1-Pvs25 and anti-Pvs25 elicited in rabbits (Covance)

TABLE 2 | Immunization regimens.

Regimen	Prime day 0		Boost day 20		Boost day 40	
	Protein	Dose	Protein	Dose	Protein	Dose
cPvMSP1-Pvs25	cPvMSP1-Pvs25	20 μg	cPvMSP1-Pvs25	20 μg	cPvMSP1-Pvs25	20 μg
Pvs25 protein	Pvs25 protein	20 μg	Pvs25 protein	20 μg	Pvs25 protein	20 μg
Adjuvant Control	Montanide ISA 51 VG		Montanide ISA 51 VG		Montanide ISA 51 VG	

CB6F1/J mice received subcutaneous immunizations at days 0, 20, and 40 with 20 μg of either cPvMSP1-Pvs25 or Pvs25, both emulsified at a 1:1 volume ratio with the adjuvant Montanide ISA 51 VG. An equivalent volume of PBS was emulsified at a 1:1 volume ratio for subcutaneous injections in the adjuvant control group at the same intervals.

were used. Fixed amounts of the monoclonal antibody N1-1H10 (1 μg) were tested with 2-fold dilutions of purified rabbit IgG using the recombinant Pvs25 protein as antigen. The concentration of polyclonal antibodies required for 50% inhibition of Pvs25-N1-1H10 interaction was then estimated using linear regression.

Indirect Immunofluorescence Assays (IFA)

Sera obtained from 10 C5B6F1/J mice after the third immunization with cPvMSP1-Pvs25 were pooled, and antibody reactivity against native *P. vivax* PvMSP1 protein was evaluated by indirect immunofluorescence. For assessment of antibody reactivity, an aliquot of blood was collected from a *P. vivax* infected *Saimiri boliviensis* monkey (kindly provided by Dr. Mary Galinski) into CPD tubes and washed twice using RPMI 1640 medium before the cells were adjusted to 1% hematocrit. Ten microliters of the cell suspension were added to wells of 12-well slides (ICN Biomedicals Inc) and air-dried before storage at −20°C. To evaluate reactivity, parasites slides were air-dried at RT for 30 min. Afterward, slides were incubated 90 min with mouse sera, diluted at 1:500 in PBS with 0.2% BSA in a dark, moist chamber. After the incubation, slides were washed 3 times with PBS containing Tween 20 (PBS-T), to minimize non-specific binding. Parasites were stained for 30 min at RT in a dark, moist chamber with goat anti-mouse Alexa Fluor 488 (Invitrogen) at a 1:500 dilution in Evans Blue 0.4% in PBS 1X. After staining, microscope slides were washed 3 times and allowed to dry completely. Parasite nuclei were stained using 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) included in the anti-fade mounting medium ProLong Gold (Life Technologies).

For assessment of reactivity to Pvs25, young oocysts were derived *in vitro* as described (Janse et al., 1985) and produced using the *P. berghei* transgenic parasite expressing Pvs25 (MRA-904, pv25DR, BEI Resources). Culture smears were stored at −80°C until need. Slides were allowed to air dry at room temperature for 30 min before being fixed for 10 min in 4% PFA/PBS. Slides were then washed 3 times with PBS 1X and blocked for 1 h in blocking buffer (10% v/v FBS, 1% w/v BSA in PBS). Slides were allowed to incubate overnight with sera from individual rabbits immunized with either Pvs25 or cPvMSP1-Pvs25 at 1:500 in 1% (w/v) BSA in PBS at 4°C in a wet chamber. The following day, slides were washed 3 times in PBS and then incubated with Alexa Fluor 488-conjugated goat anti-rabbit (H+L) IgG (ThermoFisher) at 1:500 in 0.4% Evans Blue in PBS 1X for 60 min. After washing 3 times, slides were allowed to dry completely (8 h) and mounted with ProLong Gold anti-fade reagent with DAPI (Life Technologies).

ELISpot Assays

Ninety-six-well plates were coated with 5 μg/ml Pvs25, cPvMSP1-Pvs25, and cPvMSP1 and blocked with complete RPMI (10% FBS, 1% penicillin/ streptavidin, 1% HEPES, and 50 μM 2-mercaptoethanol). Bone marrow and splenic cells were then isolated from the CB6FJ/1 mice 2 years post-immunization with Pvs25, cPvMSP1-Pvs25, or the Montanide adjuvant control. To isolate bone marrow cells from the mice, femurs were removed after the mice were euthanized following Emory IACUC approved procedures. Femurs were then placed in complete RPMI, and the ends of the bones were clipped off with sterile surgical scissors. The bone marrow was then flushed from the femur with RPMI using a syringe into a new sterile conical tube. The bone marrow was then passed through the syringe needle several times to resuspend the cells. Similarly, spleens were removed post-mortem and then mashed through a cell strainer using the plunger end of a syringe. Cells were washed and used immediately with no further processing. Cells were serially diluted on prepared plates and incubated for 16 h at 37°C. The plates were then treated with anti-IgG-biotin (Southern Biotechnology) followed by incubation with streptavidin-alkaline phosphatase (Sigma). Plates were then developed with 5-bromo-4-chloro-3-indolylphosphate (Sigma) until spots appeared, and spots counted with CTL ImmunoSpot software. Results were then normalized to adjuvant control mice.

Flow Cytometry Assays

Flow cytometry analyses of cPvMSP1-Pvs25 or Pvs25 specific T cells were conducted to simultaneously analyze IFN-γ at the single-cell level in T cells derived from splenocytes obtained 5 days after the final boosting immunization. Mice were euthanized according to the Emory IACUC approved protocols and spleens were removed. Spleens were transferred into complete media, composed of DMEM, 1% non-essential amino acids, 2 mM L-glutamine, 5% inactivated FBS, 50 μM 2-mercaptoethanol, 10 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin. Spleens were then homogenized under sterile conditions, and the homogenized fluid was passed through 200 μm nylon strainer to remove clumps and large pieces of

tissue. Red blood cells were lysed using 2 ml of BD PharmLyse buffer incubated with for 3 min before centrifugation at 400 g for 5 min and washing with 5 ml of flow cytometry buffer. Cells were counted and the concentration was adjusted to 10^7 cells/ml. 100 μl was then placed in individual round bottom tubes. Cells were stimulated for 6 h with peptide pools at 2 μg/ml at 37°C, in the presence of GolgiPlug (BD Biosciences). Cells were then incubated with Live/Dead Aqua Stain (Life Technologies) followed by surface staining with α-CD3 (PerCP Cy5.5), α-CD4 (Alexa Fluor 700), and α-CD8α (APC-Cy7) for 30 min. The cells were then fixed, permeabilized and stained with antibodies against IFN-γ (APC). All the monoclonal antibodies were obtained from BioLegend. Flow cytometry analyses were performed using an LSRII flow cytometer (BD Biosciences), and data were analyzed using FlowJo software version 10.1. The lymphocytes were initially gated on the Live/Dead channel, and then $CD3^+$, and then $CD4^+$ and $CD8^+$ populations. Antigen-specific cytokine-secreting T cells were identified within both the $CD4^+$ and $CD8^+$ populations. The frequency of antigen-specific cytokine-producing cells was determined by subtracting the percentage of cytokine-producing T cells after incubation with medium alone from the percentage of cytokine-producing T cells after incubation with the peptide pools. Samples that did not meet this requirement were set to zero.

Transmission-Blocking Assays

The transmission-blocking activity of sera derived from rabbits immunized with Pvs25 or cPvMSP1-Pvs25 was measured by direct membrane feeding assays as described elsewhere (Arevalo-Herrera et al., 2005, 2015; Vallejo et al., 2016). Briefly, 150 μl of infected RBCs from *P. vivax* infected patients were washed twice with RPMI 1640 medium (Sigma Aldrich) and diluted in a 150 μl of sera from rabbits that were not heat-inactivated obtained after three immunizations with 20 μg of cPvMSP1-Pvs25 or Pvs25 to feed 100 adults (2–3 day old) *An. albimanus* mosquitoes. Pre-immune sera from the same rabbits were used as a negative control. After 30 min of feeding, unfed mosquitoes were removed from the cages, and fed mosquitoes maintained at 27°C and 80–90% relative humidity. All procedures were performed at 37°C. Seven days after feeding, 30–40 mosquitoes were dissected, midguts were stained with 2% mercurochrome, and the numbers of oocysts per mosquito midgut were counted.

Statistical Analysis

Statistical analysis and graphs were made using GraphPad Prism 5.0 software (GraphPad Software Inc.). For analysis of the antibody responses, all ELISA titers were log-transformed to conform to the normality and variance requirements of parametric testing and compared using Student's *t*-test for comparison of antibody titers between groups. Student's *t*-test was used for the comparison of antibody avidity between groups. Mann Whitney was used for the comparison of antibody subclass ratios between immunization groups. Differences in the numbers of antibody secreting cells obtained via ELISpot were analyzed using the Mann-Whitney test to compare responses obtained from the Pvs25 and cPvMSP1-Pvs25 immunization groups. Levels of cytokine IFN-γ production obtained from flow cytometry were analyzed using unpaired *t*-tests to compare immunization groups. The transmission-blocking activity of the anti-cPvMSP1-Pvs25 or anti-Pvs25 sera compared against naïve sera or between the groups was analyzed using one-way ANOVA.

RESULTS

Design, Expression, and Characterization of the Chimeric Protein cPvMSP1-Pvs25

The chimeric PvMSP1 protein used to create a bifunctional vaccine construct by genetic fusion to the Pvs25 protein has previously been reported by our group (Fonseca et al., 2016). Briefly, the protein consists of five experimentally defined promiscuous T cell epitopes (Caro-Aguilar et al., 2002) derived from several regions along the native *P. vivax* MSP1. These epitopes are arrayed in tandem, interspaced with GPGPG spacers, and genetically fused to an extended form of the $PvMSP1_{19}$ kD fragment that contains two T helper epitopes derived from $MSP1_{33}$ kD fragment. Initial immunogenicity studies showed that this protein was able to elicit robust cytophilic antibody and $CD4^+$ and CD8+ T cell responses when delivered either alone as a recombinant protein formulated in water-in-oil emulsion using homologous prime-boost immunization regimens (Fonseca et al., 2016) or via heterologous prime-boost immunization regimens using recombinant adenoviral vectors for priming immunization (Fonseca et al., 2018). To test the feasibility of developing a hybrid vaccine with the potential of inducing blood and mosquito stage-specific immunity, we expressed cPvMSP1-Pvs25 and Pvs25 as recombinant proteins in *E. coli* (**Figures 1A,B**).

To confirm the biochemical identity of the cPvMSP1-Pvs25 protein, western blot analysis using monoclonal antibodies targeting specific regions of the cPvMSP1 and Pvs25 protein components was conducted (**Figure 1C**). The monoclonal antibody N1-1H10 was used for assessment of both the cPvMSP1-Pvs25 and recombinant Pvs25 proteins, as it recognizes a conformational-dependent epitope (Hisaeda et al., 2001) present in the second epidermal growth factor-like domain of Pvs25 (Saxena et al., 2006) and is able to inhibit oocyst development in the vector (Ramjanee et al., 2007). Anti-His tag antibodies were used to identify the His-tag present at the C terminal end of the Pvs25 protein segment of the cPvMSP1-Pvs25 protein and the recombinant Pvs25. The monoclonal antibody 2A10 targets the $(NANP)_n$ repeat region derived from *P. falciparum* circumsporozoite protein, which is present in the cPvMSP1-Pvs25 protein at the carboxyl-terminal end of the chimeric PvMSP1 protein. We observed recognition of the cPvMSP1-Pvs25 protein as a single band with the expected size of ∼52 kDa by the monoclonal antibody targeting the NANP repeats (2A10), the anti-His tag monoclonal antibody, and the monoclonal antibody targeting the Pvs25 protein (N1-1H10). As expected, we also observed binding of the anti-Pvs25 monoclonal antibody (N1-1H10) and the anti-His-tag antibody to the recombinant Pvs25 protein,

shown as a ∼25 kDa band. These experiments confirm that at least the epitopes of these monoclonal antibodies remained correctly folded.

To determine if the Pvs25 functional domains are conserved within cPvMSP1-Pvs25, we used ELISA competition assays (**Figure 1D**). Purified polyclonal antibodies elicited in rabbits by immunization with the recombinant proteins were tested at different concentrations and co-incubated with fixed amounts of the monoclonal antibody N1-1H10 (Ramjanee et al., 2007). The polyclonal antibodies inhibited the binding of N1-1H10 with a distinct competition pattern. The concentration of the polyclonal antibodies required for 50% inhibition of antigen-monoclonal antibody interaction was estimated in 32 μg for anti-Pvs25 and 13 μg for anti-cPvMSP1-Pvs25. These results suggest that antibodies elicited by immunization with the protein expressed in *E. coli* have transmission-blocking potential.

Antibody Response Induced by cPvMSP1-Pvs25

Hybrid CB6F1/J mice were immunized with Pvs25 or cPvMSP1-Pvs25 on days 0, 20, and 40 (**Table 2**). Sera were obtained 20 days after each immunization. When we assessed the anti-cPvMSP1-Pvs25 antibody titers at day 20 and 40, we observed significantly higher titers in the cPvMSP1-Pvs25 immunized group than in the Pvs25 group at both time points ($P = 0.0017$ and 0.0262, respectively, **Figure 2A**). At day 60, 20 days after the last immunization, mice immunized with cPvMSP1-Pvs25 had mean antibody titers of 3.4×10^6 against the chimeric recombinant protein, significantly higher ($P = 0.0414$) than those in mice immunized with Pvs25 which had mean antibody titers of 2.4×10^6 (**Figure 2B**). At day 730, 2 years after the first immunization, the group immunized with cPvMSP1-Pvs25 had mean antibody titers of 1.7×10^6 against cPvMSP1-Pvs25, a reduction of 26.8%. This observation is in sharp contrast with the group of mice immunized with Pvs25 that had mean antibody titers of 3.7×10^5 against cPvMSP1-Pvs25, a reduction of 89.2% ($P = 0.0107$, **Figure 2B**). The data suggest that unlike Pvs25 the antibody response induced by cPvMSP1-Pvs25 is long-lasting in mice.

To assess if antigenic competition occurs in mice immunized with the chimeric protein, antibody titers against the individual components of the bifunctional chimeric protein (Pvs25 and cPvMSP1) were also measured (**Figure 3**). The cPvMSP1-Pvs25 immunization group was able to recognize Pvs25 at day 60 with mean antibody titers of 2.4×10^6, which were similar to levels in mice immunized with Pvs25 ($P = 0.9617$) (**Figure 3A**). Assessment of anti-Pvs25 titers at day 730 in mice immunized with cPvMSP1-Pvs25 revealed a titers reduction of 47.2%, while the antibody titers in mice immunized with Pvs25 alone were reduced by 86.0%, a significant difference between the groups ($P = 0.0402$) (**Figure 3A**). As expected, mice immunized with Pvs25 alone did not produce antibodies against PvMSP1, showing only minimal reaction at the highest concentration of sera used. In contrast, mice immunized with cPvMSP1-Pvs25 had mean anti-PvMSP1 antibody titers of 6.6×10^6 at 20 days after the final immunization, and 1.0×10^6 two years later ($P = 0.0027$) (**Figure 3D**); the later titers at day 730, remained significantly higher than the Pvs25 immunized group, with a reduction of 84.8% ($P = 0.003$).

Characteristics of Anti-Pvs25 and Anti-MSP1 Antibodies

The average avidity index of anti-Pvs25 antibodies induced by immunization with cPvMSP1-Pvs25 at 20 days after the final immunization was significantly lower than that induced by immunization with Pvs25 (0.79 and 0.95, respectively, $P = 0.0038$) (**Figure 3B**). In contrast, by day 730 the respective values were 0.68 and 0.50 ($P = 0.0108$), indicating a significantly higher avidity index for antibodies induced by cPvMSP1-Pvs25 compared with Pvs25 alone.

The characteristics of the antibodies against chimeric PvMSP1 were only analyzed in the group immunized with cPvMSP1-Pvs25 since only these mice recognize this antigen. The average avidity index of the cPvMSP1-Pvs25 induced anti-MSP1 antibodies 20 days after the final immunization was 0.74 and remained similar after 2 years (0.71, $P = 0.6381$) (**Figure 3E**).

The IgG subclasses induced by vaccination with Pvs25 and cPvMSP1-Pvs25 was assessed in order to determine antibody quality, as cytophilic antibodies against MSP1, which correspond to IgG2a in mice, have previously been reported to be associated with protection (Stanisic et al., 2009). Measurement of the Pvs25-specific IgG2a and IgG1 subclasses, expressed as the IgG2a/IgG1 ratio, revealed a higher ratio for the cPvMSP1-Pvs25 immunized mice at both day 60 and day 730, however only at day 730 was the IgG2a/IgG1 subclass ratio of the cPvMSP1-Pvs25 group significantly higher than that of Pvs25 immunized mice ($P = 0.0131$) (**Figure 3C**). Assessment of the IgG subclass ratios for anti-PvMSP1 responses induced by vaccination with cPvMSP-Pvs25 revealed a different pattern, with the IgG2a/IgG1 ratio of the anti-MSP1 antibodies significantly higher at 60 days than at 730 days after the first immunization ($P = 0.0010$) (**Figure 3F**), showing a shift toward a Th2 phenotype over time.

Anti-cPvMSP1-Pvs25 Induced Antibodies Recognize the Native Parasite Proteins

The ability of the antibodies induced by vaccination with cPvMSP1-Pvs25 or Pvs25 to recognize the native antigens on the surface of oocysts and schizonts was assessed by immunofluorescence (**Figure 4**). Transgenic *P. berghei* parasites (MRA-904 parasites) expressing Pvs25 on the surface of young oocysts were used to assess the ability of antibodies from cPvMSP1-Pvs25 or Pvs25-immunized rabbits to recognize native Pvs25. As expected, sera from the cPvMSP1-Pvs25 or Pvs25-immunized rabbits were able to bind to the surface of *P. berghei* Pvs25 transgenic parasites (**Figures 4A,B**). Thin smears made from infected red blood cells from a *P. vivax* infected *Saimiri* monkey were used to assess the ability of antibodies from cPvMSP1-Pvs25 immunized mice to recognize

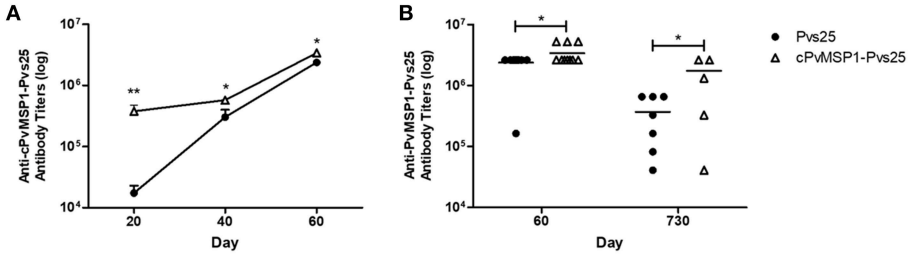


FIGURE 2 | Antibody responses to the chimeric PvMSP1-Pvs25 protein. Antibody responses against the chimeric PvMSP1-Pvs25 protein were assessed in CB6F1/J mice following immunization with either Pvs25 (closed circles) or cPvMSP1-Pvs25 (open triangles). **(A)** Time course of antibody responses to cPvMSP1-Pvs25 on days 20, 40, and 60 ($n = 10$). **(B)** Comparison of antibody titers between day 60 ($n = 10$) and at 2 years (730 days after the first immunization, $n = 7$). The titers against PvMSP1-Pvs25 are shown. Statistical analysis was conducted using Mann Whitney tests. Statistically significant differences are denoted by $^*p < 0.05$ and $^{**}p < 0.01$.

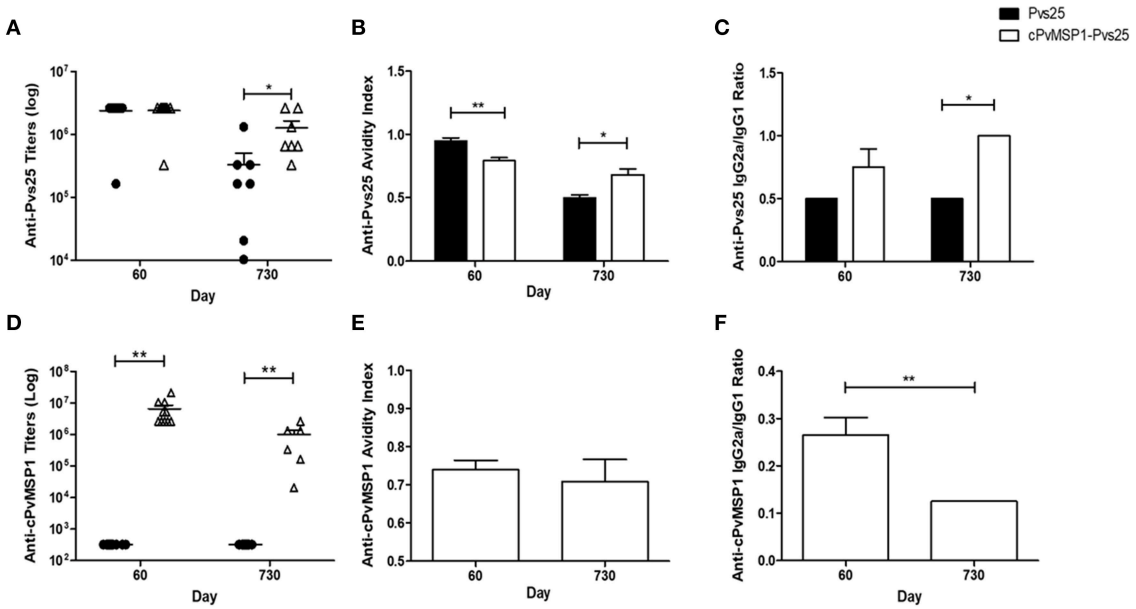


FIGURE 3 | Antibody responses to cPvMSP1 and Pvs25. Antibody responses against the recombinant cPvMSP1 and Pvs25 proteins that form the recombinant cPvMSP1-Pvs25 protein were assessed in CB6F1/J mice following immunization with either Pvs25 (closed circles or bars) or cPvMSP1-Pvs25 (open triangles or bars) on day 60 and 730 after the first immunization. The titers against Pvs25 and cPvMSP1 are shown in **(A,D)**, respectively. Results are presented for $n = 10$ mice for day 60 and $n = 7$ mice for day 730. Avidity indices for anti-Pvs25 and anti-cPvMSP1 are shown in **(B,E)**. IgG subclass responses, displayed as the IgG2a to IgG1 ratio, are shown in **(C)** for Pvs25 and **(F)** for cPvMSP1. Avidity indices and subclass results are presented as the mean values obtained from sera pooled from the 10 mice at day 60 or the 7 surviving mice at day 730, responses were averaged from four technical replicates. Statistical analysis was conducted using unpaired t-tests. Statistically significant differences are denoted by $^*p < 0.05$ and $^{**}p < 0.01$.

the native PvMSP1 protein. Pooled sera from the cPvMSP1-Pvs25 immunized group obtained at day 60 recognized *P. vivax* schizonts (**Figure 4C**). Combined, these data indicate that chimeric cPvMSP1-Pvs25 immunization elicited antibodies capable of binding the native structure of Pvs25 and the native *P. vivax* MSP1.

cPvMSP1-Pvs25 Plasma Cell Induction

To confirm that the long-lasting antibody responses observed at 2 years post-immunization were related to the induction of long-lived plasma cells (LLPCs), antigen-specific IgG plasma cells were measured in CB6F1/J mice immunized with Pvs25, cPvMSP1-Pvs25, or Montanide via ELISPOT 2 years post-immunization. Mice immunized with cPvMSP1-Pvs25 generated a robust, long-lived IgG plasma cell response not only to the cPvMSP1-Pvs25 protein itself but also to Pvs25 and cPvMSP1 (**Figure 5**). This response lasts for the lifetime of the animal. The number of long-lived IgG plasma cells in the bone marrow specific to cPvMSP1-Pvs25 was significantly higher ($P = 0.0456$) in mice immunized with cPvMSP1-Pvs25 than those immunized with Pvs25 alone (**Figure 5A**). Furthermore, the group immunized with Pvs25 was unable to generate a plasma cell response significantly above that of the adjuvant-only control. Mice immunized with cPvMSP1-Pvs25 also generated significantly greater numbers of IgG ASCs specific to Pvs25 (**Figure 5B**), and PvMSP1 (**Figure 5C**) compared to mice immunized with Pvs25 ($P = 0.0022$ and

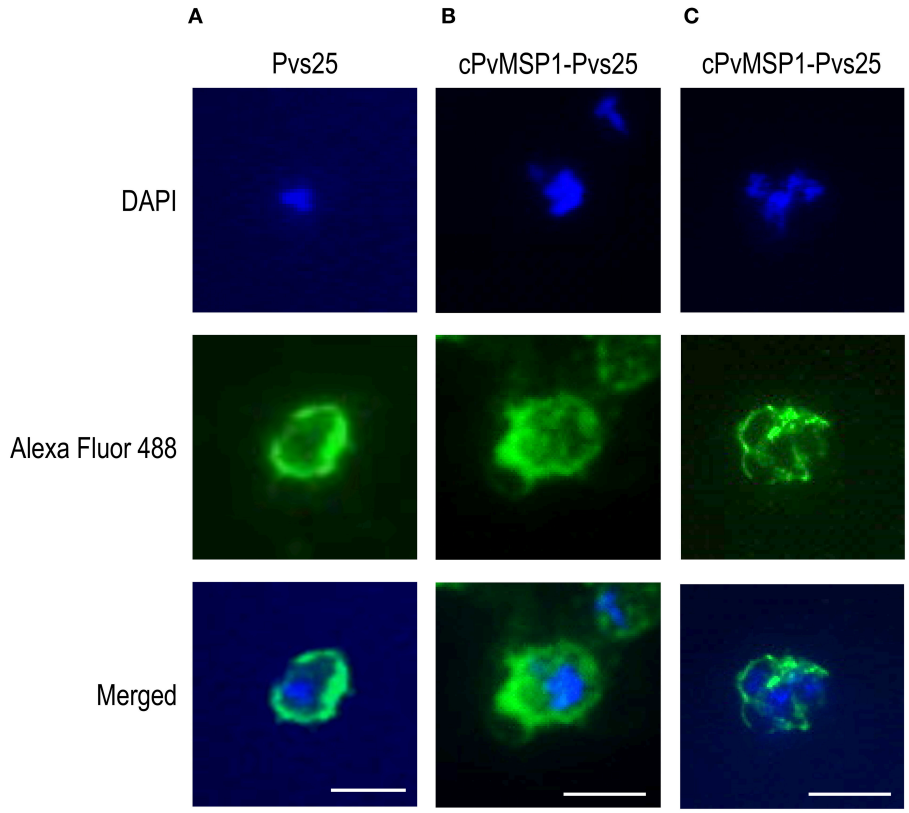


FIGURE 4 | Immunofluorescence assays of young oocysts and schizonts. Sera from individual rabbits immunized with either Pvs25 or cPvMSP1-Pvs25 was used to assess reactivity against *in vitro* derived oocysts using the *P. berghei* transgenic parasite expressing Pvs25 **(A, B)**. A pool of sera obtained from CB6F1/J mice 20 days after the final immunization with cPvMSP1-Pvs25 was used to assess reactivity against blood-stage *P. vivax* parasites **(C)**. The upper panels show staining with DAPI, the middle panels show staining with either goat-anti-rabbit or goat-anti-mouse IgG (H+L) Alexa Fluor 488 secondary antibody, and the bottom panels show the merged images. All images are shown at 100x magnification: scale bars, 5 μm.

$P = 0.0092$, respectively). This increase in long-lived IgG plasma cells was most pronounced in the bone marrow, though antigen-specific IgG plasma cells persist in the spleen at lower numbers. Overall, immunization with cPvMSP1-Pvs25 was more efficient, as it was able to stimulate long-lived, *Plasmodium*-specific IgG plasma cells when compared to standard Pvs25 immunization.

Cellular Response Induced by cPvMSP1-Pvs25

Following the assessment of the humoral response induced by vaccination with cPvMSP1-Pvs25 as compared to Pvs25, we sought to determine if cPvMSP1-Pvs25 was able to induce a cellular response able to recognize individual components of the bifunctional chimeric protein. CB6F1/J mice were immunized 3 times with 20 μg of either the chimeric cPvMSP1-Pvs25 or the recombinant Pvs25 on day 0 and 20, as described in **Table 2**. Mice were euthanized 5 days after the second immunization and splenocytes were stimulated with peptide pools representing the recombinant Pvs25 and cPvMSP1 proteins (**Table 1**) to analyze the production of IFN-γ by both $CD4^+$ and $CD8^+$ T cells (**Figure 6**). A sample gating strategy is shown in **Supplementary Figure 2**. We observed no difference in the production of IFN-γ between the immunization groups in either the $CD4^+$ or $CD8^+$ T cell populations in response to stimulation with the Pvs25 peptide pools (**Figures 6A,B**). Following stimulation with Pool 1 of cPvMSP1, we found that $CD4^+$ T cells from mice immunized with cPvMSP1-Pvs25 produced significantly higher levels of IFN-γ than those immunized with Pvs25 ($P = 0.0010$) (**Figure 6C**). Similarly, we found that following stimulation with Pool 2 of PvMSP1, both $CD4^+$ and $CD8^+$ T cells from mice immunized with the cPvMSP1-Pvs25 protein produced significantly more IFN-γ than the Pvs25 immunization group ($P = 0.0043$ for $CD4^+$, $P = 0.0138$ for $CD8^+$, **Figures 6C,D**).

Transmission-Blocking Activity of Anti-cPvMSP1-Pvs25 Antibodies

Sera samples, obtained from rabbits after three immunizations with either cPvMSP1-Pvs25 or Pvs25, were tested for transmission-blocking activity using three different *P. vivax* isolates in independent direct membrane feeding assays. The transmission blocking activity and the number of oocysts counted were assessed for both groups (**Table 3**). The activity of immune sera was compared to pre-immune control sera

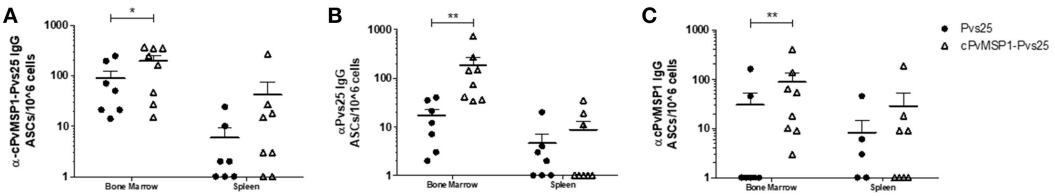


FIGURE 5 | IgG-producing long-lived plasma cells. Antigen-specific IgG plasma cells present in the bone marrow or spleen of CB6F1/J mice immunized with Pvs25 (closed circles, $n = 7$), cPvMSP1-Pvs25 (open triangles, $n = 8$) were analyzed at two years post first immunization. IgG long-lived plasma cells specific to **(A)** cPvMSP1-Pvs25, **(B)** Pvs25, and **(C)** cPvMSP1 are shown. Statistical analysis was conducted using Mann Whitney. Statistically significant differences are denoted by $^{*}p < 0.05$ and $^{**}p < 0.01$.

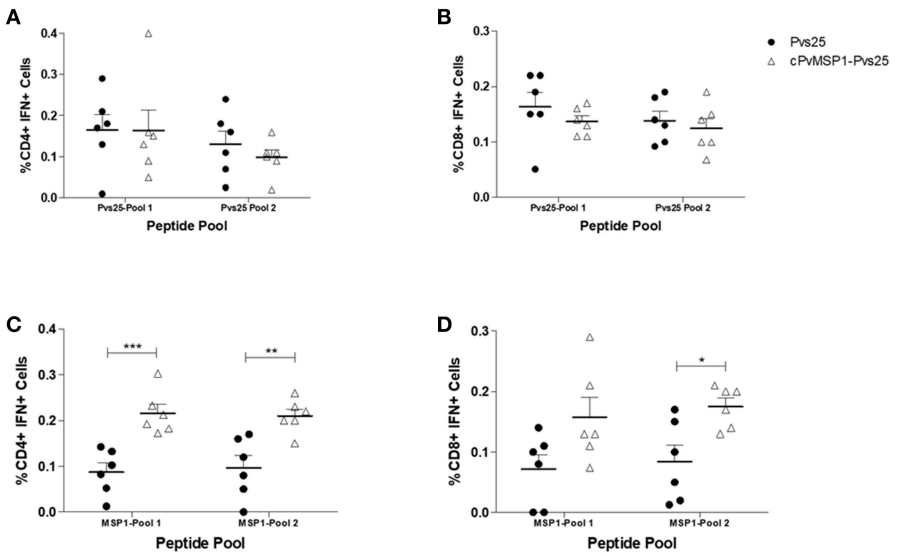


FIGURE 6 | IFN-γ production by $CD4^+$ and $CD8^+$ T cells following stimulation with Pvs25 and cPvMSP1 peptide pools. Splenocytes obtained from CB6F1/J mice immunized with either Pvs25 (closed circles, $n = 6$) or cPvMSP1-Pvs25 (open triangles, $n = 6$) 5 days after the final immunization were stimulated with peptide pools representing either MSP1 or Pvs25 at 2μg/ml for 2 h. Production of interferon-γ in response to stimulation with Pvs25 peptide pools by **(A)** $CD4^+$ T cells and **(B)** $CD8^+$ T cells in response to stimulation with cPvMSP1 peptide pools by **(C)** $CD4^+$ and **(D)** $CD8^+$ T cells are shown. Statistical analysis was conducted using unpaired *t*-tests. Statistically significant differences are denoted by $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

(**Table 3**). Upon dissection of mosquitoes, we found that sera obtained from both Pvs25 and cPvMSP1-Pvs25 resulted in significantly lower percentages of infected mosquitoes when compared to the pre-immune sera samples. Similarly, we found significantly lower numbers of oocysts present in the mosquitoes that had fed on the Pvs25 and cPvMSP1-Pvs25 samples as compared to the pre-immune rabbit sera controls. Overall, our results indicate that antibodies induced by cPvMSP1-Pvs25 are able to block the infection in 90% of exposed mosquitos, and in infected mosquitos, the parasite load is 98% lower than in mosquitoes feeding on a non-immunized source.

DISCUSSION

Development of novel malaria intervention tools, such as transmission-blocking vaccines (TBVs), is essential to address the increased reports of insecticide and drug resistance (Corbel et al., 2007; Dondorp et al., 2009), as they could increase the efficiency and sustainability of these existing malaria control methods (Sauerwein, 2007). TBVs rely on the generation of antibodies that block the development of *Plasmodium* parasites within the mosquito midgut. We propose that the ideal TBV formulation should consist of a transmission-blocking component combined with a prophylactic vaccine in order to simultaneously provide protection against disease to the recipient and reduce transmission. Under natural conditions, the human host is not exposed to the post-fertilization antigens. Since there would not be a natural boosting effect during infection, a vaccine targeting a post-fertilization antigen should be highly immunogenic to produce effective antibody responses (Saul, 2007). Here we present evidence that a chimeric protein designed based on one of the best-characterized post-fertilization antigens, P25, conjugated to a chimeric *P. vivax* MSP1 (cPvMSP1-Pvs25), elicits long-lasting antibody responses against both proteins, without immune interference, while inducing robust cellular responses to PvMSP1 in CB6F1/J, a F1 hybrid of BALB/c, and

TABLE 3 | Transmission-blocking activity.

Group	Sample	Antibody titers by ELISA	Experiment 1			Experiment 2			Experiment 3			*P*-value[e]
			No. infected (total dissected)[a]	Average number of oocyst[b]	TBA (%)[c]	No. infected (total dissected)[a]	Average number of oocyst[b]	TBA (%)[c]	No. infected (total dissected)[a]	Average number of oocyst[b]	TBA (%)[c]	
cPvMSP1-Pvs25	Control PI 1	640	20 (36)	155 ± 7.8	85.0	23 (32)	299 ± 13	65.2	10 (30)	29 ± 2.9	100	0.01
	Immune sera 1	1310720	3 (40)	26 ± 6.8		8 (32)	13 ± 1.6		7 (30)	0		
	Control PI 2	640	17 (40)	115 ± 6.8	88.2	23 (30)	262 ± 11.4	69.6	12 (30)	35 ± 2.9	100	0.01
	Immune sera 2	1310720	6 (33)	2 ± 1		7 (30)	29 ± 4.14		0 (30)	0		
Pvs25	Control PI 1	1280	23 (40)	175 ± 7.6	100	19 (30)	262 ± 13.7	63.2	11 (30)	30 ± 2.72	100	0.01
	Immune sera 1	1310720	0 (40)	0		7 (30)	34 ± 4.8		0 (30)	0		
	Immune sera 2[d]	1310720	3 (30)	3 ± 1	87.0	3 (30)	8 ± 2.5	84.2	0 (30)	0	100	0.01
Negative control	AB human serum	NA	20 (36)	657 ± 17.8	NA	23 (32)	413 ± 16.5	NA	18 (30)	320 ± 10.5	NA	

NA, not applicable.

[a]*Number of mosquitoes infected (total number of mosquitoes dissected).*

[b]*Average calculated as the total of oocyst /total of mosquitoes dissected ± standard deviation.*

[c]*Percent inhibition of mean mosquitoes with rabbit sera post-third immunization compared to pre-immune rabbit sera in each independent assay calculated as (1 – [mean mosquitoes in normal rabbit sera/mean mosquitoes post 3rd immunization sera] x 100). A pool of AB normal human sera was used as a negative control.*

[d]*Pre-immune serum from this rabbit was not tested for TBA, antibody titer was 1280.*

[e]*Statistical significance P < 0.05.*

Sera samples from immunized rabbits (cPvMSP1-Pvs25 and Pvs25) were tested in direct membrane-feeding assays (three different experiments) using blood samples collected from naturally infected patients as described (Vallejo et al., 2016).

C57BL/6 mice, comparable to those we have previously reported for cPvMSP1 alone in the parental strains (Fonseca et al., 2016).

Assessment of the quality and longevity of the antibody response induced revealed that immunization with cPvMSP1-Pvs25, as compared to Pvs25, resulted in the induction of an antibody response against the immunogens as well as against the individual components, which lasted for the lifetime of the animal with a much lower reduction in antibody titers at 2 years post-immunization. Most critically, we observed a lower reduction in anti-Pvs25 antibody titers at 2 years post-immunization in groups of mice immunized with the bifunctional chimeric protein as compared to the Pvs25 group. These results are significant as an ideal transmission-blocking vaccine candidate must be capable of inducing functional and durable antibodies using a simplified immunization regimen for use in mass administration (Nunes et al., 2014).

For the proof-of-concept studies reported here, we selected Montanide ISA 51 VG, a water-in-oil based adjuvant (Aucouturier et al., 2002) that has been tested before in clinical trials (Baumgaertner et al., 2016; Caballero et al., 2017; Saavedra and Crombet, 2017). Our results are consistent with the reported high efficiency of this adjuvant to induce robust antibody responses given its ability to promote polarization of naïve T cells into T follicular helper cells (Riteau et al., 2016). However, safety concerns have arisen from a Phase I clinical trial of Pfs25 and Pvs25 formulated in Montanide 51 showing systemic adverse events compatible with erythema nodosum (Wu et al., 2008). Recent comparisons of *P. falciparum* Pfs25 formulated in novel oil-in-water based adjuvants compared with alum-based adjuvants found that oil-in-water-based adjuvants EM081 and EM082 were more efficient in eliciting high titers of anti-Pfs25 IgG antibodies than the alum adjuvants (Patra et al., 2015). Interestingly, this study also found that an adjuvant composed of oil-in-water-glucopyranosyl lipid A-induced high-affinity antibodies that effectively blocked infection of mosquitoes with *P. falciparum* and demonstrated that avidity could provide a surrogate measure of efficacy beyond the antibody titers (Patra et al., 2015). The strong adjuvant effect was also confirmed by a Phase I clinical trial of a Pfs25 vaccine candidate paired with the aluminum adjuvant Alhydrogel, which observed a rapid decline in antibody responses after vaccination (Talaat et al., 2016). Overall, these experiments corroborate the adjuvant-dependency on the magnitude and durability of the antibody response of P25-based vaccines (Mlambo et al., 2008; Radtke et al., 2017). The long-lasting immunity induced by cPvMSP1-Pvs25 is therefore very encouraging compared to previous studies and warrant further investigation regarding the best adjuvant system to deliver the bifunctional chimeric protein.

Due to the importance of long-lived plasma cells (LLPC) in maintaining protective antibody levels for years (Radtke et al., 2017), LLPC induced by vaccination with cPvMSP1-Pvs25 were assessed. We were able to confirm high levels of bone marrow and spleen LLPCs at 2 years post-immunization. We observed that immunization with cPvMSP1-Pvs25 was more efficient at generating long-lived *Plasmodium*-specific IgG plasma cells when compared to Pvs25, with significantly more Pvs25-specific

LLPCs also observed in the bone marrow. This is in contrast with the poor immunogenicity reported for the native P25 (Tomas et al., 2001; Radtke et al., 2017). Chemical conjugation to carrier proteins, a strategy used for glycoconjugate vaccines to enhance the immunogenicity of bacterial polysaccharides (Rappuoli, 2018), has been tested to improve the immune responses induced by post-fertilization antigens. Clinical trials of the conjugated Pfs25-exoprotein A (EPA) vaccine showed excellent safety profile, but the antibody responses induced by immunization were short lasting with poor responses elicited in volunteers living in endemic areas compared to non-endemic areas (Talaat et al., 2016; Sagara et al., 2018). More recently, chemical conjugation of Pfs25 to tetanus toxoid (TT), a carrier protein used in existing polysaccharide vaccines, resulted in a significant improvement in immunogenicity and longevity (Radtke et al., 2017). TT contains CD4+ T cell epitopes that enhance humoral immunity compensating for the lack of CD4 T cells epitopes within Pfs25 resulting in the generation of T follicular helper (Tfh) cells (Radtke et al., 2017). Tfh cells are required for germinal center B cell differentiation into LLPC essential for long-lasting protection. Similar findings were subsequently reported by Parzych et al. with the *P. falciparum* merozoite surface protein 8 (MSP8) tested as a carrier to deliver Pfs25 (Parzych et al., 2017). These reports highlighted the relevance of T cell epitopes present in the carrier proteins to promote robust immune responses. The likely mechanism of action of the cPvMSP1 within cPvMSP1-Pvs25 is to serve as a carrier protein to the recruitment of *Plasmodium*-specific CD4+ T cells, which ultimately promote a robust humoral immune response to the hybrid vaccine and induction of the anti-Pvs25 LLPC response.

Avidity of the antibodies induced by vaccination with either Pvs25 or cPvMSP1-Pvs25 was also assessed as a measure of immunogenicity. At 20 days after the final immunization, anti-Pvs25 antibody avidity indices were 0.95 for Pvs25 and 0.79 for cPvMSP1-Pvs25 immunization groups. By 2 years post-immunization, the anti-Pvs25 avidity index of the Pvs25 group had fallen to 0.50, significantly lower than the avidity index of 0.68 at 2 years induced by immunization with cPvMSP1-Pvs25. Consistent with the maintenance of antibody avidity against Pvs25 by the cPvMSP1-Pvs25 immunization regimen, the avidity indices of the anti-cPvMSP1 response were not significantly different at 60 days than at 2 years post-immunization. The high avidity antibodies elicited by cPvMSP1-Pvs25 are, therefore, encouraging given their relevance as a biomarker of efficacy of TBVs beyond the antibody titers (Patra et al., 2015). We can also conclude that this outcome is likely an effect of the improved LLPC response induced by the cPvMSP1-Pvs25 vaccination regimen, allowing for the maintenance of higher avidity antibodies over time.

Cytophilic IgG antibody subclass responses directed against $MSP1_{19}$, as well as other merozoite surface antigens, have previously been found to be associated with control of parasitemia and protection from symptomatic illness in children in *P. falciparum* endemic areas (Stanisic et al., 2009). The cytophilic antibody subclasses correspond to IgG1 and IgG3 in humans and IgG2a in mice. We observed a shift in the IgG2a/IgG1 ratio of the anti-cPvMSP1 antibody response between day 60 and 2 years post-prime, with a significant reduction in the IgG2a subclass response. In contrast, there was a significantly higher IgG2a/IgG1 subclass ratio for anti-Pvs25 antibodies in cPvMSP1-Pvs25 immunized mice compared to Pvs25 immunized mice at the 2-year time point. Although we observed higher levels of IgG1 than IgG2a in the anti-cPvMSP1 response, the significantly higher IgG2a/IgG1 ratio observed for the anti-Pvs25 response elicited by cPvMSP1-Pvs25 vaccination is promising and may have biological significance due to differences in the ability of IgG subclasses to activate the classical complement pathway. Parasites present in the mosquito midgut after the female *Anopheles* mosquito ingests infected red blood cells are exposed to multiple plasma elements present in the mosquito blood meal including complement, granulocytes, and the host-derived antibodies (Sauerwein, 2007; Saul, 2007). These components can all affect parasite development within the mosquito and ultimately reduce transmission. Studies have shown that human sera with anti-Pvs25 antibodies have reduced killing activity after heat inactivation indicating that complement may be necessary to block transmission with Pvs25 vaccine-induced antibodies (Malkin et al., 2005). The classical complement pathway involves the binding of complement molecule C1q to the Fc region of the antigen bound antibody. However, IgG subclasses differ in their ability to activate complement, as human cytophilic antibodies IgG3 and IgG1 have been found to more effectively activate the classical complement pathway than the IgG2 subclass. This study suggests that cPvMSP1-Pvs25 immunization is more effective at inducing cytophilic IgG2a subclass antibodies in mice.

The ability of antibodies induced by individual components of the bifunctional chimeric protein in this study to bind the native structures expressed by young oocysts and schizonts is essential for their functional activities. We confirmed by IFAs that antibodies induced by immunization with cPvMSP1-Pvs25 recognized the native structure on the surface of transgenic oocysts expressing Pvs25 as well as the native structure of PvMSP1 in *P. vivax* blood stage schizonts. We have previously shown that passive immunization using antibodies elicited by the orthologous *P. yoelii* chimeric MSP1 protein, based on sequences from the *P. yoelii* 17X strain, protect naïve mice against heterologous challenge with the *P. yoelii nigeriensis* N67 isolates (Singh et al., 2010). Although we were unable to repeat similar passive transfer experiments in this study, future studies using transgenic parasites expressing *P. vivax* MSP1 are warranted. Additionally, the binding capability of anti-cPvMSP1-Pvs25 antibodies and the robust transmission blocking activity elicited by immunization as determined by direct membrane feeding assays, support further studies on the functionality of the anti-Pvs25 and anti-cPvMSP1 antibodies induced by vaccination with cPvMSP1-Pvs25. Furthermore, while no differences in the IFN-γ response were observed between the immunization groups following stimulation with the Pvs25 peptide pools, as expected, stimulation with cPvMSP1 peptide pools revealed that cPvMSP1-Pvs25 induced high levels of IFN-γ $CD4^+$ and $CD8^+$ T cells as compared to Pvs25-immunized mice. The ability of cPvMSP1-Pvs25 to maintain robust IFN-γ responses to cPvMSP1 when

conjugated to the Pvs25 protein is encouraging for the blood stage component of this vaccine candidate, as several reports have indicated that high frequencies of IFN-γ secreting $CD4^+$ T cells provide protection from malaria in humans (Roestenberg et al., 2009; White et al., 2013; King and Lamb, 2015).

Direct membrane feeding assays allow the assessment of transmission-blocking activity to determine the functionality of antibodies elicited by vaccination (Bompard et al., 2017). Currently, transmission-blocking assays are considered the most epidemiologically important method of evaluating transmission blocking vaccine candidates (Bompard et al., 2017). We found that sera obtained from both Pvs25 and cPvMSP1-Pvs25-immunized rabbits had significantly high transmission blocking activity, as shown by the lower percentages of infected mosquitoes and the number of oocysts compared with pre-immune sera. Unlike *P. falciparum* membrane feeding experiments, which can be done from parasite cultures, these results are promising as they came from wild *P. vivax* isolates, allowing for better prediction of the transmission blocking potential these antibodies might achieve if tested in clinical trials in endemic areas. These results support further development of cPvMSP1-Pvs25 as an effective transmission-blocking vaccine.

The development of licensed vaccines that reduce malaria transmission of *P. falciparum* and *P. vivax* is one of two main strategic goals of the 2030 Strategic Goals of Malaria Vaccine Technology published by the WHO; the other is a malaria vaccine with at least 75% efficacy against clinical malaria (Moorthy et al., 2013). While there is controversy concerning the use of transmission-blocking vaccines, including the fact that transmission-blocking antigens alone will only reduce transmission but will not protect vaccinated populations from disease (Jamrozik et al., 2015), the combination of transmission blocking antigens with antigens targeting the clinical stages of malaria could be used to prevent both disease and transmission. Studies evaluating the impact of vector control measures have demonstrated that the reduction of transmission in medium to high transmission areas induces a decrease in all-cause mortality, with the youngest age groups of the population benefiting the most (Smith et al., 2001; Rowe and Steketee, 2007; Larsen et al., 2011). We report here that the addition of an anti-erythrocytic stage antigen in the form of the chimeric PvMSP1 protein genetically conjugated to Pvs25 improves the immunogenicity of the TBV candidate while preserving the functionality of Pvs25 induced antibodies. Our results provide support for the continued development of cPvMSP1-Pvs25 and other multi-stage malaria vaccine candidates to address the need for effective vaccines targeting both the parasite stage responsible for the clinical manifestations and simultaneously the sexual stage responsible of transmission.

AUTHOR CONTRIBUTIONS

AM conceived the study. JF, BS, JJ, MA-H, and AM designed the experiments. JM, JF, BS, MA-H, MC-M, and CB performed the experiments. JM, JF, and AM performed data analysis. JM, JF, and AM wrote the manuscript. All the authors approved the final version of the manuscript.

FUNDING

This research was supported by the National Institutes of Health, NIAID grants R56-AI103382-01A1, R21 AI094402-01A1, and R21AI135711-01. This project was funded in part by ORIP/OD P51OD011132.

ACKNOWLEDGMENTS

The following reagents were obtained through BEI Resources Repository, NIAID, NIH: *Plasmodium berghei*, Strain pv25DR (15cy1Pb25-/PD28-), MRA-904, contributed by Robert Sinden, and Hybridoma N1-1H10 anti-*Plasmodium vivax* Ookinete Surface Protein 25 (Pvs25), MRA-471 contributed by Carole A. Long. The authors would like to thank Dr. Oskar Laur for the cloning of the hybrid *cPvMSP1-Pvs25* protein gene, Kelly Rubiano and Andres Amado from Caucaseco Scientific Research Center at Cali, Colombia for supervision of *P. vivax* transmission blocking assays and Dr. Mary Galinski for providing our group with *P. vivax* infected *Saimiri boliviensis* blood aliquot for IFA slides.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2019.00135/full#supplementary-material

Supplementary Figure 1 | A full scan of the entire original Western blot analysis of the purified cPvMSP1-Pvs25 **(left)** and the purified Pvs25 proteins **(right)**. Samples were incubated with the specified antibodies: monoclonal antibody N1-1H10 which targets Pvs25; anti-His-Tag monoclonal antibody targeting the C terminal tags of the cPvMSP1-Pvs25 and Pvs25 proteins; or the monoclonal antibody 2A10 which targets the cPvMSP1 C terminal tag. The molecular weight markers (BioRad) are included.

Supplementary Figure 2 | Gating strategy for $CD4^+$ and $CD8^+$ IFN-γ-producing murine T cells. The gating strategy for identifying IFN-γ $CD4^+$ and $CD8^+$ T cells from murine splenocytes isolated 5 days post boosting includes: FSC singlets, viability gating, $CD3^+$ cells, $CD4^+$ vs. $CD8^+$ T cells, and $IFN\gamma^+$ cells. Data shown represent IFN-γ gating in $CD4^+$ T cells of mice receiving Pvs25 or cPvMSP1-Pvs25.

REFERENCES

Arevalo-Herrera, M., Solarte, Y., Zamora, F., Mendez, F., Yasnot, M. F., Rocha, L., et al. (2005). *Plasmodium vivax*: transmission-blocking immunity in a malaria-endemic area of Colombia. *Am. J. Trop. Med. Hyg.* 73, 38–43. doi: 10.4269/ajtmh.2005.73.5_suppl.0730038

Arevalo-Herrera, M., Vallejo, A. F., Rubiano, K., Solarte, Y., Marin, C., Castellanos, A., et al. (2015). Recombinant Pvs48/45 antigen expressed in E. coli generates antibodies that block malaria transmission in *Anopheles albimanus* mosquitoes. *PLoS ONE* 10:e0119335. doi: 10.1371/journal.pone.0119335

Arrighi, R. B., and Hurd, H. (2002). The role of *Plasmodium berghei* ookinete proteins in binding to basal lamina components and transformation into oocysts. *Int. J. Parasitol.* 32, 91–98. doi: 10.1016/S0020-7519(01)00298-3

Aucouturier, J., Dupuis, L., Deville, S., Ascarateil, S., and Ganne, V. (2002). Montanide ISA 720 and 51: a new generation of water in oil emulsions as adjuvants for human vaccines. *Expert Rev. Vaccines* 1, 111–118. doi: 10.1586/14760584.1.1.111

Battle, K. E., Gething, P. W., Elyazar, I. R., Moyes, C. L., Sinka, M. E., Howes, R. E., et al. (2012). The global public health significance of *Plasmodium vivax*. *Adv. Parasitol.* 80, 1–111. doi: 10.1016/B978-0-12-397900-1.00001-3

Baumgaertner, P., Costa Nunes, C., Cachot, A., Maby-El Hajjami, H., Cagnon, L., Braun, M., et al. (2016). Vaccination of stage III/IV melanoma patients with long NY-ESO-1 peptide and CpG-B elicits robust CD8(+) and CD4(+) T-cell responses with multiple specificities including a novel DR7-restricted epitope. *Oncoimmunology* 5, e1216290–e1216290. doi: 10.1080/2162402X.2016.1216290

Blagborough, A. M., Musiychuk, K., Bi, H., Jones, R. M., Chichester, J. A., Streatfield, S., et al. (2016). Transmission blocking potency and immunogenicity of a plant-produced Pvs25-based subunit vaccine against *Plasmodium vivax*. *Vaccine* 34, 3252–3259. doi: 10.1016/j.vaccine.2016.05.007

Bockarie, M. J., and Dagoro, H. (2006). Are insecticide-treated bednets more protective against *Plasmodium falciparum* than *Plasmodium vivax*-infected mosquitoes? *Malar. J.* 5:15. doi: 10.1186/1475-2875-5-15

Bompard, A., Da, D. F., Yerbanga, R. S., Biswas, S., Kapulu, M., Bousema, T., et al. (2017). Evaluation of two lead malaria transmission blocking vaccine candidate antibodies in natural parasite-vector combinations. *Sci. Rep.* 7:6766. doi: 10.1038/s41598-017-06130-1

Caballero, I., Aira, L. E., Lavastida, A., Popa, X., Rivero, J., González, J., et al. (2017). Safety and immunogenicity of a human epidermal growth factor receptor 1 (HER1)-based vaccine in prostate castration-resistant carcinoma patients: a dose-escalation phase I study trial. *Front. Pharmacol.* 8:263. doi: 10.3389/fphar.2017.00263

Caro-Aguilar, I., Rodriguez, A., Calvo-Calle, J. M., Guzman, F., De la Vega, P., Patarroyo, M. E., et al. (2002). *Plasmodium vivax* promiscuous T-helper epitopes defined and evaluated as linear peptide chimera immunogens. *Infect. Immun.* 70, 3479–3492. doi: 10.1128/IAI.70.7.3479-3492.2002

Corbel, V., N'Guessan, R., Brengues, C., Chandre, F., Djogbenou, L., Martin, T., et al. (2007). Multiple insecticide resistance mechanisms in *Anopheles gambiae* and *Culex quinquefasciatus* from Benin, West Africa. *Acta Trop.* 101, 207–216. doi: 10.1016/j.actatropica.2007.01.005

Dondorp, A. M., Nosten, F., Yi, P., Das, D., Phyo, A. P., Tarning, J., et al. (2009). Artemisinin resistance in *Plasmodium falciparum* malaria. *N. Engl. J. Med.* 361, 455–467. doi: 10.1056/NEJMoa0808859

Fonseca, J. A., Cabrera-Mora, M., Singh, B., Oliveira-Ferreira, J., da Costa Lima-Junior, J., Calvo-Calle, J. M., et al. (2016). A chimeric protein-based malaria vaccine candidate induces robust T cell responses against *Plasmodium vivax* MSP119. *Sci. Rep.* 6:34527. doi: 10.1038/srep34527

Fonseca, J. A., McCaffery, J. N., Caceres, J., Kashentseva, E., Singh, B., Dmitriev, I. P., et al. (2018). Inclusion of the murine IgGkappa signal peptide increases the cellular immunogenicity of a simian adenoviral vectored *Plasmodium vivax* multistage vaccine. *Vaccine* 36, 2799–2808. doi: 10.1016/j.vaccine.2018.03.091

Gass, R. F., and Yeates, R. A. (1979). *In vitro* damage of cultured ookinetes of *Plasmodium gallinaceum* by digestive proteinases from susceptible *Aedes aegypti*. *Acta Trop.* 36, 243–252.

Gething, P. W., Elyazar, I. R., Moyes, C. L., Smith, D. L., Battle, K. E., Guerra, C. A., et al. (2012). A long neglected world malaria map: *Plasmodium vivax* endemicity in 2010. *PLoS Negl. Trop. Dis.* 6:e1814. doi: 10.1371/journal.pntd.0001814

Guerra, C. A., Howes, R. E., Patil, A. P., Gething, P. W., Van Boeckel, T. P., Temperley, W. H., et al. (2010). The international limits and population at risk of *Plasmodium vivax* Transmission in 2009. *Plos. Neglect. Trop. D* 4:e774. doi: 10.1371/journal.pntd.0000774

Haldar, K., Bhattacharjee, S., and Safeukui, I. (2018). Drug resistance in plasmodium. *Nat. Rev. Microbiol.* 16, 156–170. doi: 10.1038/nrmicro.2017.161

Han, Y. S., Thompson, J., Kafatos, F. C., and Barillas-Mury, C. (2000). Molecular interactions between *Anopheles stephensi* midgut cells and *Plasmodium berghei*: the time bomb theory of ookinete invasion of mosquitoes. *EMBO J.* 19, 6030–6040. doi: 10.1093/emboj/19.22.6030

Hisaeda, H., Collins, W. E., Saul, A., and Stowers, A. W. (2001). Antibodies to *Plasmodium vivax* transmission-blocking vaccine candidate antigens Pvs25 and Pvs28 do not show synergism. *Vaccine* 20, 763–770. doi: 10.1016/S0264-410X(01)00402-9

Jamrozik, E., de la Fuente-Nunez, V., Reis, A., Ringwald, P., and Selgelid, M. J. (2015). Ethical aspects of malaria control and research. *Malar. J.* 14:518. doi: 10.1186/s12936-015-1042-3

Janse, C. J., Mons, B., Rouwenhorst, R. J., Van der Klooster, P.F., Overdulve, J. P., and Van der Kaay, H.J. (1985). *In vitro* formation of ookinetes and functional maturity of *Plasmodium berghei* gametocytes. *Parasitology* 91(Pt 1), 19–29. doi: 10.1017/S0031182000056481

King, T., and Lamb, T. (2015). Interferon-gamma: the Jekyll and Hyde of Malaria. *PLoS Pathog.* 11:e1005118. doi: 10.1371/journal.ppat.1005118

Krotoski, W. A., Garnham, P. C., Bray, R. S., Krotoski, D. M., Killick-Kendrick, R., Draper, C. C., et al. (1982). Observations on early and late post-sporozoite tissue stages in primate malaria. I. Discovery of a new latent form of *Plasmodium cynomolgi* (the hypnozoite), and failure to detect hepatic forms within the first 24 hours after infection. *Am. J. Trop. Med. Hyg.* 31, 24–35. doi: 10.4269/ajtmh.1982.31.24

Larsen, D. A., Friberg, I. K., and Eisele, T. P. (2011). Comparison of Lives Saved Tool model child mortality estimates against measured data from vector control studies in sub-Saharan Africa. *BMC Public Health* 11(Suppl. 3):S34. doi: 10.1186/1471-2458-11-S3-S34

Malkin, E. M., Durbin, A. P., Diemert, D. J., Sattabongkot, J., Wu, Y., Miura, K., et al. (2005). Phase 1 vaccine trial of Pvs25H: a transmission blocking vaccine for *Plasmodium vivax* malaria. *Vaccine* 23, 3131–3138. doi: 10.1016/j.vaccine.2004.12.019

Mendis, K., Rietveld, A., Warsame, M., Bosman, A., Greenwood, B., and Wernsdorfer, W. H. (2009). From malaria control to eradication: the WHO perspective. *Trop. Med. Int. Health* 14, 802–809. doi: 10.1111/j.1365-3156.2009.02287.x

Mlambo, G., Maciel, J., and Kumar, N. (2008). Murine model for assessment of *Plasmodium falciparum* transmission-blocking vaccine using transgenic *Plasmodium berghei* parasites expressing the target antigen Pfs25. *Infect. Immun.* 76, 2018–2024. doi: 10.1128/IAI.01409-07

Moorthy, V. S., Newman, R. D., and Okwo-Bele, J. M. (2013). Malaria vaccine technology roadmap. *Lancet* 382, 1700–1701. doi: 10.1016/S0140-6736(13)62238-2

Nunes, J. K., Woods, C., Carter, T., Raphael, T., Morin, M. J., Diallo, D., et al. (2014). Development of a transmission-blocking malaria vaccine: progress, challenges, and the path forward. *Vaccine* 32, 5531–5539. doi: 10.1016/j.vaccine.2014.07.030

Parzych, E. M., Miura, K., Ramanathan, A., Long, C. A., and Burns, J. M. Jr. (2017). Evaluation of a plasmodium-specific carrier protein to enhance production of recombinant Pfs25, a leading transmission-blocking vaccine candidate. *Infect. Immun.* 86:e00486-17. doi: 10.1128/IAI.00486-17

Patra, K. P., Li, F., Carter, D., Gregory, J. A., Baga, S., Reed, S. G., et al. (2015). Alga-produced malaria transmission-blocking vaccine candidate Pfs25 formulated with a human use-compatible potent adjuvant induces high-affinity antibodies that block *Plasmodium falciparum* infection of mosquitoes. *Infect. Immun.* 83, 1799–1808. doi: 10.1128/IAI.02980-14

Perciani, C. T., Peixoto, P. S., Dias, W. O., Kubrusly, F. S., and Tanizaki, M. M. (2007). Improved method to calculate the antibody avidity index. *J. Clin. Lab. Anal.* 21, 201–206. doi: 10.1002/jcla.20172

Qian, F., Wu, Y., Muratova, O., Zhou, H., Dobrescu, G., Duggan, P., et al. and G.Mullen, E. D. (2007). Conjugating recombinant proteins to *Pseudomonas aeruginosa* ExoProtein A: a strategy for enhancing immunogenicity of malaria vaccine candidates. *Vaccine* 25, 3923–3933. doi: 10.1016/j.vaccine.2007.02.073

Radtke, A. J., Anderson, C. F., Riteau, N., Rausch, K., Scaria, P., Kelnhofer, E. R., et al. (2017). Adjuvant and carrier protein-dependent T-cell priming promotes a robust antibody response against the *Plasmodium falciparum* Pfs25 vaccine candidate. *Sci. Rep.* 7:40312. doi: 10.1038/srep40312

Ramjanee, S., Robertson, J. S., Franke-Fayard, B., Sinha, R., Waters, A. P., Janse, C. J., et al. (2007). The use of transgenic *Plasmodium berghei* expressing the *Plasmodium vivax* antigen P25 to determine the transmission-blocking activity of sera from malaria vaccine trials. *Vaccine* 25, 886–894. doi: 10.1016/j.vaccine.2006.09.035

Rappuoli, R. (2018). Glycoconjugate vaccines: Principles and mechanisms. *Sci. Transl. Med.* 10:eaat4615. doi: 10.1126/scitranslmed.aat4615

Riteau, N., Radtke, A. J., Shenderov, K., Mittereder, L., Oland, S. D., Hieny, S., et al. (2016). Water-in-oil-only adjuvants selectively promote T follicular helper cell polarization through a type I IFN and IL-6-dependent pathway. *J. Immunol.* 197, 3884–3893. doi: 10.4049/jimmunol.1600883

Roestenberg, M., McCall, M., Hopman, J., Wiersma, J., Luty, A. J., van Gemert, G. J., et al. (2009). Protection against a malaria challenge by sporozoite inoculation. *N. Engl. J. Med.* 361, 468–477. doi: 10.1056/NEJMoa0805832

Rowe, A. K., and Steketee, R. W. (2007). Predictions of the impact of malaria control efforts on all-cause child mortality in sub-Saharan Africa. *Am. J. Trop. Med. Hyg.* 77, 48–55. doi: 10.4269/ajtmh.2007.77.48

Saavedra, D., and Crombet, T. (2017). CIMAvax-EGF: a new therapeutic vaccine for advanced non-small cell lung cancer patients. *Front. Immunol.* 8:269. doi: 10.3389/fimmu.2017.00269

Sagara, I., Healy, S. A., Assadou, M. H., Gabriel, E. E., Kone, M., Sissoko, K., et al. (2018). Safety and immunogenicity of Pfs25H-EPA/Alhydrogel, a transmission-blocking vaccine against *Plasmodium falciparum*: a randomised, double-blind, comparator-controlled, dose-escalation study in healthy Malian adults. *Lancet Infect. Dis.* 18, 969–982. doi: 10.1016/S1473-3099(18)30344-X

Sauerwein, R. W. (2007). Malaria transmission-blocking vaccines: the bonus of effective malaria control. *Microbes Infect.* 9, 792–795. doi: 10.1016/j.micinf.2007.02.011

Sauerwein, R. W., and Bousema, T. (2015). Transmission blocking malaria vaccines: assays and candidates in clinical development. *Vaccine* 33, 7476–7482. doi: 10.1016/j.vaccine.2015.08.073

Saul, A. (2007). Mosquito stage, transmission blocking vaccines for malaria. *Curr. Opin. Infect. Dis.* 20, 476–481. doi: 10.1097/QCO.0b013e3282a95e12

Saxena, A. K., Singh, K., Su, H. P., Klein, M. M., Stowers, A. W., Saul, A. J., et al. (2006). The essential mosquito-stage P25 and P28 proteins from Plasmodium form tile-like triangular prisms. *Nat. Struct. Mol. Biol.* 13, 90–91. doi: 10.1038/nsmb1024

Saxena, A. K., Wu, Y., and Garboczi, D. N. (2007). Plasmodium p25 and p28 surface proteins: potential transmission-blocking vaccines. *Eukaryotic Cell* 6, 1260–1265. doi: 10.1128/EC.00060-07

Singh, B., Cabrera-Mora, M., Jiang, J., Galinski, M., and Moreno, A. (2010). Genetic linkage of autologous T cell epitopes in a chimeric recombinant construct improves anti-parasite and anti-disease protective effect of a malaria vaccine candidate. *Vaccine* 28, 2580–2592. doi: 10.1016/j.vaccine.2010.01.019

Singh, S., Pandey, K., Chattopadhayay, R., Yazdani, S. S., Lynn, A., Bharadwaj, A., et al. (2001). Biochemical, biophysical, and functional characterization of bacterially expressed and refolded receptor binding domain of *Plasmodium vivax* duffy-binding protein. *J. Biol. Chem.* 276, 17111–17116. doi: 10.1074/jbc.M101531200

Smith, T. A., Leuenberger, R., and Lengeler, C. (2001). Child mortality and malaria transmission intensity in Africa. *Trends Parasitol.* 17, 145–149. doi: 10.1016/S1471-4922(00)01814-6

Stanisic, D. I., Richards, J. S., McCallum, F. J., Michon, P., King, C. L., Schoepflin, S., et al. (2009). Immunoglobulin G subclass-specific responses against *Plasmodium falciparum* merozoite antigens are associated with control of parasitemia and protection from symptomatic illness. *Infect. Immun.* 77, 1165–1174. doi: 10.1128/IAI.01129-08

Talaat, K. R., Ellis, R. D., Hurd, J., Hentrich, A., Gabriel, E., Hynes, N. A., et al. (2016). Safety and immunogenicity of Pfs25-EPA/alhydrogel(R), a transmission blocking vaccine against *Plasmodium falciparum*: an open label study in malaria naive adults. *PLoS ONE* 11:e0163144. doi: 10.1371/journal.pone.0163144

Thomas, D., Tazerouni, H., Sundararaj, K. G., and Cooper, J. C. (2016). Therapeutic failure of primaquine and need for new medicines in radical cure of *Plasmodium vivax*. *Acta Trop.* 160, 35–38. doi: 10.1016/j.actatropica.2016.04.009

Tomas, A. M., Margos, G., Dimopoulos, G., van Lin, L. H., de Koning-Ward, T.F., Sinha, R., et al. (2001). P25 and P28 proteins of the malaria ookinete surface have multiple and partially redundant functions. *EMBO J.* 20, 3975–3983. doi: 10.1093/emboj/20.15.3975

Tsuboi, T., Kaslow, D. C., Gozar, M. M., Tachibana, M., Cao, Y. M., and Torii, M. (1998). Sequence polymorphism in two novel *Plasmodium vivax* ookinete surface proteins, Pvs25 and Pvs28, that are malaria transmission-blocking vaccine candidates. *Mol. Med.* 4, 772–782. doi: 10.1007/BF03401770

Vallejo, A. F., Rubiano, K., Amado, A., Krystosik, A. R., Herrera, S., and Arevalo-Herrera, M. (2016). Optimization of a membrane feeding assay for *Plasmodium vivax* infection in *Anopheles albimanus*. *PLoS Negl. Trop. Dis.* 10:e0004807. doi: 10.1371/journal.pntd.0004807

Van den Eede, P., Soto-Calle, V. E., Delgado, C., Gamboa, D., Grande, T., Rodriguez, H., et al. (2011). *Plasmodium vivax* sub-patent infections after radical treatment are common in Peruvian patients: results of a 1-year prospective cohort study. *PLoS ONE* 6:e16257. doi: 10.1371/journal.pone.0016257

Vlachou, D., Lycett, G., Siden-Kiamos, I., Blass, C., Sinden, R. E., and Louis, C. (2001). *Anopheles gambiae* laminin interacts with the P25 surface protein of *Plasmodium berghei* ookinetes. *Mol. Biochem. Parasitol.* 112, 229–237. doi: 10.1016/S0166-6851(00)00371-6

Vlachou, D., Zimmermann, T., Cantera, R., Janse, C. J., Waters, A. P., and Kafatos, F. C. (2004). Real-time, *in vivo* analysis of malaria ookinete locomotion and mosquito midgut invasion. *Cell. Microbiol.* 6, 671–685. doi: 10.1111/j.1462-5822.2004.00394.x

White, M. T., Bejon, P., Olotu, A., Griffin, J. T., Riley, E. M., Kester, K. E., et al. (2013). The relationship between RTS,S vaccine-induced antibodies, CD4(+) T cell responses and protection against *Plasmodium falciparum* infection. *PLoS ONE* 8:e61395. doi: 10.1371/journal.pone.0061395

World Health Organization (2014). *World Malaria Report 2014*. Geneva: World Health Organization.

World Health Organization (2018). *World Malaria Report 2018*. (Geneva: W. H. Organization).

Wu, Y., Ellis, R. D., Shaffer, D., Fontes, E., Malkin, E. M., Mahanty, S., et al. (2008). Phase 1 trial of malaria transmission blocking vaccine candidates Pfs25 and Pvs25 formulated with montanide ISA 51. *PLoS ONE* 3:e2636. doi: 10.1371/journal.pone.0002636

Zieler, H., and Dvorak, J. A. (2000). Invasion *in vitro* of mosquito midgut cells by the malaria parasite proceeds by a conserved mechanism and results in death of the invaded midgut cells. *Proc. Natl. Acad. Sci. U.S.A.* 97, 11516–11521. doi: 10.1073/pnas.97.21.11516

Conflict of Interest Statement: 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 Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 19 June 2019
doi: 10.3389/fcimb.2019.00215

Chimeric Murine Polyomavirus Virus-Like Particles Induce *Plasmodium* Antigen-Specific CD8$^+$ T Cell and Antibody Responses

David J. Pattinson[1,2], Simon H. Apte[1], Nani Wibowo[3], Yap P. Chuan[3], Tania Rivera-Hernandez[3], Penny L. Groves[1], Linda H. Lua[4], Anton P. J. Middelberg[3] and Denise L. Doolan[1,2]**

[1] Infectious Diseases Programme, QIMR Berghofer Medical Research Institute, Brisbane, QLD, Australia, [2] Centre for Molecular Therapeutics, Australian Institute of Tropical Health and Medicine, James Cook University, Cairns, QLD, Australia, [3] Australian Institute for Bioengineering and Nanotechnology, University of Queensland, Brisbane, QLD, Australia, [4] Protein Expression Facility, University of Queensland, Brisbane, QLD, Australia

OPEN ACCESS

Edited by:
Alberto Moreno,
Emory University School of Medicine,
United States

Reviewed by:
Giampietro Corradin,
Université de Lausanne, Switzerland
Kai Huang,
University of Texas Medical Branch at Galveston, United States
Sean C. Murphy,
University of Washington,
United States

***Correspondence:**
David J. Pattinson
david.pattinson@jcu.edu.au
Denise L. Doolan
denise.doolan@jcu.edu.au

Specialty section:
This article was submitted to Clinical Microbiology, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: 20 February 2019
Accepted: 03 June 2019
Published: 19 June 2019

Citation:
Pattinson DJ, Apte SH, Wibowo N, Chuan YP, Rivera-Hernandez T, Groves PL, Lua LH, Middelberg APJ and Doolan DL (2019) Chimeric Murine Polyomavirus Virus-Like Particles Induce Plasmodium Antigen-Specific CD8$^+$ T Cell and Antibody Responses. Front. Cell. Infect. Microbiol. 9:215. doi: 10.3389/fcimb.2019.00215

An effective vaccine against the *Plasmodium* parasite is likely to require the induction of robust antibody and T cell responses. Chimeric virus-like particles are an effective vaccine platform for induction of antibody responses, but their capacity to induce robust cellular responses and cell-mediated protection against pathogen challenge has not been established. To evaluate this, we produced chimeric constructs using the murine polyomavirus structural protein with surface-exposed CD8$^+$ or CD4$^+$ T cell or B cell repeat epitopes derived from the *Plasmodium yoelii* circumsporozoite protein, and assessed immunogenicity and protective capacity in a murine model. Robust CD8$^+$ T cell responses were induced by immunization with the chimeric CD8$^+$ T cell epitope virus-like particles, however CD4$^+$ T cell responses were very low. The B cell chimeric construct induced robust antibody responses but there was no apparent synergy when T cell and B cell constructs were administered as a pool. A heterologous prime/boost regimen using plasmid DNA priming followed by a VLP boost was more effective than homologous VLP immunization for cellular immunity and protection. These data show that chimeric murine polyomavirus virus-like particles are a good platform for induction of CD8$^+$ T cell responses as well as antibody responses.

Keywords: malaria, vaccine, circumsporozoite protein, virus-like particle, murine polyomavirus, cellular immunity, T cell responses, *Plasmodium yoelii*

INTRODUCTION

Although annual malaria related mortality rates have decreased by approximately 25% since 2010, improvements in morbidity and mortality have stabilized in recent years (World Malaria Report, 2017) and rebounds have been reported in some countries (Alonso et al., 2011). This is despite an annual financial investment of approximately US$2.7 billion, which includes malarial preventative measures such as residual spraying, insecticide-treated mosquito nets and preventative therapies (World Malaria Report, 2017). An effective vaccine is considered by many to be an essential tool for malaria disease control and eradication (Alonso et al., 2011). The ideal vaccine would target the pre-erythrocytic stage to induce sterile infection-blocking immunity which either prevents sporozoite invasion of the hepatocyte and/or halts the development of the parasite at the liver-stage; this would stop the development

of clinical symptoms of malaria which manifest during the blood-stage, as well as the transmission of malaria which occurs during the sexual stage. A partially effective vaccine which reduced liver-stage parasite burden and thus lowered blood-stage parasitemia to potentially below a threshold associated with clinical disease would, however, also be a useful tool.

Recently, GlaxoSmithKline achieved a significant milestone with Mosquirix™ (also known as RTS,S) by receiving a positive scientific opinion from the European Medicines Agency as the first malaria vaccine for the immunization of children aged 6 weeks to 17 months. This sub-unit virus-like particle (VLP) based vaccine targets the *P. falciparum* circumsporozoite protein (*Pf* CSP) by combining a protein which includes multiple *Pf* CSP B cell repeats and T cell epitopes fused with recombinant hepatitis B surface antigen (RTS), and recombinant wild-type hepatitis B surface antigen (S) (Gordon et al., 1995). These recombinant proteins combine to form stable VLPs, which are co-administered with the AS01 adjuvant (Agnandji et al., 2012). Disappointingly, clinical studies have shown that although RTS,S provided some protection in the first year after vaccination, this efficacy was very low and waned quickly (Tinto et al., 2015; Olotu et al., 2016) with negative efficacy and a rebound in later years demonstrated in a long-term follow-up study where RTS,S immunized 5–17 month children were more likely to be infected 5 years following vaccination (Olotu et al., 2016). These data emphasize the urgent need to identify new vaccine targets and vaccine delivery platforms that enhance immunogenicity and provide durable protection.

The CSP remains the most advanced subunit vaccine candidate, as a target for neutralizing antibodies prior to liver infection (Charoenvit et al., 1991; Mishra et al., 2012) and for T cell responses whilst in the hepatocyte (Grillot et al., 1990; Weiss et al., 1990, 1992; Franke et al., 1997, 2000). The induction of T cell or antibody mediated protection against sporozoite challenge has been demonstrated in murine and non-human primate models as well as humans by various vaccine platforms based on the CSP (Sedegah et al., 1994; Walsh et al., 2006; Mettens et al., 2008; Tewari et al., 2010; Tamminga et al., 2011; Noe et al., 2014; Janitzek et al., 2016; Collins et al., 2017; Yoshida et al., 2018). Thus, the CSP is widely considered the antigen of choice for evaluating novel vaccine delivery platforms targeting the pre-erythrocytic stage of the parasite life cycle.

Whilst antibody responses generated by chimeric VLP immunizations using various VLP platforms with antigens from various pathogens in clinical studies are well-documented (reviewed in Kushnir et al., 2012), little research has been done to evaluate their efficacy at generating cellular responses. Chimeric VLPs using hamster and simian virus 40 polyomavirus structural proteins have shown antigen-specific cytotoxic T lymphocyte (CTL) responses with viral epitopes (Mazeike et al., 2012; Kawano et al., 2014), and a murine polyomavirus VLP with large HER-2_{1-683} oncogene insert mouse study showed rejection and inhibition of antigen expressing tumor cells *in vivo* (Tegerstedt et al., 2005). Furthermore, both HPV16 L1 VLPs and their subunit component capsomeres are potent inducers of CTL responses capable of tumor regression without adjuvants (Ohlschläger et al., 2003).

In this study, we produced chimeric VLPs using the murine polyomavirus (MuPyV) (Salunke et al., 1986) by genomic insertion of either *Plasmodium yoelii* CSP $CD8^+_{(280-288)}$ (Weiss et al., 1992) or $CD4^+_{(59-79)}$ (Grillot et al., 1990) T cell epitopes, or the B cell repeat $epitope_{(QGPGAPx2)}$ into a surface-exposed region of the VP1 structural protein (Middelberg et al., 2011). We selected the MuPyV-VP1 platform which has been extensively developed by our group, and can be produced in bacterial expression systems which can be purified at gram per liter levels (Liew et al., 2010). The proteins form pentameric capsomeres (Salunke et al., 1986) which can be chemically induced *in vitro* to self-assemble into VLPs (Chuan et al., 2008a; Middelberg et al., 2011; Rivera-Hernandez et al., 2013). We comprehensively evaluated antibody and T cell responses as well as protection from *Plasmodium yoelii* 17XNL sporozoite challenge using chimeric *Py*CSP surface exposed on murine polyomavirus VLP constructs delivered separately or pooled.

Consistent with previous results with the murine polyomavirus VLPs (Anggraeni et al., 2013; Rivera-Hernandez et al., 2013; Wibowo et al., 2014; Seth et al., 2016; Tekewe et al., 2017), the *Plasmodium* B cell VLP was a strong inducer of antigen-specific antibody responses. Additionally, we showed that the VLP platform was capable of inducing robust VLP-induced $CD8^+$ T cell responses. However, the VLPs did not stimulate a good $CD4^+$ T cell response, and there was no apparent help provided by the CD4 VLP to the VLP-induced $CD8^+$ T cell responses nor to the induced antibody titers. The VLP-induced $CD8^+$ T cell responses were however enhanced using a heterologous DNA prime-boost regimen.

MATERIALS AND METHODS

Plasmid Construction

The plasmid pGEX-4T-1 (GE Healthcare Biosciences, UK) containing the murine polyomavirus VP1 sequence (M34958) was provided by Professor Robert Garcea (University of Colorado, USA). The VP1 sequence was modified by inserting an *Afe*I restriction enzyme site flanked with $Glycine_4$-Serine linker sequences at position 293 and designated pGEX-VP1-S4-G4S (Middelberg et al., 2011). This allows for antigens to be inserted into a surface-exposed region of the VP1 protein with the linker sequences added to reduce structural interference from the insert on VP1 formation. Codon optimized sequences representing defined *Py*CSP $CD8^+_{(280-288)}$ (Weiss et al., 1992) and $CD4^+_{(59-79)}$ (Grillot et al., 1990) T cell epitopes and the B cell repeat epitope $_{(QGPGAPx2)}$ (Charoenvit et al., 1991) were separately inserted into the *Afe*I site in pGEX-VP1-S4-G4S using standard molecular biology techniques to generate chimeric $CD8^+$ or $CD4^+$ T cell or B cell VLP constructs. All constructs were confirmed by Sanger sequencing.

Protein Expression, Purification, and VLP Assembly

Wild-type pGEX-VP1-S4-G4S, or the chimeric $CD8^+$, $CD4^+$, or B cell constructs were individually transformed into chemically competent *E. coli* Rosetta DE3 pLysS bacteria (Novagen, CA,

USA). The GST-tagged VP1 proteins were expressed and purified as previously described (Chuan et al., 2008a; Middelberg et al., 2011). Briefly, bacteria inoculated Terrific Broth was incubated at 37°C and 180 RPM until the OD_{600} reached approximately 0.5, after which the culture was cooled to 26°C and induced by adding 0.2 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated overnight at 26°C and 180 RPM. The cultures were centrifuged and pellets lysed by sonication then filtered lysate was passed through a GSTrap HP affinity column (GE Healthcare, UK) and the purified protein was cleaved from the GST tag using thrombin (GE Healthcare, UK) and polished using a Superdex 200 10/300 GL column (GE Healthcare, UK). Endotoxin levels were reduced (<5 EU/ml) using Vivapure Q maxi H ion exchange columns (Sartorius Stedim, Germany) and confirmed with an Endosafe PTS reader (Charles River Laboratory, USA) as previously described (Middelberg et al., 2011). VP1 capsomeres were assembled into VLPs by dialysis against an assembly buffer then against PBS (Chuan et al., 2008a; Liew et al., 2010; Middelberg et al., 2011). The resultant VLPs were analyzed by asymmetric flow field-flow fractionation coupled to multi angle light scattering (AF4-MALS) using an Eclipse 2 AFFFF system coupled with a Dawn EOS MALS system (Wyatt Technology Corporation, Santa Barbara, USA), and transmission electron microscopy (TEM) using a JEOL 1010 (JEOL Ltd., Tokyo, Japan) to assess size distribution as previously described (Chuan et al., 2008b; Lipin et al., 2008).

Immunization of Mice

All animal experiments were approved by the QIMR Berghofer 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). BALB/c mice (n = 10/group) aged 7–8 weeks (Animal Resources Center, WA, Australia) were immunized with the chimeric VLP constructs three times at 3-week intervals by subcutaneous injection at the base of the tail. VLPs were administered at two doses, 10 or 30 μg in independent experiments, and individually or pooled. VLPs were administered without adjuvant as this platform is considered self-adjuvating (Chackerian et al., 2002; Stanley, 2006). Synthetic peptides corresponding to the epitopes presented by the chimeric VLP co-administered with 50 μg of high molecular weight poly (I:C) adjuvant (Invivogen, USA) were included as comparator groups in each experiment. In heterologous prime-boost regimens, mice received two doses of 100 μg of *Py*CSP plasmid DNA (pVR2516, Vical Inc, CA, USA) at 3-week intervals by intramuscular injection into the tibialis anterior muscle, followed by a booster dose of pooled VLP or synthetic peptide in poly(I:C) adjuvant as previously described. In the second experiment, one group received three homologous immunizations with *Py*CSP plasmid DNA at 100 μg per dose. Control groups included wild-type VLPs, ovalbumin $CD8^{+}_{257-264}$ and $CD4^{+}_{323-339}$ pooled peptides with 50 μg poly(I:C), and PBS only (naïve). Immunized mice were split into two groups (n = 5/group) to evaluate either immunogenicity using splenocytes for T cell assays or protective efficacy by sporozoite challenge (**Figure 1**).

Sporozoite Challenge and Protection

Two weeks after the final immunization, mice were challenged with 1,000 cryopreserved *P. yoelii* 17 XNL sporozoites (Sanaria Inc., MD, USA) in 200 μl PBS with 2% naive mouse serum administered intravenously into the tail vein. Livers were harvested 42 h post-challenge to assess liver-stage parasite burden as previously described (Schussek et al., 2013). Briefly, livers were homogenized in 5 ml of RLT buffer (Qiagen, Netherlands) supplemented with 1% β-2-mercaptoethanol (Sigma-Aldrich), then RNA was extracted using an RNeasy Mini kit (Qiagen) following the manufacturer's protocol. Using 2.5 μg of RNA, cDNA was synthesized using a Super Script VILO reverse-transcriptase cDNA synthesis kit (Life Technologies, USA) according to manufacturer's guidelines. Then the *P. yoelii* 18S rRNA and murine housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA were quantified using quantitative reverse transcriptase-PCR (qRT-PCR) using a Rotor-gene 3000 (Corbett Research, Mortlake, Australia) with acquisition on the FAM channel. For *Py*18S cDNA quantification, a Taqman Fast Advanced master mix (Applied Biosystems, Australia) with a custom made Taqman probe [250 nm] (6FAM-CTGGCCCTTTGAGAGCCCACTGATT-BHQ-1) and primers [1 um] (5″- CTTGGCTCCGCCTCGATAT and 3″- TCAAAGTAACGAGAGCCCAATG) (Applied Biosystems). A GAPDH kit (Applied Biosystems) combined with platinum Taq polymerase, PCR buffers (Invitrogen) was used for GAPDH quantification. The data are presented as a ratio of copies of *Py*18S rRNA per 10^5 copies of GAPDH.

Splenocyte Harvesting and Stimulation for T Cell Assays

Ten days after the final immunization, spleens were removed and single cell suspensions were generated by mechanical disruption followed by red blood cell lysis. Splenocytes were then co-incubated with gamma irradiated (16,666 cGy) mouse B cell lymphoma A20 cells (ATCC TIB-208) which were transfected, peptide stimulated, or untreated. Transfections with *Py*CSP encoded plasmid DNA pVR2516 (Vical, USA) or empty vector pVR1020 (Vical, USA) plasmid DNA was achieved the AMAXA Nucleofector system (Lonza, Switzerland) using Kit V and program C-25 with 5 x 10^6 A20 cells per cuvette, following the manufacturer's protocol. Peptide stimulation used either *Py*CSP $CD8^{+}_{(280-288)}$ or $CD4^{+}_{(59-79)}$ T cell epitope synthesized peptides, or these peptides combined with the B cell repeat epitope (QGPGAPQGPGAP) peptide at 10 μg/ml. For this assay, 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. Each well-contained 5 x 10^5 splenocytes and 1.5 × 10^5 A20 cells in 200 μl of media. Background responses were removed by subtracting transfected pVR1020 wells from pVR2516 wells, and untreated A20 wells from peptide-pulse wells for ELISpot, cytometric bead array (CBA) and intracellular cytokine staining (ICS) assays.

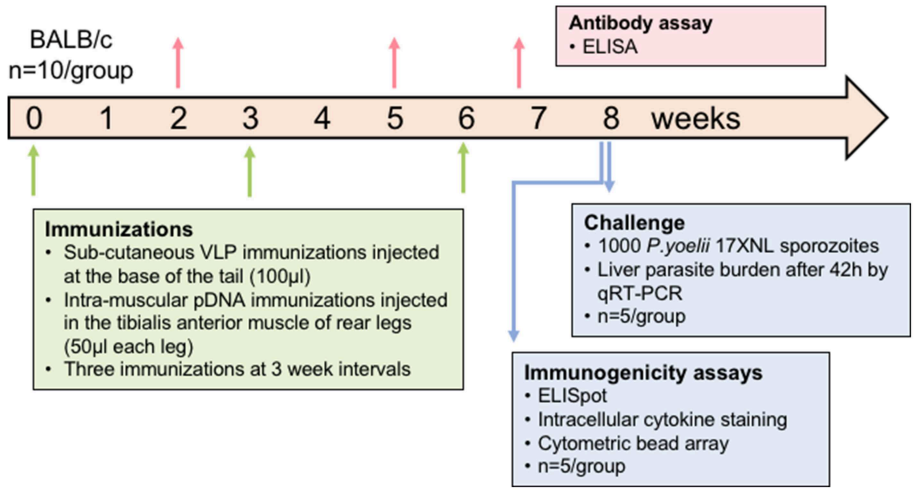


FIGURE 1 | Schematic of experimental design. In each experiment, mice ($n = 10$/group) were immunized three times at a 3-week interval. Plasmid DNA injections were intramuscular into the tibialis anterior whilst VLP immunizations were subcutaneous on the back near the base of the tail. Blood samples were collected 14 days after doses 1 and 2 and then 5 days after the final dose to assess antibody responses. Half of the mice were euthanized at 10 days after the final dose and splenocytes used for immunogenicity assays. The other half were challenged 2 weeks after the final dose by injecting 1,000 *P. yoelii* sporozoites into a tail vein, and livers removed 42 h later to assess parasite load by qRT-PCR. In one experiment, the VLP doses were 30 µg for individual VLPs or 10 µg of each in the pooled VLP group. In another experiment, the pooled VLP dose was increased to 30 µg of each VLP and a DNA-only control was included.

IFN-γ ELISpot Assay

IFN-γ ELISpot assays were conducted as previously described (Schussek et al., 2017). Briefly, MSIPS4510 multiscreen ELISpot plate (Merck Millipore, Germany) well were pre-coated with 100 µg/ml anti-mouse IFN-γ (BD Biosciences, USA) then blocked with KD-MEM supplemented with 10% FCS. Quadruplicate wells were used for each stimulation. Plates were incubated for 40 h at 37°C and 5% CO_2. After plate washing, IFN-γ secreting cells were stained with 2 µg/ml biotinylated anti-mouse IFN-γ (BD Biosciences, USA) followed by 1 µg/ml streptavidin-HRP (BD Biosciences, USA). The assay was developed using AEC substrate (BD Biosciences, USA), and spots counted using the AID ELISpot reader system (Autoimmun Diagnostika GmbH, Germany).

Cytometric Bead Array

Splenocyte/A20 cultures were incubated at 37°C and 5% CO_2 for 72 h. Culture supernatant was collected and secreted IFN-γ, TNF, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, and IL-13 cytokines assayed using the mouse cytometric bead array flex kit (BD Biosciences, USA) following the manufacturer's protocol. Samples were analyzed using a FACSArray instrument (BD Biosciences, USA) using the CBA array software (BD Biosciences, USA).

Intracellular Cytokine Staining

To detect monofunctional or polyfunctional T cell responses, splenocyte/A20 cultures were incubated with 0.1% Golgi Plug (BD Biosciences, USA) for 6 h at 37°C and 5% CO_2. Cells were stained with anti-$CD8^+$ (53-6.7) and anti-$CD4^+$ (RM4.5) antibodies before being fixed with 1% paraformaldehyde. Cells were then stained with anti-IFN-γ (XMG1.2), anti-IL-2 (JES6-5H4), and anti-TNF-α (MP6-XT22) diluted in permwash buffer (BD Biosciences, USA). All antibodies were purchased from Biolegend. 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

Antigen-specific antibody responses were detected by ELISA using the *Py*CSP B cell repeat peptide linked to a polystyrene binding tag (Kumada et al., 2010) with a $glycine_4$ spacer (Kogot et al., 2012) (PST-B cell, RIIIRRIRGGGG-QGPGAPx3) (Mimotopes, Australia). The PST-tag with a glycine spacer was incorporated to enhance attachment of the peptide to the plate and expose the B cell repeat for antibody recognition. Nunc maxisorp plates (Thermo Fisher Scientific, USA) were coated overnight with the PST-B cell peptide (5 µg/ml) in a carbonate coating buffer, then blocked with PBS containing 2% BSA. Triplicate wells of sera from mice were titrated by 2-fold dilutions in PBS-BSA 0.1%, and then incubated with biotinylated donkey α-mouse IgG (Jackson ImmunoResearch Laboratories, USA) diluted in PBS-BSA 0.1%, followed by an incubation with streptavidin-HRP (BD Biosciences, USA) diluted 1:1,000 in PBS-BSA 0.1 and 0.2% Tween20. Plates 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). Positive results were recorded if OD_{450} values were > 3 standard deviations above the mean blank (no serum) values.

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 displayed as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

RESULTS

VLP Construction

In-frame genomic insertion of antigen-derived epitopes into the MuPyV-VP1 protein was confirmed by sequencing. After the VLPs were assembled *in vitro*, analysis using AF4-MALS showed only small amounts of protein aggregation and TEM images confirmed that the chimeras had a similar morphology to wild-type VLPs (**Figure 2**). The mean radius of each of the chimeric VLPs was similar to that of the wild type 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)].

VLP-Induced T Cell Immune Responses

Mice were immunized with chimeric CD8 or CD4 VLPs to assess their capacity independently to induce $CD8^{+}$ or $CD4^{+}$ T cell responses, and to evaluate if pooling of the VLPs would result in an additive or synergistic response. Pooled VLPs were evaluated in independent experiments at 10 μg per dose and at 30 μg per dose to dose-match the independent VLPs (**Figure 1**).

IFN-γ responses detected by ELISpot showed that homologous immunization with the CD8 VLP induced significant IFN-γ spot forming cells (SFCs) when stimulated with pooled peptide or *Py*CSP transfected A20 cells ($p < 0.001$) (**Figures 3A**, **4A**). The magnitude of VLP-induced $CD8^{+}$ T cell response was similar to that induced by CD8 peptide immunization when stimulated by peptide (but not transfected A20 cells). In contrast, the CD4 VLP failed to induce a significant IFN-γ response (**Figures 3A**, **4A**); robust responses against peptide ($p < 0.0001$) and transfected stimulation ($p < 0.001$) were observed in mice immunized in parallel with CD4 peptides in adjuvant (**Figure 3A**) and similarly in the subsequent experiment (**Figure 4A**).

Immunization with pooled CD8 plus CD4 VLPs resulted in a similar IFN-γ profile to that induced by the individual VLPs, with a robust response to the CD8 peptide and negligible response to the CD4 peptide (**Figure 4A**).

Heterologous prime-boost immunization with DNA prime/VLP boost induced a stronger response than homologous VLP immunization (**Figures 3A–C**). This was attributed to the plasmid DNA prime with the homologous DNA regimen inducing the most robust response (**Figures 4A–C**). The reduction in responses between the DNA only and DNA prime/VLP boost could be due to an inhibition caused by VLPs, but more likely that the third dose of DNA was more immunogenic than the VLPs.

The profile of IFN-γ responses detected with both CBA and ICS readouts was similar to that shown by ELIspot for the CD8 and CD4 VLPs (**Figures 3A–C**, **4A–C**). Robust IFN-γ responses induced by the CD8 VLP were similar to that induced by peptide in adjuvant. Negligible $CD4^{+}$ T cell responses were induced by CD4 VLP immunizations, as opposed to the robust $CD4^{+}$

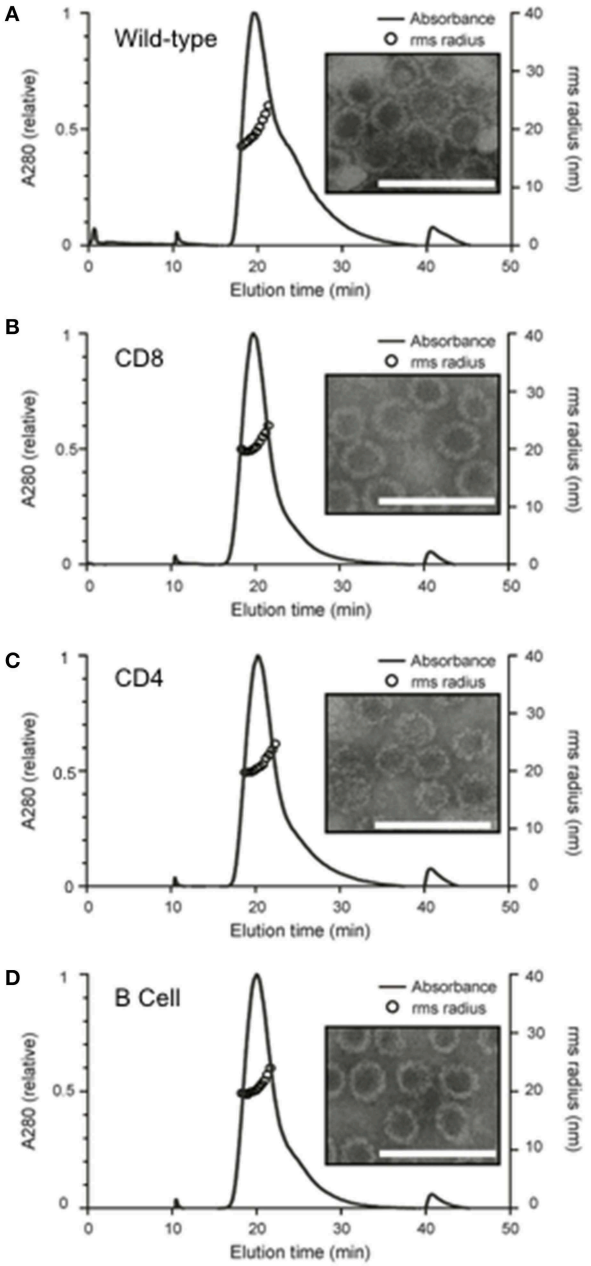


FIGURE 2 | Structural analysis of *in vitro* assembled chimeric murine polyomavirus virus-like particles. Wild and chimeric MuPyVP1-G4S proteins were expressed in *E. coli* and purified by liquid chromatography. Proteins were dialyzed against an assembly buffer to form VLPs and then against PBS. Post-assembly solutions were analyzed to detect the formation of VLPs using asymmetrical flow field- flow fractionation coupled with multi-angled lights scattering (AF4-MALS) and transmission electron microscopy of **(A)** wild-type VLPs, **(B)** CD8 VLP chimeras, **(C)** CD4 VLP chimeras, and **(D)** B cell VLP chimeras. UV_{280} absorbance is displayed relative to peak absorbance (solid line) for each sample, and particle size is shown as root-square-radius (open circles). Scale bar is 100 nm.

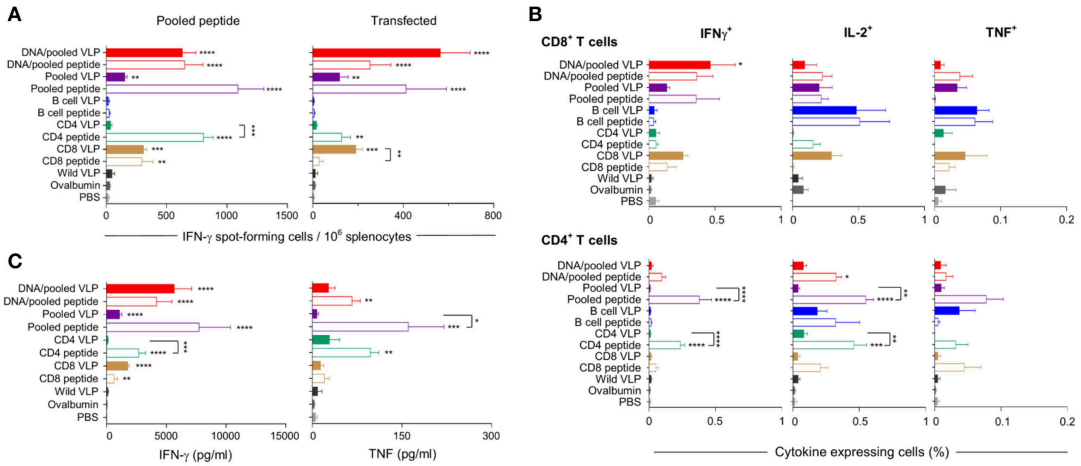


FIGURE 3 | Cellular immune responses induced by individual (30 μg) or pooled (10 μg each) chimeric VLP immunizations. BALB/c mice ($n = 5$/group) were immunized with a prime/boost regimen using two i.m., priming immunizations with *Py*CSP plasmid DNA (100 μg) followed by a single s.c. boost using either pooled *Py*CSP VLPs (10 μg each) or pooled *Py*CSP peptides (30 μg each) with poly(I:C); or with three homologous s.c doses of pooled *Py*CSP VLPs (10 ug each) or individual *Py*CSP VLPs (30 ug); or pooled or individual *Py*CSP peptides (30 μg each) with poly(I:C); wild type VLPs (30 μg); pooled ovalbumin peptides (30 μg each) with poly(I:C); or PBS only. Ten days after the final boost, splenocytes (5×10^5/well) were cultured with irradiated A20 cells pulsed with pooled *Py*CSP $CD8_{280-288}$, $CD4_{59-79}$, or B cell peptides (1.5×10^5/well), or with irradiated A20 cells transfected with pVR2516 *Py*CSP plasmid DNA (5×10^4/well), for assessment of T cell specific immune responses. **(A)** IFN-γ ELISpot was used to determine IFN-γ spot forming cells/million splenocytes following 40 h incubation with pooled $CD8_{280-288}$ plus $CD4_{59-79}$ peptides (left) or DNA transfected A20 cells (right). **(B)** ICS was used to determine $CD8^+$ and $CD4^+$ T cell, IFN-γ, IL-2, and TNF cytokine responses following 18 h stimulation with pooled peptides, with the final 6 h supplemented with GolgiPlug. **(C)** Cytometric Bead Array (CBA) was used to quantify cytokine concentrations in 72 h culture supernatant following pooled peptide stimulation. All data are presented as group means + SEM (media only subtracted). Statistical comparisons are made to the PBS control group, and between homologous VLP and peptide immunization groups using log-transformed data with significance determined using one-way ANOVA followed by Bonferroni's *post-hoc* test. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

T cell responses which were evident following CD4 peptide immunization. This differentiation is likely due to the reduced actual peptide load in the CD4 VLPs of approximately 0.52 μg.

TNF was induced in mice immunized with the CD4 peptide individually or in pools (**Figures 3C**, **4C**). TNF was also significantly induced by DNA immunizations in response to pooled peptide ($p < 0.01$) and CD4 peptide stimulation ($p < 0.0001$). No VLP immunizations resulted in significant TNF or IL-2 production from stimulated splenocytes. Our CBA assessed a range of cytokines specifically: IFN-γ, L-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13 and TNF. However, with the exception of IFN-γ and TNF, we only detected a very low level of cytokines induced by our VLP immunizations (data not shown).

VLP-Induced Antibody Responses

The B cell VLP was predicted to induce strong antibody responses since it comprised the dominant *Py*CSP B cell linear epitope exposed on the surface of the VLP in a repetitive array. Responses to the B cell VLP were assessed in parallel individually as well as in a pool with the CD8 and CD4 VLPs since it was anticipated that the inclusion of the CD4 VLP would enhance the epitope-specific antibody titer.

Robust anti-*Py*CSP B cell peptide IgG responses were induced by all groups containing the B cell VLP ($p < 0.0001$ relative to naive) (**Figure 5**). In the homologous VLP immunizations, there were no significant differences between any groups indicating no additive effect by including the CD4 VLP. VLP-induced antibody responses were significantly higher than homologous DNA immunizations ($p < 0.0001$). In the DNA prime/VLP boost regimen, the response induced by the 10 μg/construct boost was significant higher as compared to the 30 μg/construct boost ($p < 0.01$); conversely in the homologous VLP only groups, the 30 μg per dose group higher than the 10 μg per dose, although not significantly different.

Protection From Sporozoite Challenge

To evaluate the protective efficacy of chimeric VLPs, VLP-immunized mice ($n = 5$/group) were challenged with 1,000 *P. yoelii* 17XNL sporozoites. In the first experiment, 40% (2/5) mice immunized with DNA prime / VLP boost had no parasite RNA detected in their liver and there was a 38% reduction in liver-stage parasite burden relative to the PBS control group overall in the group; these differences were not significant given the relatively small group sizes. When the experiment was repeated using the higher dose pooled VLPs (30 μg/construct), a significant reduction in parasite burden was noted in mice immunized with the heterologous DNA prime / VLP pooled VLP regimen ($p < 0.01$) (**Figure 6**). This equates to a mean 72% reduction in the liver parasite burden relative to the PBS immunized group. In the pooled VLP immunized mice, there was a mean 57% reduction in liver-stage parasite burden relative to the PBS control, although this did not reach statistical significance.

DISCUSSION

Development of an efficacious malaria vaccine has been challenging, with even the most advanced candidate Mosquirix™ showing very poor efficacy despite very

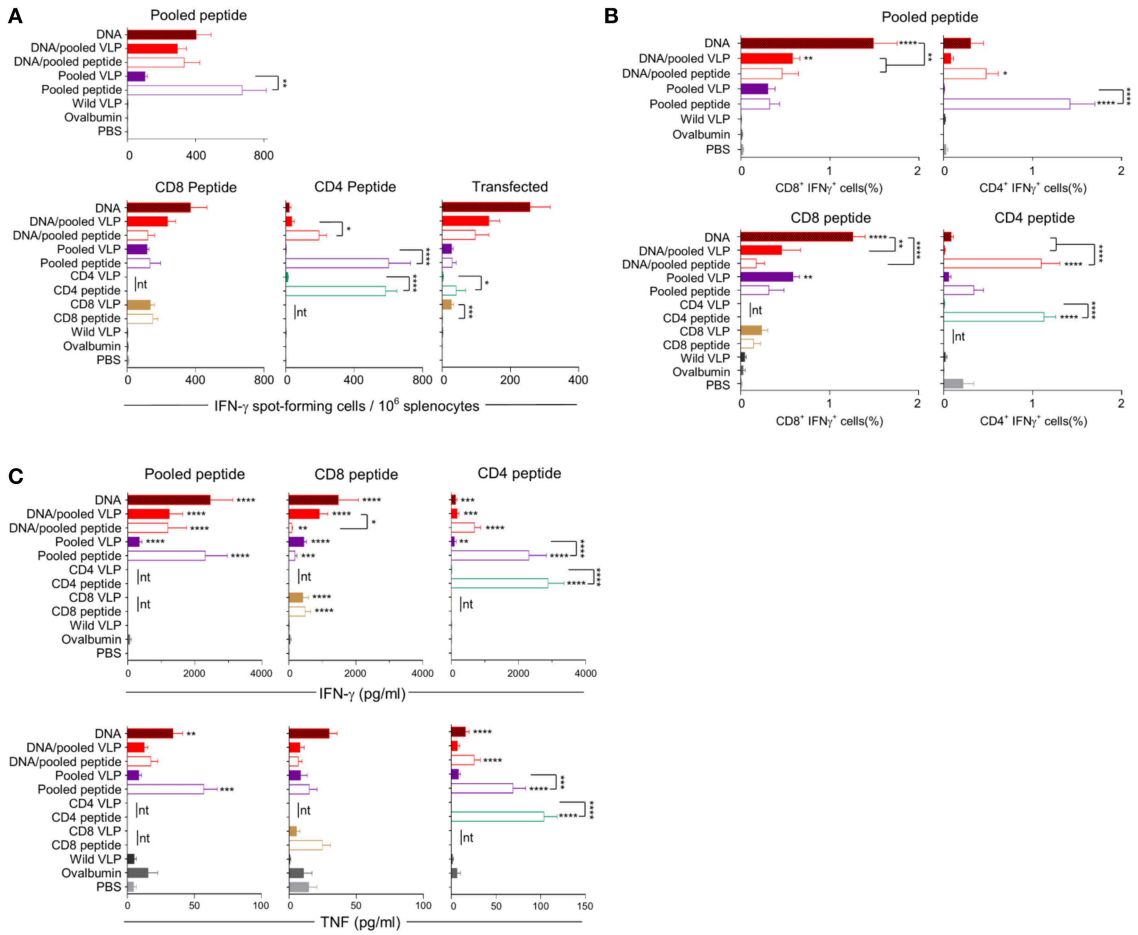


FIGURE 4 | Cellular immune responses induced by individual (30 μg) or pooled (30 μg each) chimeric VLP immunizations. BALB/c mice ($n = 5$/group) received three immunizations at 3 week intervals with three homologous i.m., *Py*CSP plasmid DNA (100 μg) immunizations or a prime/boost regimen using two i.m., priming immunizations with *Py*CSP plasmid DNA (100 μg) followed by a single s.c. boost using either pooled *Py*CSP VLPs (30 μg each) or pooled *Py*CSP peptides (30 μg each) with poly(I:C); or three homologous s.c doses of pooled or individual *Py*CSP VLPs (30 μg each) or *Py*CSP peptides (30 μg each) with poly(I:C); wild type VLPs (30 μg); pooled ovalbumin peptides (30 μg each) with poly(I:C); or PBS only. **(A)** IFN-γ ELIspot, **(B)** ICS, and **(C)** CBA responses were assayed as described in the legend to **Figure 3**, using individual CD8 peptides or CD4 peptides, pooled peptides, or DNA-transfected A20 cells. All data sets are presented as group means + SEM (media only subtracted). Statistical comparisons are made to the PBS control group, and between homologous VLP and peptide immunization groups using log-transformed data with significance determined using one-way ANOVA followed by Bonferroni's *post-hoc* test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ (nt, not tested).

extensive refinement during preclinical development and early stage clinical testing (Tinto et al., 2015; Olotu et al., 2016). Mosquirix™ is a chimeric VLP construct expressing the central repeat region plus the entire C-terminal flanking region (amino acids 207–395) of the *P. falciparum* CSP fused to the hepatitis B virus surface antigen, HBsAg. Protection has been associated with antibodies or $CD4^+$ T cell responses, but this vaccine fails to induce $CD8^+$ T cell responses which are thought to be required for protection against intracellular pathogens such as the *Plasmodium* spp. parasite (Seder and Hill, 2000). Various *Plasmodium* targets have been evaluated using a range of subunit vaccine delivery platforms including recombinant protein, recombinant viruses, synthetic peptides, plasmid DNA, nanoparticles, and VLPs [reviewed in Draper et al. (2015)]. However, none of these platforms have induced robust $CD8^+$ and $CD4^+$ T cell responses as well as antibody responses sufficient for protection against malaria. In other disease models, VLP vaccines show potential for induction of broad antibody and cellular immune responses.

Protection directed against the pre-erythrocytic stage of the *Plasmodium* lifecycle following sporozoite challenge can be achieved primarily by antibody responses which inhibit sporozoite infection of the hepatocyte (Majarian et al., 1984; Charoenvit et al., 1991) or $CD8^+$ T cell responses which act directly or indirectly upon the liver-stage parasite (Weiss et al., 1988, 1990, 1992; Doolan and Hoffman, 2000); moreover, there is evidence that induction of $CD4^+$ T cell responses is required for optimal efficacy (Weiss et al., 1993). There is increasing interest in immunization regimens which induce liver-resident memory T cells as the parasite-infected liver is considered the primary site

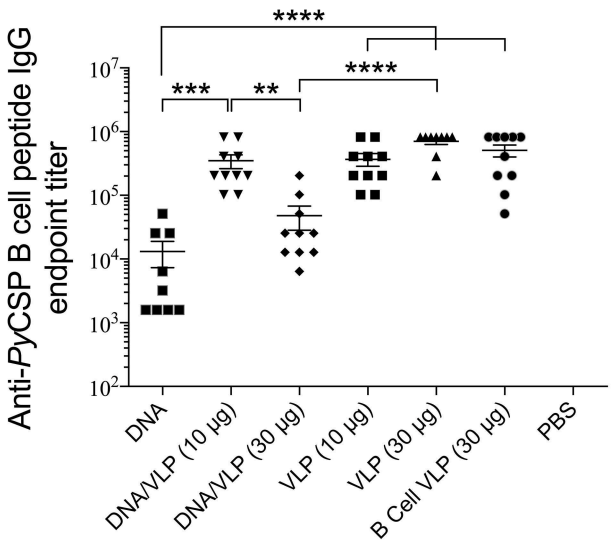


FIGURE 5 | Anti-*Py*CSP B cell peptide IgG responses induced by immunization with individual or pooled VLP immunizations. In separate series of experiments, mice ($n = 10$/group) were immunized with individual VLPs (30 µg/dose) or pooled VLPs (either 10 µg/construct or 30 µg/construct), as described in the legends to **Figures 3**, **4**. Sera collected 5 days following the final immunization were batch analyzed by ELISA to determine the anti-B cell peptide-specific IgG endpoint titer. Data is presented as mean endpoint titer + SEM. Statistical comparisons between immunization groups used log-transformed data with significance determined using one-way ANOVA followed by Bonferroni's *post-hoc* test. $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

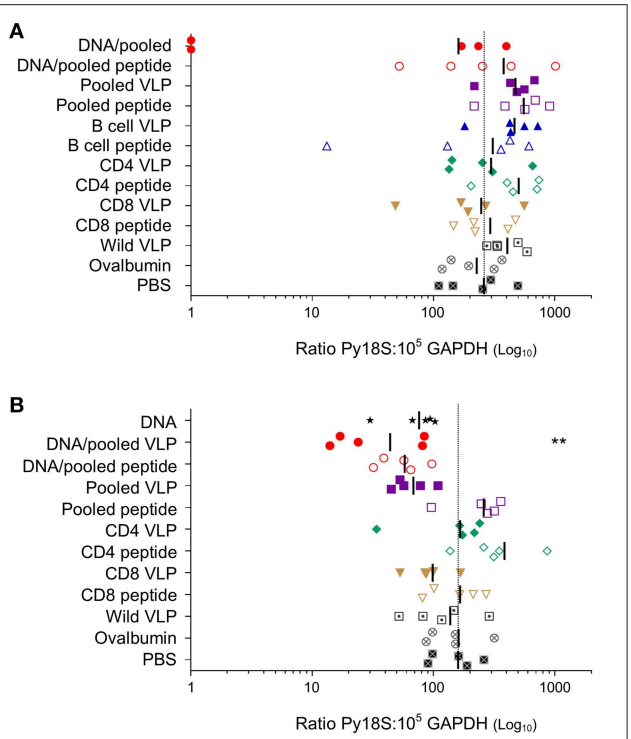


FIGURE 6 | Protective capacity induced by individual or pooled chimeric VLPs using homologous or heterologous immunization regimens. In two independent experiments **(A)** BALB/c mice ($n = 5$/group) were immunized with three immunizations at 3-week intervals (as in **Figure 3**) with a prime-boost regimen using two i.m. *Py*CSP plasmid DNA (100 µg) primes followed by a s.c. boost of either pooled *Py*CSP VLPs (10 µg each) or pooled *Py*CSP peptides (30 µg each); or three homologous s.c. immunizations with pooled *Py*CSP VLPs (10 µg each) or pooled PyCSP peptides (30 µg each) in poly(I:C); or individual *Py*CSP B cell, CD4 or CD8 VLPs (30 µg) or corresponding peptides (30 µg) with poly(I:C); or wild-type VLPs (30 µg); or ovalbumin pooled peptides (30 µg each) with poly(I:C); or PBS only. (B) BALB/c mice ($n = 5$/group) were immunized with three immunizations at 3-week intervals (as in **Figure 4**) with three i.m immunizations of *Py*CSP plasmid DNA (100 µg); a prime/boost regimen or homologous regimens as above with dosing modifications to pooled *Py*CSP VLPs increasing to 30 µg per VLP. In both experiments, mice were challenged 2 weeks after the final immunization by i.v., injection of 1,000 *P. yoelii* 17XNL cryopreserved sporozoites. Parasite burden was assessed by qRT-PCR analysis of *Py*18s rRNA extracted from livers harvested 42 h post-challenge and calculated as a ratio to the GAPDH housekeeping-gene mRNA. Results for individual mice are shown, with bars representing the group mean. The dotted line represents the naïve mouse mean. Log-transformed ratio data was analyzed using one-way ANOVA followed by Bonferroni's *post-hoc* test. $^{**}p < 0.01$.

for protective T cell mediated immune responses which act to reduce liver-stage parasite burden (Fernandez-Ruiz et al., 2016; Gola et al., 2018; Olsen et al., 2018). It is possible that immune responses measured in the periphery or in splenocytes may not accurately reflect the vaccine-induced responses in the liver, so future studies should consider evaluation of liver-resident memory T cells in addition to the more traditional readouts from splenocytes.

Many VLP vaccine platforms have been developed, using structural proteins from various viral species. VLPs based on the murine polyomavirus are considered a promising VLP for vaccine development because of their reported humoral immunogenicity and their ability to be produced reproducibly with high yields (Chuan et al., 2008a; Liew et al., 2010, 2012; Middelberg et al., 2011; Anggraeni et al., 2013; Rivera-Hernandez et al., 2013; Wibowo et al., 2014; Seth et al., 2016; Tekewe et al., 2017). However, their capacity to induce robust antibody plus $CD4^+$ and $CD8^+$ T cell responses has not been thoroughly investigated. Accordingly, we evaluated both cellular and antibody responses using chimeric murine polyomavirus VLPs incorporating the CSP as a vaccine delivery platform, in the *P. yoelii* murine model.

It is well-established that VLPs are very effective at inducing antigen-specific antibody responses [reviewed in Grgacic and Anderson (2006)] likely due to their ability cross-link B cell surface receptors (Thönes et al., 2008) acting in a T cell independent manner (Snapper and Mond, 1993; Szomolanyi-Tsuda and Welsh, 1998). Consistent with that, herein we showed that our B cell VLP induced high IgG titres against the *Py*CSP B cell epitope. There was a significant decrease in antibody titers in the DNA prime/VLP boost group when the pooled VLP component was increased from 10 to 30 µg per VLP (**Figure 5**). This variation was not seen in the VLP only immunized mice, but those mice received three VLP doses and the additional doses may have normalized the responses.

We established that immunizations with either chimeric CD8 VLPs alone, or as pooled with a B cell VLP and CD4 T cell VLP

were able to induce moderate levels of IFN-γ directed against the $CD8^+$ T cell epitope presented as a synthetic peptide or in the context of whole antigen transfectant, with both ELISpot and ICS (**Figures 3A,C**, **4A,C**). This antigen-specific recognition confirms that the CD8 chimeric VLP presented the incorporated $CD8^+$ T cell epitope *in vivo* to naive $CD8^+$ T cells, and that this presentation was via MHC class I molecules (Heath et al., 2004). These results are consistent with previous studies reporting that chimeric VLPs incorporating a single $CD8^+$ T cell epitope from viral or ovalbumin targets induced CTL activity mediated by $CD8^+$ T cells (Sedlik et al., 1997; Crisci et al., 2009). It is possible that our immunizations with pooled VLPs using 30 μg of each construct failed to protect mice from challenge as the induced $CD8^+$ T cell response was insufficient to protect (Schmidt et al., 2008) with a mean frequency of 0.59% of $CD8^+$ T cells expressing IFN-γ (**Figure 4B**).

We had anticipated that immunizing with a pool of VLPs which included the CD4 T cell peptide might result in an additive or synergistic response, as noted for immunization with a pool of the synthetic peptides. However, there was no evidence of any additive or synergistic effects on $CD8^+$ T cell responses when VLPs were administered as a pool with the CD4 VLP; this was likely due to the very low responses induced by the CD4 VLP. The carrier MuPyV VP1 protein is likely to contain $CD4^+$ T helper epitopes similar to those in the HBV core protein (Schödel et al., 1992; Bickert et al., 2007) and may provide carrier-related T cell help for antibody responses, but those would provide no antigen-specific help against challenge by the *Plasmodium* parasite. Poly (I:C) was selected to adjuvant the synthetic peptides *in vivo* because it is a known inducer of type I (Salem et al., 2005; Qiu and Cui, 2007; Coffman et al., 2010; Schneider-Ohrum et al., 2011) and II (Tewari et al., 2010; Kastenmüller et al., 2013) cellular responses. However, VLPs are generally considered self-adjuvating due to their viral structure (Stanley et al., 2006; Chackerian, 2007). There are however contradictory reports, with some reports showing that adjuvants are essential (Storni et al., 2002), beneficial (Freyschmidt et al., 2004; Ding et al., 2009) or not necessary (Mazeike et al., 2012; Rivera-Hernandez et al., 2013; Kawano et al., 2014). In our studies, we elected to exclude adjuvants so as to better understand the immunogenicity of our chimeric VLPs. We cannot however rule out the possibility that administration of the CD4 VLP with poly(I:C) could have enhanced the VLP-induced T cell response, but other data generated in our laboratory showed no enhancement of $CD4^+$ T cell when pooled VLPs were co-administered with poly(I:C) (manuscript in preparation).

In our VLP constructs, each T cell epitope was inserted into surface-exposed regions of the VP1 protein, since we predicted that epitopes inserted into this region would be less likely to interfere with VLP formation. It is possible that this location may not have been optimal for the induction of cellular immune responses and that placing the epitopes on terminal regions may have been beneficial, as reported in one study in a chimeric calicivirus-like VLP system with ovalbumin CD8 epitopes showing significantly higher IFN-γ responses, target-specific lysis and protection (Crisci et al., 2009). It is also possible that the lack of responses to the CD4 epitope may have been a dose related issue; the CD4 peptide component of the 30 μg VLP dose was approximately 0.52 μg peptide epitope equivalent, whereas the peptide immunized mice received nearly 60 times the epitope per dose. Our immunogenicity assessments used lymphocytes taken from spleen and not draining lymph nodes, but the strong responses detected in splenocytes from the peptide-immunized mice would be expected to accurately reflect a response in the draining lymph nodes; if the VLPs induced a robust $CD4^+$ T cell response then this should also have been detected in splenocytes.

To evaluate the protective capacity of our chimeric VLPs, we harvested livers 42 h post- sporozoite challenge, at the late liver-stage of the *P. yoelii* lifecycle before parasites were expected to release into the blood (Baer et al., 2007). This timepoint maximized the opportunity for VLP-induced protective immune responses to clear parasite-infected cells. Liver-stage protection has previously been achieved by adoptively transferring a $CD8^+_{280-288}$ T cell clone (Weiss et al., 1988), or by passive transfer of monoclonal antibodies specific to the *Py*CSP central repeat (Charoenvit et al., 1991). These protective antigenic epitopes were incorporated within our VLP chimeras, and generated epitope-specific $CD8^+$ T cell and antibody responses. In our study, there was evidence that VLPs could induce protection against sporozoite challenge since sterile protection was seen in 2 of 5 mice and there was a reduction in parasite load; however, this protection was suboptimal. Our immunological data supports the induction of the type of immune response required to protect against *Plasmodium* sporozoite challenge, but the magnitude of those VLP-induced responses may have been insufficient to reach a protective threshold (Schmidt et al., 2010). This is consistent with other studies where protection correlated with the number of adoptively transferred clonal $CD8^+_{280-288}$ T cells where 20×10^6 cells were required for sterile protection (Weiss et al., 1992), or with the number of memory $CD8^+$ T cells stimulated by immunizations (Schmidt et al., 2008, 2010), or with the amount of passive transferred monoclonal antibodies (Charoenvit et al., 1991).

Indeed, we found that more robust antibody and cellular responses were induced by a prime/boost regimen as compared with homologous VLPs, and we anticipate that this would be further enhanced with the inclusion of a second VLP boost.

Overall, our data showed that the MuPyV chimeric VLP platform is capable of inducing antigen-specific antibody responses, and established for the first time for this vaccine platform, their capacity to induce $CD8^+$ T cell immune responses. Future studies could evaluate alternative epitope insertion sites within the MuPyV VP1 protein to determine if other sites enhance cellular immune responses, or the inclusion of adjuvants in the vaccination regimen. Additional regions of the target antigens (e.g., repetitive regions targeted by antibody responses, together with T helper epitopes) could also be included. Alternative target antigens could be also evaluated since studies have suggested that multiple antigen targets may be required for protection from *Plasmodium* challenge (Doolan et al., 1996; John et al., 2005; Osier et al., 2008). With approximately 2,000 active genes, and 816 identified proteins present during *Plasmodium* liver-stage of infection (Tarun et al.,

2008) part of the challenge is to identify the most optimal target antigens for induction of vaccine-induced protective immunity (Schussek et al., 2017).

DATA AVAILABILITY

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

ETHICS STATEMENT

All animal experiments were approved by the QIMR Berghofer 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).

AUTHOR CONTRIBUTIONS

DP, SA, DD, and AM contributed conception and design of the study. DP, NW, YC, TR-H, LL, and AM contributed to construction of virus-like particles. DP, SA, and PG conducted mouse experiments. DP performed the statistical analysis. DP wrote the first draft of the manuscript. DD and SA edited the manuscript. All authors contributed to manuscript revision, 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 Robert Garcea (University of Colorado, CO, USA) for the plasmid containing the MuPyV-VP1 protein, and Dr. Stephen Hoffman and colleagues (Sanaria Inc., Rockville, MD, USA) for providing cryopreserved sporozoites. TEM images were taken by Alice Lei Yu from the University of Queensland, Australia.

REFERENCES

Agnandji, S. T., Lell, B., Fernandes, J. F., Abossolo, B. P., Methogo, B. G., Methogo, B. G., et al. (2012). A phase 3 trial of RTS,S/AS01 malaria vaccine in African infants. *N. Engl. J. Med.* 367, 2284–2295. doi: 10.1056/NEJMoa1208394

Alonso, P. L., Brown, G., Arevalo-Herrera, M., Binka, F., Chitnis, C., Collins, F., et al. (2011). A research agenda for malaria eradication: vaccines. *PLoS Med.* 8:e1000398. doi: 10.1371/journal.pmed.1000406

Anggraeni, M. R., Connors, N. K., Wu, Y., Chuan, Y. P., Lua, L. H., and Middelberg, A. P. (2013). Sensitivity of immune response quality to influenza helix 190 antigen structure displayed on a modular virus-like particle. *Vaccine* 31, 4428–4435. doi: 10.1016/j.vaccine.2013.06.087

Baer, K., Klotz, C., Kappe, S. H., Schnieder, T., and Frevert, U. (2007). Release of hepatic *Plasmodium yoelii* merozoites into the pulmonary microvasculature. *PLoS Pathog.* 3:e171. doi: 10.1371/journal.ppat.0030171

Bickert, T., Wohlleben, G., Brinkman, M., Trujillo-Vargas, C. M., Ruehland, C., Reiser, C. O., et al. (2007). Murine polyomavirus-like particles induce maturation of bone marrow-derived dendritic cells and proliferation of T cells. *Med. Microbiol. Immunol.* 196, 31–39. doi: 10.1007/s00430-006-0026-x

Chackerian, B. (2007). Virus-like particles: flexible platforms for vaccine development. *Expert Rev Vaccines* 6, 381–390. doi: 10.1586/14760584.6.3.381

Chackerian, B., Lenz, P., Lowy, D. R., and Schiller, J. T. (2002). Determinants of autoantibody induction by conjugated papillomavirus virus-like particles. *J. Immunol.* 169, 6120–6126. doi: 10.4049/jimmunol.169.11.6120

Charoenvit, Y., Mellouk, S., Cole, C., Bechara, R., Leef, M. F., Sedegah, M., et al. (1991). Monoclonal, but not polyclonal, antibodies protect against Plasmodium-yoelii sporozoites. *J. Immunol.* 146, 1020–1025.

Chuan, Y. P., Fan, Y. Y., Lua, L., and Middelberg, A. P. (2008b). Quantitative analysis of virus-like particle size and distribution by field-flow fractionation. *Biotechnol. Bioeng.* 99, 1425–1433. doi: 10.1002/bit.21710

Chuan, Y. P., Lua, L. H., Middelberg, A. P.,Chuan, Y. P., Lua, L. H., and Middelberg, A. P. (2008a). High-level expression of soluble viral structural protein in *Escherichia coli*. *J. Biotechnol.* 134, 64–71. doi: 10.1016/j.jbiotec.2007.12.004

Coffman, R. L., Sher, A., and Seder, R. A. (2010). Vaccine adjuvants: putting innate immunity to work. *Immunity.* 33, 492–503. doi: 10.1016/j.immuni.2010.10.002

Collins, K. A., Snaith, R., Cottingham, M. G., Gilbert, S. C., and Hill, A. V. S. (2017). Enhancing protective immunity to malaria with a highly immunogenic virus-like particle vaccine. *Sci. Rep.* 7:46621. doi: 10.1038/srep46621

Crisci, E., Almanza, H., Mena, I., Córdoba, L., Gómez-Casado, E., Castón, J. R., et al. (2009). Chimeric calicivirus-like particles elicit protective anti-viral cytotoxic responses without adjuvant. *Virology* 387, 303–312. doi: 10.1016/j.virol.2009.02.045

Ding, F. X., Wang, F., Lu, Y. M., Li, K., Wang, K. H., He, X. W., et al. (2009). Multiepitope peptide-loaded virus-like particles as a vaccine against hepatitis B virus-related hepatocellular carcinoma. *Hepatology* 49, 1492–1502. doi: 10.1002/hep.22816

Doolan, D. L., and Hoffman, S. L. (2000). The complexity of protective immunity against liver-stage malaria. *J. Immunol.* 165, 1453–1462. doi: 10.4049/jimmunol.165.3.1453

Doolan, D. L., Sedegah, M., Hedstrom, R. C., Hobart, P., Charoenvit, Y., and Hoffman, S. L. (1996). Circumventing genetic restriction of protection against malaria with multigene DNA immunization: CD8(+) T cell-, interferon gamma-, and nitric oxide-dependent immunity. *J. Exp. Med.* 183, 1739–1746. doi: 10.1084/jem.183.4.1739

Draper, S. J., Angov, E., Horii, T., Miller, L. H., Srinivasan, P., Theisen, M., et al. (2015). Recent advances in recombinant protein-based malaria vaccines. *Vaccine* 33, 7433–7443. doi: 10.1016/j.vaccine.2015.09.093

Fernandez-Ruiz, D., Ng, W. Y., Holz, L. E., Ma, J. Z., Zaid, A., Wong, Y. C., et al. (2016). Liver-resident memory CD8(+) T cells form a front-line defense against malaria liver-stage infection. *Immunity* 45, 889–902. doi: 10.1016/j.immuni.2016.08.011

Franke, E. D., Corradin, G., and Hoffman, S. L. (1997). Induction of protective CTL responses against the *Plasmodium yoelii* circumsporozoite protein by immunization with peptides. *J. Immunol.* 159, 3424–3433.

Franke, E. D., Sette, A., Sacci, J., Southwood, S., Corradin, G., and Hoffman, S. L. (2000). A subdominant CD8(+) cytotoxic T lymphocyte (CTL) epitope from the *Plasmodium yoelii* circumsporozoite protein induces CTLs that eliminate infected hepatocytes from culture. *Infect. Immun.* 68, 3403–3411. doi: 10.1128/IAI.68.6.3403-3411.2000

Freyschmidt, E. J., Alonso, A., Hartmann, G., and Gissmann, L. (2004). Activation of dendritic cells and induction of T cell responses by HPV 16 L1/E7 chimeric virus-like particles are enhanced by CpG ODN or sorbitol. *Antivir. Ther.* 9, 479–489.

Gola, A., Silman, D., Walters, A. A., Sridhar, S., Uderhardt, S., and Salman, A., M., et al. (2018). Prime and target immunization protects against liver-stage malaria in mice. *Sci. Transl. Med*. 10:460. doi: 10.1126/scitranslmed.aap9128

Gordon, D. M., McGovern, T. W., Krzych, U., Cohen, J. C., Schneider, I., LaChance, R., et al. (1995). Safety, immunogenicity, and efficacy of a recombinantly produced Plasmodium-falciparum circumsporozoite-protein hepatitis-b surface-antigen subunit vaccine. *J. Infect. Dis.* 171, 1576–1585. doi: 10.1093/infdis/171.6.1576

Grgacic, E. V., and Anderson, D. A. (2006). Virus-like particles: passport to immune recognition. *Methods* 40, 60–65. doi: 10.1016/j.ymeth.2006.07.018

Grillot, D., Michel, M., Müller, I., Tougne, C., Rènia, L., Mazier, D., et al. (1990). Immune responses to defined epitopes of the circumsporozoite protein of the murine malaria parasite, *Plasmodium yoelii*. *Eur. J. Immunol*. 20, 1215–1222. doi: 10.1002/eji.1830200604

Heath, W. R., Belz, G. T., Behrens, G. M., Smith, C. M., Forehan, S. P., Parish, I. A., et al. (2004). Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens. *Immunol. Rev*. 199, 9–26. doi: 10.1111/j.0105-2896.2004.00142.x

Janitzek, C. M., Matondo, S., Thrane, S., Nielsen, M. A., Kavishe, R., Mwakalinga, S. B., et al. (2016). Bacterial superglue generates a full-length circumsporozoite protein virus-like particle vaccine capable of inducing high and durable antibody responses. *Malar. J*. 15:545. doi: 10.1186/s12936-016-1574-1

John, C. C., Moormann, A. M., Pregibon, D. C., Sumba, P. O., McHugh, M. M., Narum, D. L., et al. (2005). Correlation of high levels of antibodies to multiple pre-erythrocytic *Plasmodium falciparum* antigens and protection from infection. *Am. J. Trop. Med. Hyg*. 73, 222–228. doi: 10.4269/ajtmh.2005.73.222

Kastenmüller, K., Espinosa, D. A., Trager, L., Stoyanov, C., Salazar, A. M., Pokalwar, S., et al. (2013). Full-length *Plasmodium falciparum* circumsporozoite protein administered with long-chain poly (I.C) or the Toll-like receptor 4 agonist glucopyranosyl lipid adjuvant-stable emulsion elicits potent antibody and CD4+ T cell immunity and protection in mice. *Infect. Immun*. 81, 789–800. doi: 10.1128/IAI.01108-12

Kawano, M., Morikawa, K., Suda, T., Ohno, N., Matsushita, S., Akatsuka, T., et al. (2014). Chimeric SV40 virus-like particles induce specific cytotoxicity and protective immunity against influenza A virus without the need of adjuvants. *Virology* 448, 159–167. doi: 10.1016/j.virol.2013.10.010

Kogot, J. M., Sarkes, D. A., Val-Addo, I., Pellegrino, P. M., and Stratis-Cullum, D. N. (2012). Increased affinity and solubility of peptides used for direct peptide ELISA on polystyrene surfaces through fusion with a polystyrene-binding peptide tag. *Biotechniques* 52, 95–102. doi: 10.2144/000113810

Kumada, Y., Kuroki, D., Yasui, H., Ohse, T., and Kishimoto, M. (2010). Characterization of polystyrene-binding peptides (PS-tags) for site-specific immobilization of proteins. *J. Biosci. Bioeng.* 109, 583–587. doi: 10.1016/j.jbiosc.2009.11.005

Kushnir, N., Streatfield, S. J., and Yusibov, V. (2012). Virus-like particles as a highly efficient vaccine platform: diversity of targets and production systems and advances in clinical development. *Vaccine* 31, 58–83. doi: 10.1016/j.vaccine.2012.10.083

Liew, M. W., Rajendran, A., and Middelberg, A. P. (2010). Microbial production of virus-like particle vaccine protein at gram-per-litre levels. *J. Biotechnol*. 150, 224–231. doi: 10.1016/j.jbiotec.2010.08.010

Liew, M. W. O., Chuan, Y. P., and Middelberg, A. P. J. (2012). High-yield and scalable cell-free assembly of virus-like particles by dilution. *Biochem. Eng. J.* 67, 88–96. doi: 10.1016/j.bej.2012.05.007

Lipin, D. I., Lua, L. H., and Middelberg, A. P. (2008). Quaternary size distribution of soluble aggregates of glutathione-S-transferase-purified viral protein as determined by asymmetrical flow field flow fractionation and dynamic light scattering. *J. Chromatogr. A* 1190, 204–214. doi: 10.1016/j.chroma.2008.03.032

Majarian, W. R., Daly, T. M., Weidanz, W. P., and Long, C. A. (1984). Passive immunization against murine malaria with an IgG3 monoclonal antibody. J. *Immunol*. 132, 3131–3137.

Mazeike, E., Gedvilaite, A., and Blohm, U. (2012). Induction of insert-specific immune response in mice by hamster polyomavirus VP1 derived virus-like particles carrying LCMV GP33 CTL epitope. *Virus Res*. 163, 2–10. doi: 10.1016/j.virusres.2011.08.003

Mettens, P., Dubois, P. M., Demoitié, M. A., Bayat, B., Donner, M. N., Bourguignon, P., et al. (2008). Improved T cell responses to *Plasmodium falciparum* circumsporozoite protein in mice and monkeys induced by a novel formulation of RTS,S vaccine antigen. *Vaccine* 26, 1072–1082. doi: 10.1016/j.vaccine.2007.12.018

Middelberg, A. P., Rivera-Hernandez, T., Wibowo, N., Lua, L. H., Fan, Y., Magor, G., et al. (2011). A microbial platform for rapid and low-cost virus-like particle and capsomere vaccines. *Vaccine* 29, 7154–7162. doi: 10.1016/j.vaccine.2011.05.075

Mishra, S., Nussenzweig, R. S., and Nussenzweig, V. (2012). Antibodies to Plasmodium circumsporozoite protein (CSP) inhibit sporozoite's cell traversal activity. *J. Immunol. Methods*. 377, 47–52. doi: 10.1016/j.jim.2012.01.009

Noe, A. R., Espinosa, D., Li, X., Coelho-Dos-Reis, J. G., Funakoshi, R., Giardina, S., et al. (2014). A full-length *Plasmodium falciparum* recombinant circumsporozoite protein expressed by *Pseudomonas fluorescens* platform as a malaria vaccine candidate. *PLoS ONE* 9:e107764. doi: 10.1371/journal.pone.0107764

Ohlschläger, P., Osen, W., Dell, K., Faath, S., Garcea, R. L., Jochmus, I., et al. (2003). Human papillomavirus type 16 L1 capsomeres induce L1-specific cytotoxic T lymphocytes and tumor regression in C57BL/6 mice. *J. Virol*. 77, 4635–4645. doi: 10.1128/JVI.77.8.4635-4645.2003

Olotu, A., Fegan, G., Wambua, J., Nyangweso, G., Leach, A., Lievens, M., et al. (2016). Seven-year efficacy of RTS,S/AS01 malaria vaccine among young african children. *N. Engl. J. Med*. 374, 2519–2529. doi: 10.1056/NEJMoa1515257

Olsen, T. M., Stone, B. C., Chuenchob, V., and Murphy, S. C. (2018). Prime-and-trap malaria vaccination to generate protective CD8(+) liver-resident memory T cells. *J. Immunol*. 201, 1984–1993. doi: 10.4049/jimmunol.1800740

Osier, F. H., Fegan, G., Polley, S. D., Murungi, L., Verra, F., Tetteh, K. K., et al. (2008). Breadth and magnitude of antibody responses to multiple *Plasmodium falciparum* merozoite antigens are associated with protection from clinical malaria. *Infect. Immun*. 76, 2240–2248. doi: 10.1128/IAI.01585-07

Qiu, F., and Cui, Z. (2007). CD4+ T helper cell response is required for memory in CD8+ T lymphocytes induced by a poly(I:C)-adjuvanted MHC I-restricted peptide epitope. *J. Immunother*. 30, 180–189. doi: 10.1097/01.cji.0000211330.61019.6f

Rivera-Hernandez, T., Hartas, J., Wu, Y., Chuan, Y. P., Lua, L. H., Good, M., et al. (2013). Self-adjuvanting modular virus-like particles for mucosal vaccination against group A *streptococcus* (GAS). *Vaccine* 31, 1950–1955. doi: 10.1016/j.vaccine.2013.02.013

Salem, M. L., Kadima, A. N., Cole, D. J., and Gillanders, W. E. (2005). Defining the antigen-specific T-Cell response to vaccination and poly (I:C)/TLR3 signaling - evidence of enhanced primary and memory CD8 T-Cell responses and antitumor immunity. *J. Immunother*. 28, 220–228. doi: 10.1097/01.cji.0000156828.75196.0d

Salunke, D. M., Caspar, D. L., and Garcea, R. L. (1986). Self-assembly of purified polyomavirus capsid protein VP1. *Cell* 46, 895–904. doi: 10.1016/0092-8674(86)90071-1

Schmidt, N. W., Butler, N. S., Badovinac, V. P., and Harty, J. T. (2010). Extreme CD8 T cell requirements for anti-malarial liver-stage immunity following immunization with radiation attenuated sporozoites. *PLoS Pathog*. 6:e1000998. doi: 10.1371/journal.ppat.1000998

Schmidt, N. W., Podyminogin, R. L., Butler, N. S., Badovinac, V. P., Tucker, B. J., Bahjat, K. S., et al. (2008). Memory CD8 T cell responses exceeding a large but definable threshold provide long-term immunity to malaria. *Proc. Natl. Acad. Sci. U.S.A*. 105, 14017–14022. doi: 10.1073/pnas.0805452105

Schneider-Ohrum, K., Giles, B. M., Weirback, H. K., Williams, B. L., DeAlmeida, D. R., and Ross, T. M. (2011). Adjuvants that stimulate TLR3 or NLPR3 pathways enhance the efficiency of influenza virus-like particle vaccines in aged mice. *Vaccine* 29, 9081–9092. doi: 10.1016/j.vaccine.2011.09.051

Schödel, F., Moriarty, A. M., Peterson, D. L., Zheng, J. A., Hughes, J. L., Will, H., et al. (1992). The position of heterologous epitopes inserted in hepatitis-B Virus core particles determines their immunogenicity. *J. Virol*. 66, 106–114.

Schussek, S., Groves, P. L., Apte, S. H., and Doolan, D. L. (2013). Highly sensitive quantitative real-time PCR for the detection of Plasmodium liver-stage parasite burden following low-dose sporozoite challenge. *PLoS ONE* 8:e77811. doi: 10.1371/journal.pone.0077811

Schussek, S., Trieu, A., Apte, S. H., Sidney, J., Sette, A., and Doolan, D. L. (2017). Novel *Plasmodium antigens* identified via genome-based antibody

screen induce protection associated with polyfunctional T cell responses. *Sci. Rep*. 7:18. doi: 10.1038/s41598-017-15354-0

Sedegah, M., Hedstrom, R., Hobart, P., and Hoffman, S. L. (1994). Protection against malaria by immunization with plasmid DNA encoding circumsporozoite protein. *Proc. Natl. Acad. Sci. U.S.A*. 91, 9866–9870. doi: 10.1073/pnas.91.21.9866

Seder, R. A., and Hill, A. V. (2000). Vaccines against intracellular infections requiring cellular immunity. *Nature* 406, 793–798. doi: 10.1038/35021239

Sedlik, C., Saron, M., Sarraseca, J., Casal, I., and Leclerc, C. (1997). Recombinant parvovirus-like particles as an antigen carrier: a novel nonreplicative exogenous antigen to elicit protective antiviral cytotoxic T cells. *Proc. Natl. Acad. Sci. U.S.A*. 94, 7503–7508. doi: 10.1073/pnas.94.14.7503

Seth, A., Kong, I. G., Lee, S. H., Yang, J. Y., Lee, Y. S., Kim, Y., et al. (2016). Modular virus-like particles for sublingual vaccination against group A *streptococcus*. *Vaccine* 34, 6472–6480. doi: 10.1016/j.vaccine.2016.11.008

Snapper, C. M., and Mond, J. J. (1993). Towards a comprehensive view of immunoglobulin class switching. *Immunol. Today* 14, 15–17. doi: 10.1016/0167-5699(93)90318-F

Stanley, M., Lowy, D. R., and Frazer, I. (2006). Chapter 12: prophylactic HPV vaccines: underlying mechanisms. *Vaccine* 24(Suppl 3), 106–113. doi: 10.1016/j.vaccine.2006.05.110

Stanley, M. A. (2006). Human papillomavirus vaccines. *Rev. Med. Virol*. 16, 139–149. doi: 10.1002/rmv.498

Storni, T., Lechner, F., Erdmann, I., Bächi, T., Jegerlehner, A., Dumrese, T., et al. (2002). Critical role for activation of antigen-presenting cells in priming of cytotoxic T cell responses after vaccination with virus-like particles. *J. Immunol*. 168, 2880–2886. doi: 10.4049/jimmunol.168.6.2880

Szomolanyi-Tsuda, E., and Welsh, R. M. (1998). T-cell-independent antiviral antibody responses. *Curr. Opin. Immunol*. 10, 431–435. doi: 10.1016/S0952-7915(98)80117-9

Tamminga, C., Sedegah, M., Regis, D., Chuang, I., Epstein, J. E., Spring, M., et al. (2011). Adenovirus-5-vectored *P. falciparum* vaccine expressing CSP and AMA1. Part B: safety, immunogenicity and protective efficacy of the CSP component. *PLoS ONE* 6:e25868. doi: 10.1371/journal.pone.0025868

Tarun, A. S., Peng, X., Dumpit, R. F., Ogata, Y., Silva-Rivera, H., Camargo, N., et al. (2008). A combined transcriptome and proteome survey of malaria parasite liver stages. *Proc. Natl. Acad. Sci. U.S.A*. 105, 305–310. doi: 10.1073/pnas.0710780104

Tegerstedt, K., Lindencrona, J. A., Curcio, C., Andreasson, K., Tullus, C., Forni, G., et al. (2005). A single vaccination with polyomavirus VP1/VP2Her2 virus-like particles prevents outgrowth of HER-2/neu-expressing tumors. *Cancer Res*. 65, 5953–5957. doi: 10.1158/0008-5472.CAN-05-0335

Tekewe, A., Fan, Y., Tan, E., Middelberg, A. P., and Lua, L. H. (2017). Integrated molecular and bioprocess engineering for bacterially produced immunogenic modular virus-like particle vaccine displaying 18 kDa rotavirus antigen. *Biotechnol. Bioeng*. 114, 397–406. doi: 10.1002/bit.26068

Tewari, K., Flynn, B. J., Boscardin, S. B., Kastenmueller, K., Salazar, A. M., Anderson, C. A., et al. (2010). Poly(I:C) is an effective adjuvant for antibody and multi-functional CD4+ T cell responses to *Plasmodium falciparum* circumsporozoite protein (CSP) and alphaDEC-CSP in non human primates. *Vaccine* 28, 7256–7266. doi: 10.1016/j.vaccine.2010.08.098

Thönes, N., Herreiner, A., Schädlich, L., Piuko, K., and Müller, M. (2008). A direct comparison of human papillomavirus type 16 L1 particles reveals a lower immunogenicity of capsomeres than viruslike particles with respect to the induced antibody response. *J. Virol.* 82, 5472–5485. doi: 10.1128/JVI.02482-07

Tinto, H., D'alessandro, U., Sorgho, H., Valea, I., Tahita, M. C., Kabore, W., et al. (2015). Efficacy and safety of RTS,S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: final results of a phase 3, individually randomised, controlled trial. *Lancet* 386, 31–45. doi: 10.1016/S0140-6736(15)60721-8

Walsh, D. S., Gettayacamin, M., Leitner, W. W., Lyon, J. A., Stewart, V. A., Marit, G., et al. (2006). Heterologous prime-boost immunization in rhesus macaques by two, optimally spaced particle-mediated epidermal deliveries of *Plasmodium falciparum* circumsporozoite protein-encoding DNA, followed by intramuscular RTS,S/AS02A. *Vaccine* 24, 4167–4178. doi: 10.1016/j.vaccine.2006.02.041

Weiss, W. R., Berzofsky, J. A., Houghten, R. A., Sedegah, M., Hollindale, M., and Hoffman, S. L. (1992). A T-cell clone directed at the circumsporozoite protein which protects mice against both Plasmodium-yoelii and Plasmodium-berghei. *J. Immunol*. 149, 2103–2109.

Weiss, W. R., Mellouk, S., Houghten, R. A., Sedegah, M., Kumar, S., Good, M. F., et al. (1990). Cytotoxic T cells recognize a peptide from the circumsporozoite protein on malaria-infected hepatocytes. *J. Exp. Med*. 171, 763–773. doi: 10.1084/jem.171.3.763

Weiss, W. R., Sedegah, M., Beaudoin, R. L., Miller, L. H., and Good, M. F. (1988). CD8+ T cells (cytotoxic/suppressors) are required for protection in mice immunized with malaria sporozoites. *Proc. Natl. Acad. Sci. U.S.A*. 85, 573–576. doi: 10.1073/pnas.85.2.573

Weiss, W. R., Sedegah, M., Berzofsky, J. A., and Hoffman, S. L. (1993). The role of CD4+ T cells in immunity to malaria sporozoites. *J. Immunol*. 151, 2690–2698.

Wibowo, N., Hughes, F. K., Fairmaid, E. J., Lua, L. H., Brown, L. E., and Middelberg, A. P. (2014). Protective efficacy of a bacterially produced modular capsomere presenting M2e from influenza: extending the potential of broadly cross-protecting epitopes. *Vaccine* 32, 3651–3655. doi: 10.1016/j.vaccine.2014.04.062

World Malaria Report (2017). World Health Organization. Available online at: http://www.who.int/malaria/publications/world-malaria-report-2017/en/ (accessed January 25, 2019).

Yoshida, K., Iyori, M., Blagborough, A. M., Salman, A. M., Dulal, P., Sala, K. A., et al. (2018). Adenovirus-prime and baculovirus-boost heterologous immunization achieves sterile protection against malaria sporozoite challenge in a murine model. *Sci. Rep*. 8:3896. doi: 10.1038/s41598-018-21369-y

Conflict of Interest Statement: The University of Queensland (UQ) filed patents on the use of MuPyV as a vaccine platform. LL and AM contributed to those patents and, through their employment with UQ, hold an indirect interest in this intellectual property.

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 Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 20 June 2019
doi: 10.3389/fcimb.2019.00203

Delineating the Plausible Molecular Vaccine Candidates and Drug Targets of Multidrug-Resistant *Acinetobacter baumannii*

Shama Mujawar[1†], *Rohit Mishra*[2†], *Shrikant Pawar*[3,4], *Derek Gatherer*[5] *and Chandrajit Lahiri*[1*]

[1] Department of Biological Sciences, Sunway University, Petaling Jaya, Malaysia, [2] Department of Bioinformatics, University of Mumbai, Mumbai, India, [3] Department of Computer Science, Georgia State University, Atlanta, GA, United States, [4] Department of Biology, Georgia State University, Atlanta, GA, United States, [5] Division of Biomedical and Life Sciences, Lancaster University, Lancaster, United Kingdom

OPEN ACCESS

Edited by:
Alberto Moreno,
Emory University School of Medicine,
United States

Reviewed by:
Gyanendra Prakash Dubey,
Institut Pasteur, France
Muhammad Ammar Zafar,
Wake Forest School of Medicine,
United States

***Correspondence:**
Chandrajit Lahiri
chandrajitl@sunway.edu.my

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Clinical Microbiology, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: 01 February 2019
Accepted: 23 May 2019
Published: 20 June 2019

Citation:
Mujawar S, Mishra R, Pawar S, Gatherer D and Lahiri C (2019) Delineating the Plausible Molecular Vaccine Candidates and Drug Targets of Multidrug-Resistant Acinetobacter baumannii. Front. Cell. Infect. Microbiol. 9:203. doi: 10.3389/fcimb.2019.00203

Nosocomial infections have become alarming with the increase of multidrug-resistant bacterial strains of *Acinetobacter baumannii*. Being the causative agent in ∼80% of the cases, these pathogenic gram-negative species could be deadly for hospitalized patients, especially in intensive care units utilizing ventilators, urinary catheters, and nasogastric tubes. Primarily infecting an immuno-compromised system, they are resistant to most antibiotics and are the root cause of various types of opportunistic infections including but not limited to septicemia, endocarditis, meningitis, pneumonia, skin, and wound sepsis and even urinary tract infections. Conventional experimental methods including typing, computational methods encompassing comparative genomics, and combined methods of reverse vaccinology and proteomics had been proposed to differentiate and develop vaccines and/or drugs for several outbreak strains. However, identifying proteins suitable enough to be posed as drug targets and/or molecular vaccines against the multidrug-resistant pathogenic bacterial strains has probably remained an open issue to address. In these cases of novel protein identification, the targets either are uncharacterized or have been unable to confer the most coveted protection either in the form of molecular vaccine candidates or as drug targets. Here, we report a strategic approach with the 3,766 proteins from the whole genome of *A. baumannii* ATCC19606 (AB) to rationally identify plausible candidates and propose them as future molecular vaccine candidates and/or drug targets. Essentially, we started with mapping the vaccine candidates (VaC) and virulence factors (ViF) of *A. baumannii* strain AYE onto strain ATCC19606 to identify them in the latter. We move on to build small networks of VaC and ViF to conceptualize their position in the network space of the whole genomic protein interactome (GPIN) and rationalize their candidature for drugs and/or molecular vaccines. To this end, we propose new sets of known proteins

unearthed from interactome built using key factors, KeF, potent enough to compete with VaC and ViF. Our method is the first of its kind to propose, *albeit* theoretically, a rational approach to identify crucial proteins and pose them for candidates of vaccines and/or drugs effective enough to combat the deadly pathogenic threats of *A. baumannii*.

Keywords: *Acinetobacter baumannii*, nosocomial infection, vaccine candidates, drug targets, network analysis

INTRODUCTION

Nosocomial or hospital-acquired infections are among the multitude of diseases caused by the opportunistic pathogen *Acinetobacter baumannii,* one of the world's six most important multidrug-resistant (MDR) microorganisms identified in hospitals (Talbot et al., 2006; Lin and Lan, 2014). While critically ill patients in the intensive care unit (ICU) accounted for up to 20% of ventilator-associated pneumonia and bloodstream infections with *A. baumannii* (Fournier and Richet, 2006; Vincent et al., 2009), the mortality rates might reach up to 35% along with endocarditis, meningitis, skin and wound sepsis, and even urinary tract infections for immunosuppressive patients (Lin and Lan, 2014; Darvishi, 2016). The cases for treatment are complicated due to the fact that the severity of the infection depends on the site and the patient's susceptibility to such diseases (Antunes et al., 2014). Moreover, there exist numerous natural reservoirs for *A. baumannii* including natural and agricultural soil, vegetables, aquaculture, and other inanimate objects outside the hospital environment (Eveillard et al., 2013).

The complications, resulting in an array of diseases caused by *A. baumannii*, arise from a plethora of virulence factors used by the pathogen to access and colonize the host system. These include, but are obviously not limited to, porins, capsular polysaccharides, lipopolysaccharides, phospholipases, outer membrane vesicles (OMVs), metal acquisition systems, and protein secretion systems (Lee et al., 2017). Besides these, β-lactamases acquisition, efflux pumps up-regulation, aminoglycosides modification, target sites alteration, and permeability defects are the crucial factors guiding the mechanism of antibiotic resistance conferred by this pathogen (Lee et al., 2017). Further threats of infection arise from colonization outside the human host, mainly on medical devices, through the mechanism of biofilm production involving the associated pathways, proteins, secretion systems, and quorum sensing (Perez et al., 2007). With such a robust antibiotic resistance mechanism entailing a barrage of proteins comprising the host invading machinery, *A. baumannii* has been able to confer extensive drug resistance (XDR). In fact, such ability has gone to the extent of evading almost every new-generation antibiotic, including carbapenems, which used to be prescribed to treat MDR organismal infections (Viehman et al., 2014).

To cater to the need of addressing the urgent and pressing issues of antibiotic threats, vaccine development has been resorted to as one of the cost-effective and most promising strategies to prevent infections. This is due to the fact that inactivated whole cells and attenuated strains are able to elicit antibodies against multiple surface proteins, which can be utilized in the form of vaccines, to combat the antibiotic threats. In fact, efforts have been tested to utilize only parts of the pathogen without the administration of whole organisms. Thus, vaccines comprising multiple proteins of the bacterial outer membrane complex (OMC), OMVs, OmpA, auto-transporter (Ata), biofilm-associated protein (Bap), K1 capsular polysaccharide, and poly-*N*-acetyl-β-(1-6)-glucosamine (PNAG) have been shown to elicit antibodies and to induce protective immunity against infection, thereby giving promising results in early human clinical trials (Bertot et al., 2007; Fransen et al., 2007; Chiang et al., 2015). Moreover, recent studies to determine potential vaccine targets have delineated a combinatorial approach of *in silico* prediction tools with reverse vaccinology through comparative genome analysis and *in vitro* proteomics (Moriel et al., 2013; Singh et al., 2017). For instance, *in silico* analysis in *A. baumannii* helped the identification of a highly conserved outer membrane protein with β-barrel assembly, BamA, as the potential target for vaccine (Singh et al., 2017). However, the advent of new and emerging XDR strains of *A. baumannii*, which possibly arises from immune selection, might lead to antigen sequence variability and a down-regulation of the target antigens, thereby conferring poor "cross-protective efficacy" (Chiang et al., 2015). Therefore, the identification of potential antigens, expressed by the new and emerging *A. baumannii* strains during infections, still keeps the issue quite complex to be addressed.

Simplifying the complexity of diseases, caused by such XDR pathogens like *A. baumannii*, is thus challenging. Therefore, to unearth plausible antigenic proteins, potential enough to elicit an antibody response, a detailed analysis of the complex interaction of the proteins, involved in the disease phenomenon, might be helpful. Earlier attempts to computationally analyze such protein interaction networks or interactomes (PIN) of infectious diseases mostly focused on network centrality parametric values for the identification of candidate drug targets (Lahiri et al., 2014; Pan et al., 2015). Generally, in biological networks, centrality measures of degree (DC), betweenness (BC), closeness (CC), and eigenvector (EC) have been used extensively (Jeong et al., 2001; Lahiri et al., 2014; Pan et al., 2015). While DC gives a very basic understanding of the number of interacting partners of a particular protein, EC relates to the essential proteins interacting with other crucial partners in the disease phenomenon (Lahiri et al., 2014). Besides BC, EC has been shown to be a good target for drugs, albeit theoretically for infectious diseases and utilized in the identification of side effect-free drug targets of idiopathic diseases like cancer (Lahiri et al., 2014; Pan et al., 2015; Ashraf et al., 2018). However, in order to gain an insight into the global scenario of the disease complexity, the PIN needs to be

inspected thoroughly for an effective analysis, potential enough to be translational in nature. Thus, the whole genome protein interactome (GPIN) has been utilized to prune and decompose to obtain a core of highly interacting proteins through the k-core analysis approach (Seidman, 1983). This coupled with the functional module-based cartographic analyses of the global network (Guimerà and Nunes Amaral, 2005a) has already been adopted to theoretically identify potential role players in bacterial infectious diseases (Pawar et al., 2017, 2018).

In this study, we have similarly delineated the relevance of the aforementioned centrality parametric measures for PIN of the claimed vaccine candidates and virulence factors (Moriel et al., 2013) of *A. baumannii*, namely, VaCAB and ViFAB. To this end, we have analyzed KeFAB, the PIN of key factors responsible for virulence and pathogenicity of *A. baumannii* (Chen et al., 2015). The top rankers of these three PINs were mapped onto the GPIN of *A. baumannii* to unravel their position in the network space and rationalize their candidature for drugs and/or molecular vaccines compared to our own sets of proposed candidates for vaccines. We consolidate our findings by the antigenic potential of these proteins along with their active sites for drug targets. In summary, we analyze the PIN of different relevant pathogenic proteins of *A. baumannii* to identify the plausible potential candidates for vaccines and/or drugs targets.

MATERIALS AND METHODS

Acronyms and Terms Utilized

The present study on *A. baumannii* comprises a conglomerate of several terms pertaining to graph theory in general and network biology in particular. **Table 1** lists them all in a comprehensive manner with an expansion of the acronym, where applicable, followed by a short description of the terms for the ease of reference of a broad interdisciplinary range of readers.

Dataset Collection and Processing

Datasets for *A. baumannii* proteins were collected in different ways. The focus was given for either the strains AYE or ATCC17978, having either substantial reports or evidences for causing MDR. For the strain AYE, 168 proteins mentioned in the **Supplementary Data 2** by Moriel et al. (2013) were collected and categorized as vaccine candidates (VaC, **Supplementary Data 1**) while 124 from **Supplementary Datas 3–5** reported by the same group were collated as virulent factors (ViF, **Supplementary Data 2**). Other proteins were collected using keywords used by default for the available strains of ATCC17978 and AYE in VFDB (Chen et al., 2015). To this end, using the same keywords, further proteins of these strains were collected through direct literature search and the sequences of all these were retrieved from UniProt database (Apweiler et al., 2004). The last category proteins from VFDB, UniProt, and literature were parked under key factors (KeF, **Supplementary Data 3**). The counts of identifiers (IDs) for the various KeF proteins were 973 for ATCC17978, 92 for AYE, and 24 for no IDs found in the literature. Nine of these IDs, belonging to t-RNA, were eliminated, leaving a final total of 1,078 KeF (**Figure 1**).

The datasets of the aforementioned VaC, ViF, and KeF proteins were converted into the counterparts of the corresponding *A. baumannii* strain ATCC19606, due to the reason of non-availability of ATCC17978 and AYE strains in the STRING 10.5 biological meta-database of protein interaction (Szklarczyk et al., 2016). A FASTA file containing a total of 3,376 protein sequences, downloaded from STRING for *A. baumannii* strain ATCC19606, was used as a database for standalone BLAST during mapping of the sequences of strain AYE onto strain 19606. Standalone BLAST is a non-graphic user interface version of BLAST that runs on command lines in Linux operating system (OS) and allows execution of BLAST locally on such OS for sequence alignment. The execution of such BLAST, returned 2,111 and 1,676 hits for VaC and ViF, respectively. A filtering of the topmost ones with the highest percentage of identities from these hits returned 168 and 123 proteins, respectively, of the strain 19606. A further threshold cutoff of 99–100% identity was set to select out the identical proteins of strain AYE in 19606 for the next set of analysis, leaving out other ambiguous ones (duplicates and/or percentage-wise less identical) to obtain 79 VaC and 78 ViF proteins of strain 19606. Similar approaches were adopted for selecting out the KeF proteins of the strain 19606 from those of the strains ATCC17978 and AYE. For this, 1,078 KeF protein sequences were executed in standalone BLAST to yield a total of 15,793 hits, from which the top-ranked 1,075 proteins, with highest percentage identity, were selected out. Final cutoff of 99–100% identity was used to remove ambiguous ones and duplicates to obtain 640 IDs for further processing (**Figure 1**).

Submission of these mapped proteins of ATCC19606 into STRING helped the retrieval of protein interaction datasets having the default medium (0.4) level confidence upon the interaction (period of access: August to September 2018). The detailed protein links file containing the interaction datasets for the whole genome of *A. baumannii* strain ATCC19606 was retrieved for the accession number 575584 in STRING. All interaction datasets for each category of VaC, ViF, and KeF proteins, hereafter used for PIN construction, have been listed in **Supplementary Datas 1–3**.

Interactome Construction

After the removal of duplicate interactions, all individual interaction data obtained as above were imported for construction and visualization of the small PINs (SPINs), which were named VaCAB, ViFAB, and KeFAB, and the whole genome PIN named GPIN, using Cytoscape version 3.6.0 (Shannon et al., 2003) and Gephi 0.9.2 (Bastian et al., 2009) (**Figure 2**; **Supplementary Datas 1–3**: Sheets 5–7). All interactomes were considered to be non-directional in nature to represent undirected graphs as $G = (V, E)$, where V are finite set of vertices and E are edges in which $e = (u,v)$ connecting two vertices (nodes), u and v, or proteins in the present context. Thus, the degree, d (v), indicates the number of interactions (physical and functional) a protein has with other proteins (Diestel, 2000).

TABLE 1 | The acronyms and terms used in the present study.

Acronyms/Terms	Description
VaC	**Va**ccine **C**andidates; proteins reported by Moriel *et al*. from *Acinetobacter baumannii* strain AYE and ATCC17978
ViF	**Vi**rulent **F**actors; proteins reported by Moriel *et al*. from *A. baumannii* strain AYE and ATCC17978
KeF	**Ke**y **F**actors; proteins retrieved from various databases and literature survey from strain AYE and ATCC17978 using common terms related to virulence
VFDB	**Vi**rulent **F**actors **D**ata**b**ase; database of several bacteria listing the known and/or predicted virulent factors as per literature
STRING	**S**earch **T**ool for the **R**etrieval of **In**teracting **G**enes/Proteins; meta-database listing known and predicted protein–protein interactions from biological organisms
Cytoscape/Gephi	Interacting Software for visualizing node and edges of a graph and integrating them together
PIN	**P**rotein **I**nteraction **N**etwork; interaction pattern of proteins in a graphical form giving a network of nodes and edges
Interactome	A network of interacting entities; in the present study, this refers to Protein Interactome or PIN
Interactors	The molecules interacting in a network; in the present study, these are proteins interacting with VaC, ViF, or KeF
VaCAB	**VaC** PIN of ***A. b****aumannii*; interactome of VaC with their interactors of strain ATCC19606 retrieved from STRING and visualized through Cytoscape/Gephi
ViFAB	**ViF** PIN of ***A. b****aumannii*; interactome of ViF with their interactors of strain ATCC19606 retrieved from STRING and visualized through Cytoscape/Gephi
KeFAB	**KeF** PIN of ***A. b****aumannii*; interactome of KeF with their interactors of strain ATCC19606 retrieved from STRING and visualized through Cytoscape/Gephi
SPIN	**S**mall PIN of ***A. b****aumannii*; this refers to VaCAB/ViFAB/KeFAB of *A. baumannii* strain ATCC19606
GPIN	**G**enomic **PIN** of *A. baumannii*; interactome of the total number of proteins of whole genome of *A. baumannii* strain ATCC19606 retrieved from STRING and visualized through Cytoscape/Gephi
Network Analyser	Java Plug-in for Cytoscape for graph theoretical analysis of the network of nodes and edges
CytoNCA	**Cyto**scape **N**etwork **C**entrality **A**nalyser; Cytoscape Java Plug-in for graph theoretical analysis of the network of nodes and edges
Centrality Measures	Graph theoretical measures for assessing the central character of nodes
BC/CC/DC/EC	Betweenness/Closeness/Degree/Eigenvector centrality measures; reflect measures for nodes of central passage/proximity/connectivity/weightage, respectively
k-Core	Maximally connected sub-graph of nodes having at least k-degree; achieved by gradually pruning all nodes of degree less than k
Modules	Sub-graphs of nodes having common aspects with respect to common biological functions
Functional Connectivity	Different classes of within-module and between-module connectivity
KFC	An approach combining k-core, functional connectivity, and centrality measures
Standalone BLAST	Offline **B**asic **L**ocal **A**lignment **S**earch **T**ool; this comprises a downloadable version of the sequence alignment tool that can be installed locally on system and can be used with command lines to find the match/mismatch/gap between the given sequences
COG	**C**luster of **O**rthologous **G**roups; similar groups of proteins having related functions

PIN Analyses

SPIN

Each of the constructed three SPINs were subsequently analyzed utilizing BC, CC, DC, and EC measures of centrality, commonly applied to biological networks (Pavlopoulos et al., 2011) (**Supplementary Datas 1–3**). This was done with the Cytoscape integrated java plugins, namely, Network Analyzer and CytoNCA (Assenov et al., 2007; Tang et al., 2015), utilizing the edge weights as the combined scores obtained from different parameters in STRING. These combined scores, which range from 0 to 1, generally convey the confidence of the protein's interaction with level of parametric evidences from gene neighborhood, gene fusion, gene co-occurrence, gene co-expression, experiments, annotated pathways, and text mining. Using the top 15 proteins appearing for each measures of analysis as mentioned above, a commonality of the proteins for all centrality measures was observed through Venny 2.1 (Oliveros, 2007–2015) (**Figure 3**).

GPIN

The whole genome protein interaction network, GPIN, was analyzed by MATLAB version 7.11 (MATLAB Statistics Toolbox Release, 2010). An idea of the simplest of the technical aspects of the GPIN, i.e., the distributions of network degree (k), was obtained by plotting it against the Complementary Cumulative Distribution Function (CCDF) (**Figure 4A**). A k-core analysis of the whole genome was done by a network decomposition method that produces a gradually increasing cohesive sequence of sub-graphs and reveals the number of proteins having at least k-degree classified in K-shell as per their interacting patterns (**Figures 4B,C**) (Seidman, 1983). Further knowledge of the connectivity and participation of each protein, with respect to their functions, was obtained through analysis of the cartographic representation of the network topology that plots the within-module degree z score of the protein against its participation coefficient, P (**Figure 5**) (Guimerà and Amaral, 2005b). The participation of each protein in a modular network, sharing common biological function, gives rise to a concept of functional module having high intra-connectivity and sparse inter-connectivity (Vella et al., 2018). Calculations of such functional modules as per Rosvall method (Rosvall and Bergstrom, 2011) led to a major classification of the proteins of GPIN into

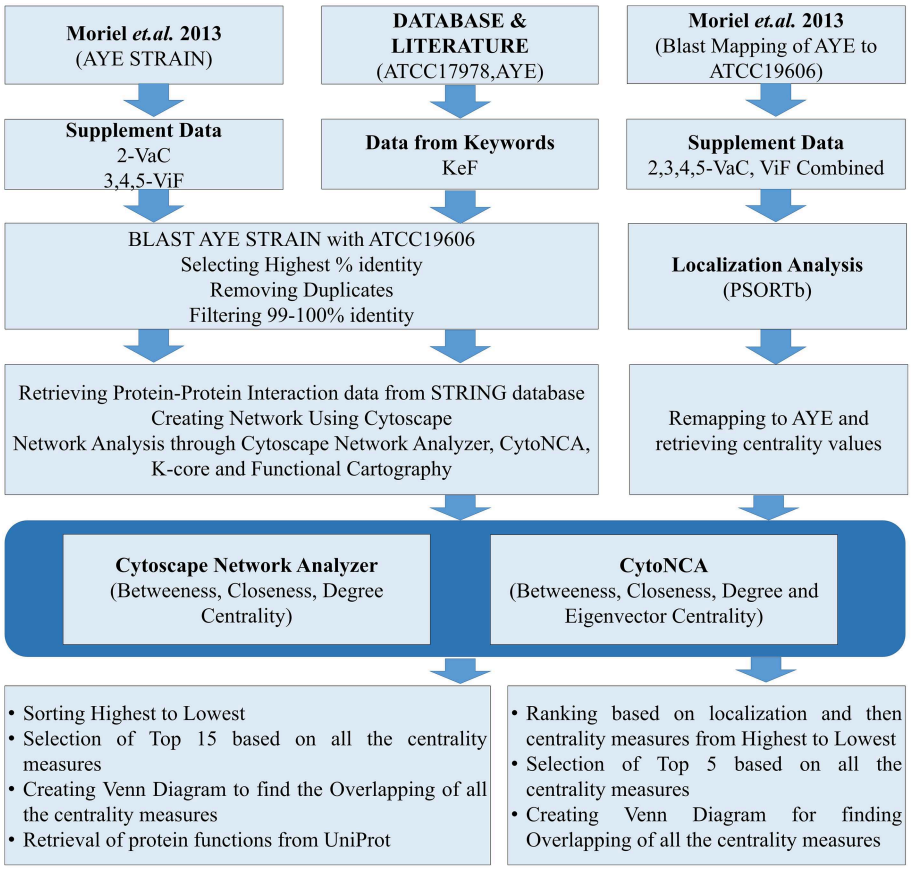


FIGURE 1 | The flow chart for the whole process of the present study.

non-hub and hub nodes, each having further sub-classes. These are ultra-peripheral (R1), peripheral (R2), non-hub connector (R3), and non-hub kinless (R4) for the former while provincial (R5), connector (R6), and kinless (R7) for the latter categories (Guimerà and Amaral, 2005b) (**Figure 5**; **Supplementary Data 4**).

Vaccine and/or Drug Candidature Prediction

The network analyzed and shortlisted VaC, ViF, and KeF proteins along with their interactors from the three SPINs, namely, VaCAB, ViFAB, and KeFAB, were subjected to further analyses for predicting the plausible vaccine and/or drug candidates. To this end, the indispensable proteins of the GPIN were also taken into consideration as per the KFC method described by Ashraf et al. (2018). All such proteins were explored for their cellular localization, signal peptide prediction, COG classification, antigenic site prediction, followed by active site prediction. Cellular localization was analyzed by PSORTb v3.0.2 (Yu et al., 2010). Location of signal peptides was predicted using the server called SignalP 4.1 Server (Petersen et al., 2011). Lipoprotein signal peptides were predicted using the LipoP 1.0 Server (Juncker et al., 2003). For uncharacterized proteins, functional annotation with classification was done through the WebMGA server (Wu et al., 2011). Such COG classification from WebMGA was performed for all proteins, however, even if they are characterized, to maintain unanimity of comparison. To predict the antigenic potential of the candidate proteins, epitope prediction was done through the immune epitope database (IEDB) resource utilizing Bepipred Linear Epitope Prediction and maintaining a threshold cutoff of 0.75 for increased sensitivity (Vita et al., 2014). Furthermore, without any solved X-ray crystallographic or NMR 3D structures for the selected proteins, they were homology modeled and validated to pursue active site prediction studies. We have used Phyre2 (Kelley et al., 2015) and SWISS MODEL (Schwede et al., 2003) protein modeling servers to generate the structures and the integrated Procheck server in the latter, to evaluate them through Ramachandran plot, Q mean score, and z score. To identify the best structure of the different models generated by the abovementioned servers, we have performed consensus studies. These consensus models were finally utilized to determine the active sites or binding pockets of the selected proteins by the CASTp server (Computer Atlas of Surface Topology of protein) (Dundas et al., 2006). A comparative account of the results obtained from this section of the analysis is shown in **Table 7**.

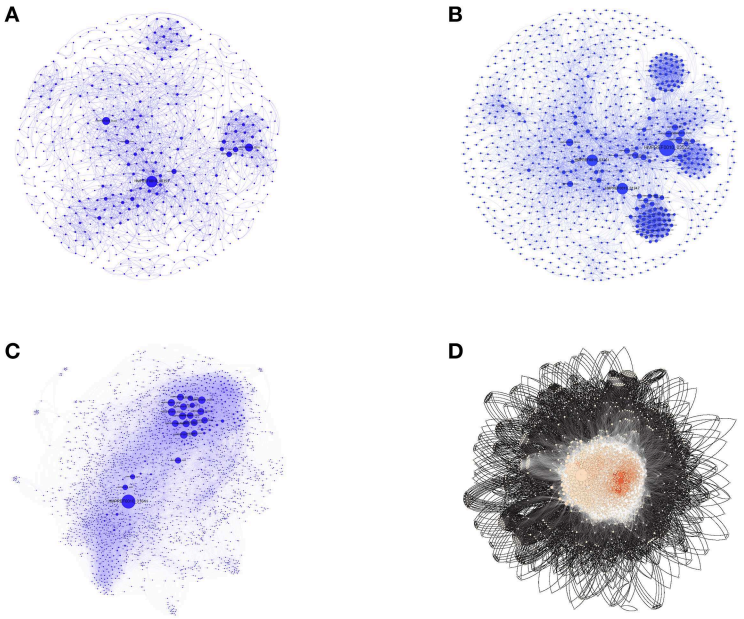


FIGURE 2 | The three SPINs and GPINs of *A. baumannii* reflecting the degree of connectivity. SPINs are represented in blue spheres connected through blue-colored curved lines for **(A)** VaCAB, having vaccine candidates; **(B)** ViFAB, with virulent factors; and **(C)** KeFAB, with key factors each with their interactors. **(D)** GPIN with proteins represented in black spheres connected with black curved lines to form the interactome.

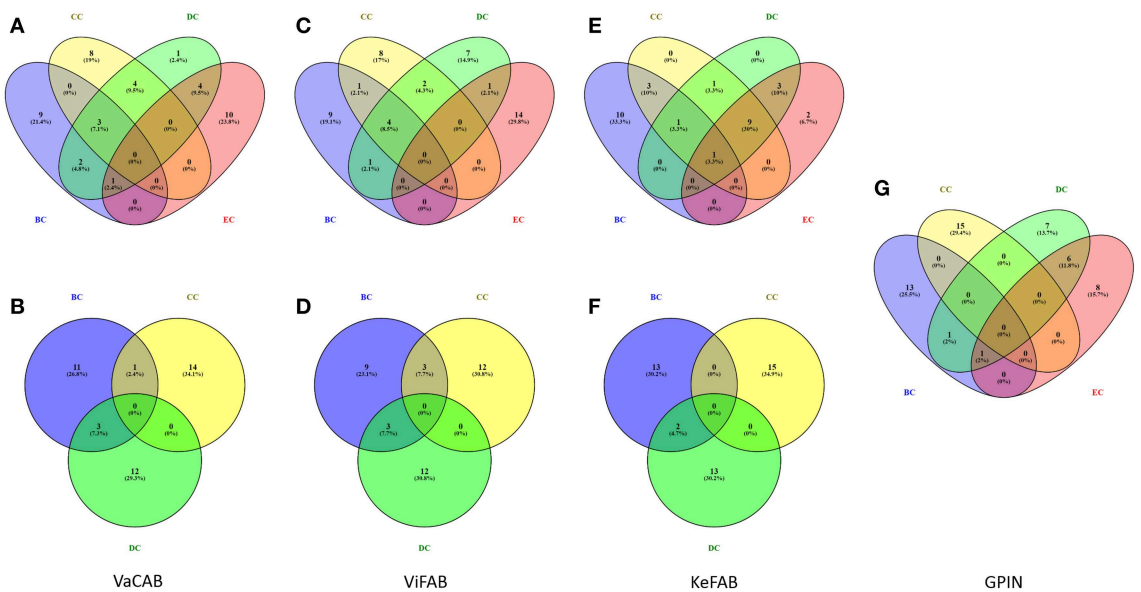


FIGURE 3 | Venn diagram representation for the top-ranking network centrality measures of SPIN and GPIN of *A. baumannii*. **(A,B)** VaCAB, **(C,D)** ViFAB, **(E,F)** KeFAB, and **(G)** GPIN. Measures of four types of centrality are from CytoNCA and three types are from Network Analyzer. BC, CC, DC, and EC denote betweenness centrality, closeness centrality, degree centrality, and eigenvector centrality, respectively.

Interactor-Free Candidature Analyses

In order to determine the important vaccine candidates among a barrage of proteins proposed by Moriel et al. (2013), filtration was used to remove the influence of the interactors on the actual candidates upon their ranking. For the same, we have initially merged the VaCAB and ViFAB together, having their individual centrality-based analyzed data. Thereafter, the VaC and ViF interactors, of the respective PIN, were removed, leaving behind the actual candidates. As the PINs (VaCAB and ViFAB) were constructed from STRING data using the strain ATCC19606, we mapped back the centrality measures of the total VaC and ViF proteins onto the AYE strain of *A. baumannii*. This was followed

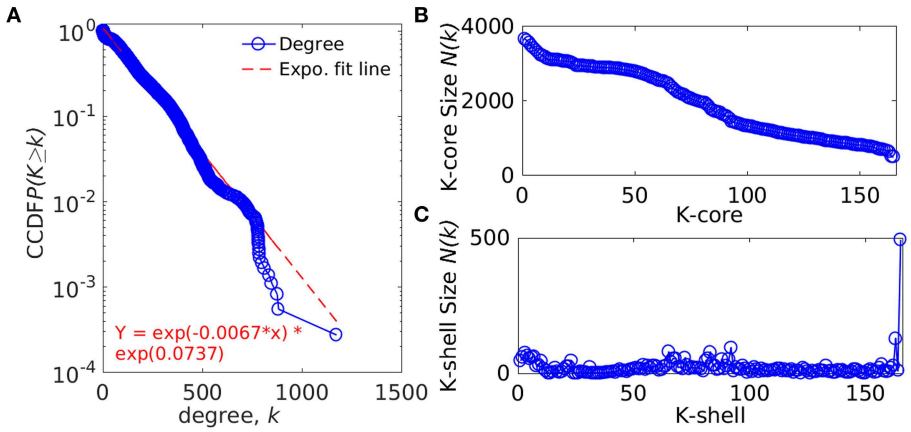


FIGURE 4 | Network topological measures for the set of proteins from the GPIN of *A. baumannii*. **(A)** The degree distribution, **(B)** *k*-core distribution, and **(C)** K-shell sizes. CCDF denotes Complementary Cumulative Distribution Function.

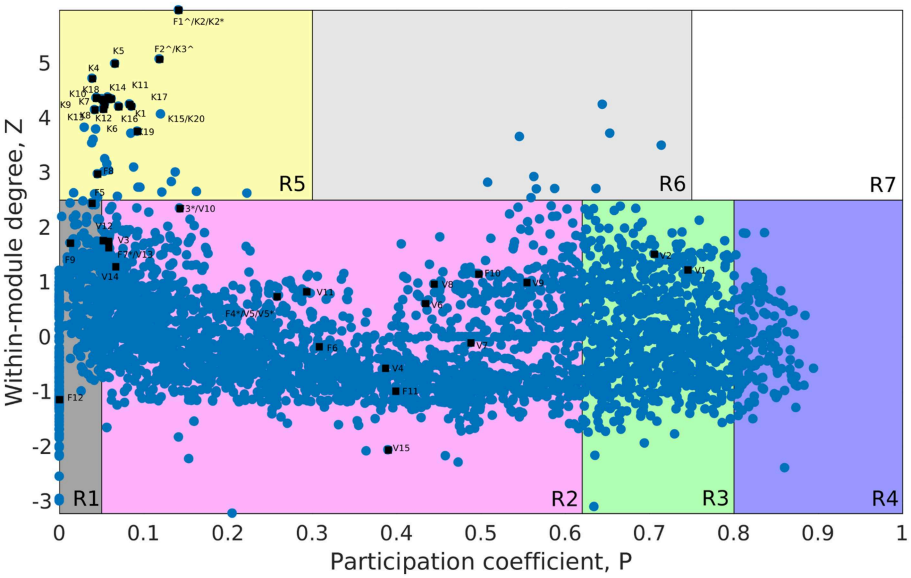


FIGURE 5 | Cartographic representation for the set of classified proteins from the GPIN of *A. baumannii*. Designated quadrants from R1 to R7 (in random colors) comprise nodes in each representing different classes of proteins. Selected Vaccine Candidates, VaC (V), Virulent Factors, ViF (F), and Key Factors, KeF (K) proteins from network centrality analyzed SPIN (Small PIN) are mapped onto different quadrants as deemed fit in GPIN (Genome PIN). ^ and * represent proteins shared between different centralities only and those also between different categories, respectively.

by assigning the localization status of the total set of candidate proteins through the usage of PSORTb. Sorting was done based on localization followed by descending order of the centrality measures. Top five rankers, in each case, were used for creating a Venn diagram to find out the most promising candidates from the intersection of the centrality measures (**Figure 1**).

RESULTS

The Candidate Proteins

In order to the identify the potential molecular vaccine candidates and drug targets of MDR *A. baumannii*, we have started with the available pathogenic strains, namely, AYE and ATCC17978, to accumulate the proclaimed proteins in categories of VaC, ViF, and KeF with counts of 168, 124, and 1,078, respectively (**Supplementary Datas 1–3**: Sheet 1). As these strains are not enlisted in the protein interaction database, STRING, we had to find the real counterparts of these proteins, from the only listed strain ATCC19606. An initial processing of the data through standalone blast from the total protein sets of ATCC19606 yielded 168, 123, and 1,075 VaC, ViF, and KeF candidates, respectively (**Supplementary Datas 1–3**: Sheet 3), which were further filtered for an exact match of 99–100% to result in 79, 78, and 640 proteins, respectively (**Supplementary Datas 1–3**: Sheet 4). Thus, all the candidate proteins were mapped onto ATCC19606 from other pathogenic

strains. All data, hereafter, refer to strain ATCC19606 only, unless otherwise stated.

The Three Individual SPINs

To identify the crucial candidates among the three aforementioned sets of VaC, ViF, and KeF proteins of *A. baumannii*, we have taken an approach of building three different SPINs, each with its respective set of interaction data (**Figure 2**; **Supplementary Datas 1–3**: Sheets 5–7). Upon analyzing these SPINs through the Cytoscape plugins CytoNCA and Network Analyzer, the top rank holders as per each centrality measures are recorded (**Supplementary Datas 1–3**: Sheets 8–11), indicating their importance. A combined result from both the plugins are presented in **Table 2**, wherein protein entries are represented by their NCBI gene accession numbers with the suffix "HMPREF0010_" being replaced by "AB_" for the ease of further use. It is to be noted that there are overlaps of these protein entries across the different centrality measures and/or the different categories of VaCAB, ViFAB, and KeFAB. An overlap of the proteins across the measures within a particular category is represented by a "caret" (^) notation while that from cross-categories is denoted with an "asterisk" (*). For instance, the protein AB_03493 is associated with the VaCAB category and is represented with V1*, due to its presence in the category of ViFAB (BC measure), despite its presence in two measures of BC and DC of VaCAB. On the contrary, AB_1947 is present in the BC, CC, and DC categories of ViFAB only and is represented by F5^. The numbers of the proteins to denote such overlaps are put randomly, however, and do not indicate the actual ordering of the rank as depicted in **Table 2**. Each one of such overlapping proteins has been indicated in bold to stand out from the rest in the same measures and/or categories and has been utilized for further mapping in the whole genome context later (**Figure 5**). It is important to note that there are more overlaps between the VaCAB and ViFAB categories of proteins as indicated by the asterisk in the entries. On the contrary, proteins of KeFAB tend to have much fewer overlaps among other categories. The numbers of the top-ranking proteins from each of the categories and measures of centralities and their overlaps are reflected with Venn diagrams (**Figure 3**). The functions of these proteins, taken from UniProt, reflect those that have been mostly characterized, with some having proven and eminent roles as vaccine and drug targets (**Supplementary Datas 1–3**: Sheet 12).

Despite the overview obtained about the importance of the proteins from a comparative account within and between each of the aforementioned categories of VaCAB, ViFAB, and KeFAB, it is to be noted that these PINs are a mixture of the actual VaC, ViF, and KeF proteins along with their interactors that were extracted from STRING. Interestingly, of the 15 top rank holders of each category of PIN, the actual proteins were 9, 3, 7, and 1 as per BC, CC, DC, and EC measures of VaCAB, and the rest were their interactors. Similarly, for ViFAB, the actual VaC proteins were 6, 6, 3, and 1 for the same measures, respectively, as mentioned above. For KeFAB, however, the numbers of KeF proteins were almost the same throughout, being 4, 5, 5, and 5 for BC, CC, DC, and EC, respectively (**Table 3**). However, to decipher the effective drug or a vaccine candidate from the global perspective of the whole genome, the total sets of proteins interacting in the whole genome of *A. baumannii* (GPIN) were analyzed as described in the next section.

The Complete GPIN

In an attempt to project the most probable candidates for vaccines and drug targets, we have further explored the GPIN. GPIN, built from the theoretically predicted and empirically found physical and functional interactions, was analyzed for its degree distribution in general and *k*-core distribution in particular. With an exponential curve for the cumulative degree distribution frequency (CCDF), the non-linear preferential attachment nature was evident (**Figure 4A**) (Vázquez, 2003). To this end, an idea of the important proteins forming the core of the genome was obtained through the *k*-core network decomposition. The shell size of the 165th innermost core had a conglomerate of 494 proteins (**Figures 4B,C**).

We have started analyzing the GPIN with the four common centrality measures as mentioned for SPIN (**Table 2**). A closer look at the top 15 from each measure reflects proteins mostly from the categories of KeF, two of which have overlaps with the ViFAB category (F1 and F2, **Table 2**). Around two-thirds of the top-ranking positions in DC are occupied by KeFAB proteins, while 99% of the top 15 EC measures comprise the KeFAB categories.

A detailed *k*-core analysis of the different proteins as per the centrality measures revealed other important facts. The numbers of VaCAB proteins belonging to the innermost core are 2, 3, 7, and 12 as per the top rank holders of BC, CC, DC, and EC measures (**Table 3**). Of these, the numbers of VaC proteins are 1, 1, 2, and 1 as per the same measures while the rest are their interactors (**Table 3**). This analysis filters out HMPREF0010_01334 as per the BC, CC, and DC measure overlaps and HMPREF0010_02233 as per EC, to be in the innermost core of the whole genome from the proclaimed VaC proteins. For the ViFAB proteins, 6, 10, 14, and 14 proteins, respectively, for BC, CC, DC, and EC measures belong to the 165th core, of which only 1, 2, 2, and 1, respectively, are the VaC proteins while the rest are their interactors (**Table 3**). This filters out HMPREF0010_03351 from BC, CC, and DC overlaps, while the new candidates HMPREF0010_01731, HMPREF0010_02683, and HMPREF0010_01948 stand out from CC, DC, and EC, respectively. The picture becomes different when it comes to the KeFAB where CC, DC, and EC all had only 165th core proteins from the top rankers while BC reflected 14 of them. Moreover, the actual KeF candidates are more in this PIN compared to the VaC and ViF proteins. The analysis filters out 4, 5, 5, and 5 KeF proteins from the BC, CC, DC, and EC measured top rankers (**Table 3**). This brings forth the candidates HMPREF0010_03512 from BC and CC, HMPREF0010_00239 from CC and DC, and HMPREF0010_00091 from DC and EC overlaps. Moreover, three candidates, namely, HMPREF0010_02437, HMPREF0010_01461, and HMPREF0010_02576, were unique in having overlaps across CC, DC, and EC measures, while HMPREF0010_01789 from EC and three candidates from BC, namely, HMPREF0010_03553, HMPREF0010_01114, and HMPREF0010_00382, were unique. It is important to note that

TABLE 2 | The topmost proteins of *A. baumannii* SPIN and GPIN as per BC, CC, DC, and EC network centrality measures.

Network	BC	CC	DC	EC
SPIN				
VaCAB	**AB_03493(V1*)**,	**AB_03704(V4^)**,	**AB_03493(V1*)**,	**AB_01255(V3*)**,
	AB_01517(V2^),	**AB_00353(V5*)**,	**AB_01517(V2^)**,	**AB_02233(V11^)**,
	AB_01255(V3*),	**AB_01334(V6^)**,	**AB_01255(V3*),**	**AB_03186(V12*)**,
	AB_03704(V4^),	**AB_00811(V7^)**,	**AB_03704(V4^)**,	**AB_03184(V13*)**,
	AB_00353(V5*),	**AB_02146(V8^)**,	**AB_00353(V5*)**,	**AB_03185(V14^)**,
	AB_01334(V6^),	**AB_00805(V9^)**,	**AB_01334(V6^)**,	AB_01226,
	AB_00252,	**AB_03351(V10*)**,	**AB_00811(V7^)**,	AB_02909,
	AB_01118,	**AB_02143(F6*)**,	**AB_02146(V8^)**,	AB_02910,
	AB_01117,	AB_02888,	**AB_00805(V9^)**,	AB_01257,
	AB_01710,	AB_02124,	**AB_03351(V10*)**,	AB_00660,
	AB_00112,	AB_02677,	**AB_02233(V11^)**,	AB_00760,
	AB_00698,	AB_02145,	**AB_03186(V12*)**,	AB_01258,
	AB_00210,	AB_01785,	**AB_03184(V13*)**,	AB_02683,
	AB_03124,	AB_03499,	**AB_03185(V14^)**,	AB_00675,
	AB_00209	AB_02178	AB_01388	AB_02914
ViFAB	**AB_01641(F1*)**,	**AB_02522(F2*)**,	**AB_01641(F1*)**,	**AB_02627(F9^)**,
	AB_02522(F2*),	**AB_03351(F3*)**,	**AB_02522(F2*)**,	AB_02354,
	AB_03351(F3*),	**AB_00353(F4*)**,	**AB_03351(F3*)**,	AB_02629,
	AB_00353(F4*),	**AB_01947(F5^)**,	**AB_00353(F4*)**,	AB_01950,
	AB_01947(F5^),	**AB_02143(F6*)**,	**AB_01947(F5^)**,	AB_03765,
	AB_02143(F6*),	**AB_03184(F7*)**,	**AB_03184(F7*)**,	AB_00169,
	AB_03493(V1*)	**AB_02069(F8^)**,	**AB_02069(F8^)**,	AB_01359,
	AB_01255(V3*),	AB_00812,	**AB_02627(F9^)**	AB_01360,
	AB_00113,	AB_02142,	**AB_03186(V12*)**,	AB_01656,
	AB_01972,	AB_01731,	AB_02909,	AB_01791,
	AB_00354,	AB_02888,	AB_01226,	AB_01948,
	AB_01713,	AB_01946,	AB_00660,	AB_01951,
	AB_01724,	AB_01248,	AB_02910,	AB_02545,
	AB_02769,	AB_02124,	AB_02683,	AB_02548,
	AB_02820	AB_01647	AB_01361	AB_02626
KeFAB	**AB_01491(K1^)**,	**AB_01491(K1^)**,	**AB_01491(K1^)**,	**AB_01491(K1^)**,
	AB_01641(K2*),	**AB_01641(K2*)**,	**AB_01641(K2*)**,	**AB_03022(K7^)**,
	AB_02522(K3*),	**AB_02522(K3*)**,	**AB_00239(K6^)**,	**AB_02437(K8^)**,
	AB_03512(K4^),	**AB_03512(K4^)**,	**AB_03022(K7^)**,	**AB_01461(K9^)**,
	AB_03207(K5^),	**AB_03207(K5^)**,	**AB_02437(K8^)**,	**AB_01350(K10^)**,
	AB_00427,	**AB_00239(K6^)**,	**AB_01461(K9^)**,	**AB_01047(K11^)**
	AB_01800,	**AB_03022(K7^)**,	**AB_01350(K10^)**,	**AB_03233(K12^)**,
	AB_03553,	**AB_02437(K8^)**,	**AB_01047(K11^)**	**AB_03046(K13^)**,
	AB_01281,	**AB_01461(K9^)**,	**AB_03233(K12^)**,	**AB_02576(K14^)**,
	AB_02769,	**AB_01350(K10^)**,	**AB_03046(K13^)**,	**AB_02252(K15^)**,
	AB_01114,	**AB_01047(K11^)**,	**AB_02576(K14^)**,	**AB_01051(K16^)**,
	AB_00382,	**AB_03233(K12^)**,	**AB_02252(K15^)**,	**AB_01740(K17^)**,
	AB_02314,	**AB_03046(K13^)**,	**AB_01051(K16^)**,	**AB_00091(K18^)**,
	AB_03088,	**AB_02576(K14^)**,	**AB_01740(K17^)**,	AB_01789,
	AB_02152	**AB_02252(K15^)**	**AB_00091(K18^)**	AB_03031
GPIN				
	AB_01641(F1*/K2*),	AB_00110,	**AB_01491(K1^)**,	**AB_01491(K1^),**
	AB_01590,	AB_01918,	**AB_01641(F1*/K2*)**,	**AB_01641(F1*/K2*)**,
	AB_02383,	AB_02296,	**AB_03512(K4^)**,	**AB_02522(F2*/K3*)**,
	AB_03076,	AB_03731,	**AB_03207(K5^)**,	**AB_03512(K4^)**,
	AB_03532,	AB_03732,	**AB_01461(K9^)**,	**AB_03207(K5^)**,
	AB_03595,	AB_03317,	**AB_03233(K12^)**,	**AB_00239(K6^)**,
	AB_02409,	AB_03318,	**AB_03046(K13^)**,	**AB_03022(K7^)**,
	AB_01611,	AB_03726,	**AB_02576(K14^)**,	**AB_02437(K8^)**,
	AB_01613,	AB_02992,	**AB_01740(K17^)**,	**AB_01461(K9^)**,
	AB_02391,	AB_03666,	**AB_00091(K18^)**,	**AB_01350(K10^)**,
	AB_03107,	AB_02700,	AB_03659,	**AB_01047(K11^)**,
	AB_03129,	AB_03651,	AB_03532,	**AB_03233(K12^)**,
	AB_01437,	AB_02374,	AB_01651,	**AB_01051(K16^)**,
	AB_03092,	AB_02371,	AB_00045,	**AB_00091(K18^)**,
	AB_00006	AB_02372	AB_01281	AB_02769

The bold cased proteins are overlapping either within same categories (^) or between others (*) in SPIN. Numbers are arbitrary.

TABLE 3 | The innermost core proteins of VaCAB, ViFAB, KeFAB, and GPIN as per the four centrality measures.

Centrality measures	Protein ID	Protein type	Core	Protein ID	Protein type	Core	Protein ID	Protein type	Core	Protein ID	Protein type	Core
BC	AB_03493	VaC	84	AB_01641	Interactor	165	AB_01491	Interactor	165	AB_01590	Genome	66
	AB_01517	VaC	84	AB_02522	Interactor	165	AB_01641	Interactor	165	AB_02383	Genome	85
	AB_01255	Interactor	165	AB_03351	ViF	165	AB_02522	Interactor	165	AB_03076	Genome	68
	AB_03704	VaC	108	AB_00353	ViF	163	AB_03512	KeF	165	AB_03532	Genome	165
	AB_00353	VaC	163	AB_01947	Interactor	165	AB_03207	Interactor	165	AB_01641	Genome	165
	AB_01334	VaC	165	AB_02143	ViF	123	AB_00427	Interactor	165	AB_03595	Genome	162
	AB_00252	VaC	68	AB_03493	ViF	86	AB_01800	Interactor	165	AB_02409	Genome	66
	AB_01118	Interactor	70	AB_01255	Interactor	165	AB_03553	KeF	165	AB_01611	Genome	22
	AB_01117	Interactor	90	AB_00113	Interactor	68	AB_01281	Interactor	165	AB_01613	Genome	66
	AB_01710	VaC	91	AB_01972	Interactor	112	AB_02769	Interactor	165	AB_02391	Genome	161
	AB_00112	Interactor	68	AB_00354	ViF	92	AB_01114	KeF	165	AB_03107	Genome	91
	AB_00698	VaC	91	AB_01713	Interactor	151	AB_00382	KeF	165	AB_03129	Genome	74
	AB_00210	Interactor	98	AB_01724	ViF	110	AB_02314	Interactor	165	AB_01437	Genome	30
	AB_03124	Interactor	92	AB_02769	Interactor	165	AB_03088	Interactor	151	AB_03092	Genome	85
	AB_00209	VaC	102	AB_02820	Interactor	147	AB_02152	Interactor	165	AB_00006	Genome	65
CC	AB_03704	VaC	108	AB_02522	Interactor	165	AB_01491	Interactor	165	AB_00110	Genome	62
	AB_00353	VaC	163	AB_03351	ViF	165	AB_01641	Interactor	165	AB_01918	Genome	5
	AB_01334	VaC	165	AB_00353	ViF	163	AB_02522	Interactor	165	AB_02296	Genome	5
	AB_00811	Interactor	124	AB_01947	Interactor	165	AB_03512	KeF	165	AB_03731	Genome	2
	AB_02146	Interactor	112	AB_02143	ViF	123	AB_03207	Interactor	165	AB_03732	Genome	2
	AB_00805	Interactor	104	AB_03184	Interactor	165	AB_00239	KeF	165	AB_03317	Genome	82
	AB_03351	Interactor	165	AB_02069	Interactor	165	AB_03022	Interactor	165	AB_03318	Genome	76
	AB_02143	Interactor	123	AB_00812	ViF	163	AB_02437	KeF	165	AB_03726	Genome	82
	AB_02888	Interactor	123	AB_02142	ViF	134	AB_01461	KeF	165	AB_02992	Genome	6
	AB_02124	Interactor	117	AB_01731	ViF	165	AB_01350	Interactor	165	AB_03666	Genome	4
	AB_02677	Interactor	96	AB_02888	Interactor	123	AB_01047	Interactor	165	AB_02700	Genome	6
	AB_02145	Interactor	104	AB_01946	Interactor	165	AB_03233	Interactor	165	AB_03651	Genome	2
	AB_01785	Interactor	103	AB_01248	Interactor	165	AB_03046	Interactor	165	AB_02374	Genome	7
	AB_03499	Interactor	131	AB_02124	Interactor	165	AB_02576	KeF	165	AB_02371	Genome	7
	AB_02178	Interactor	165	AB_01647	Interactor	165	AB_02252	Interactor	165	AB_02372	Genome	7
DC	AB_03493	VaC	84	AB_01641	Interactor	165	AB_01491	Interactor	165	AB_01641	Genome	165
	AB_01517	VaC	84	AB_02522	Interactor	165	AB_01641	Interactor	165	AB_03207	Genome	165
	AB_01255	Interactor	165	AB_03351	ViF	165	AB_00239	KeF	165	AB_03659	Genome	165
	AB_03704	VaC	108	AB_00353	ViF	163	AB_03022	Interactor	165	AB_03512	Genome	165
	AB_00353	VaC	163	AB_01947	Interactor	165	AB_02437	KeF	165	AB_03532	Genome	165
	AB_01334	VaC	165	AB_03184	Interactor	165	AB_01461	KeF	165	AB_01740	Genome	165
	AB_00811	Interactor	124	AB_02069	Interactor	165	AB_01350	Interactor	165	AB_01651	Genome	165
	AB_02146	Interactor	112	AB_02627	Interactor	165	AB_01047	Interactor	165	AB_02576	Genome	165
	AB_00805	Interactor	104	AB_03186	Interactor	165	AB_03233	Interactor	165	AB_03046	Genome	165
	AB_03351	Interactor	165	AB_02909	Interactor	165	AB_03046	Interactor	165	AB_01461	Genome	165
	AB_02233	VaC	165	AB_01226	Interactor	165	AB_02576	KeF	165	AB_03233	Genome	165
	AB_03186	Interactor	165	AB_00660	Interactor	165	AB_02252	Interactor	165	AB_01491	Genome	165
	AB_03184	Interactor	165	AB_02910	Interactor	165	AB_01051	Interactor	165	AB_00091	Genome	165
	AB_03185	Interactor	165	AB_02683	ViF	165	AB_01740	Interactor	165	AB_00045	Genome	74
	AB_01388	VaC	102	AB_01361	Interactor	165	AB_00091	KeF	165	AB_01281	Genome	165
EC	AB_01255	Interactor	165	AB_02627	Interactor	165	AB_01491	Interactor	165	AB_02522	Genome	165
	AB_02233	VaC	165	AB_02354	Interactor	165	AB_03022	Interactor	165	AB_02769	Genome	165
	AB_03186	Interactor	165	AB_02629	Interactor	165	AB_02437	KeF	165	AB_03233	Genome	165
	AB_03184	Interactor	165	AB_01950	Interactor	165	AB_01461	KeF	165	AB_00091	Genome	165
	AB_03185	Interactor	165	AB_03765	Interactor	165	AB_01350	Interactor	165	AB_03207	Genome	165

(Continued)

TABLE 3 | Continued

Centrality measures	Protein ID	Protein type	Core	Protein ID	Protein type	Core	Protein ID	Protein type	Core	Protein ID	Protein type	Core
	AB_01226	Interactor	165	AB_00169	Interactor	165	AB_01047	Interactor	165	AB_01641	Genome	165
	AB_02909	Interactor	165	AB_01359	Interactor	165	AB_03233	Interactor	165	AB_03022	Genome	165
	AB_02910	Interactor	165	AB_01360	Interactor	165	AB_03046	Interactor	165	AB_02437	Genome	165
	AB_01257	Interactor	163	AB_01656	Interactor	165	AB_02576	KeF	165	AB_01350	Genome	165
	AB_00660	Interactor	165	AB_01791	Interactor	156	AB_02252	Interactor	165	AB_01047	Genome	165
	AB_00760	Interactor	153	AB_01948	ViF	165	AB_01051	Interactor	165	AB_01461	Genome	165
	AB_01258	Interactor	153	AB_01951	Interactor	165	AB_01740	Interactor	165	AB_00239	Genome	165
	AB_02683	Interactor	165	AB_02545	Interactor	165	AB_00091	KeF	165	AB_03512	Genome	165
	AB_00675	Interactor	165	AB_02548	Interactor	165	AB_01789	KeF	165	AB_01491	Genome	165
	AB_02914	Interactor	165	AB_02626	Interactor	165	AB_03031	Interactor	165	AB_01051	Genome	165

Shades of green in light are for interactors while those in dark are the candidates from each category.

sorting of the whole genome proteins as per the four centrality measures yielded 2, 0, 14, and 15, respectively, of which 0, 0, 4, and 2 proteins, respectively, belong to either the VaC, ViF, or KeF category while the rest are their interactors (**Table 3**).

Furthermore, to identify the candidate VaC, ViF, and KeF proteins in the network topological space of *A. baumannii*, we have classified the protein sets of GPIN and represented them cartographically (**Figure 5**; **Supplementary Data 4**). Essentially, one part of such classification, namely, *z* score, is based on their regional connectivity with other similar proteins having a similar biological function, which is referred to as the functional module. The other part, namely, the participation coefficient, *P*, deals with the participation of these proteins with other functional modules, either related or non-related. Thus, there are seven such quadrants that are formed and termed R1 to R7. Noticeably, the proteins of VaCAB and ViFAB are spread throughout R1–R3 in the network space, while those of KeFAB are mostly concentrated in R5 (**Figure 5**; **Supplementary Data 4**). Interestingly, R4 and R6 did not show any mapping of the VaC, ViF, or KeF proteins or their interactors, represented by V, F, and K comprising the full VaCAB, ViFAB, and KeFAB SPIN (**Figure 5**; **Supplementary Data 4**). Furthermore, there were no proteins from the GPIN that occupied the R7 quadrant, having the highest *P* or *z* scores. With a more focused analysis to find out the most indispensable proteins of GPIN, we have performed the KFC method mentioned by Ashraf et al. (2018). These KFC proteins all belong to the innermost 165th core and had R5 as their classifying *P* vs. *z* quadrant and high EC scores (**Table 3**; **Supplementary Data 4**).

The Candidature Prediction

To ascertain the plausible candidature of the proteins of VaCAB, ViFAB, KeFAB, and GPIN as either vaccine or drug targets, we have reclassified them as VaC, ViF, and KeF, along with their interactors and KFC proteins, as mentioned in the previous section. This projects 2 VaC with 13 interactors, 4 ViF with 29 interactors, 19 KeF with 41 interactors from the 165th core and 10 KFC proteins for further analyses (**Tables 3**, **4**). Among these, the cellular localization could not be determined for one VaC, two ViF, five KeF, and two R6 proteins, respectively. Notably, one candidate from VaC, namely, AB_02233, belongs to the outer membrane, while the periplasmic and extracellular comprise the ViF category. The remaining categories of KeF, KFC, and R6 mostly comprise cytoplasmic proteins having few cytoplasmic membrane proteins for KeF and R6 as well (**Table 7**). To this end, only one KeF (AB_01691) did not reflect any COG classification for the aforementioned categories. On the contrary, 50% of R6 proteins did not reflect any COG, being uncharacterized. Interestingly, only one protein from each of the VaC, ViF, and KeF categories, namely, AB_02233, AB_03351, and AB_01758, respectively, predicted a signal peptide while KFC and R6 did not show any. Moreover, only one lipoprotein cleavage site was predicted for ViF, KeF, and R6, namely, AB_03351, B_01758, and AB_00641, respectively, while VaC has two proteins (AB_01334 and AB_02233) showing the same compared to KFC, having none. Furthermore, about 50% of the proteins of the category KeF and KFC indicated an overlap of the antigenic site with the active pocket site while proteins of VaC, ViF, and R6 had no such overlap for candidature prediction (**Table 7**).

The Final Selection

Finally, to assess for the relevance of the final set of proteins (**Table 4**), in virulence and pathogenicity of *Acinetobacter*, we have cross-examined through UniProt, PDB, and PubMed, for their role, either predictive or empirical, in *Acinetobacter* and/or other gram-negative bacterial pathogens. Two unique VaC proteins, namely, AB_01334 and AB_02233, are predicted to encode tetratricopeptide repeat protein and peptidase M16 inactive domain protein, respectively (**Table 7**). Annotations for other ViF proteins like thiol:disulfide interchange protein (AB_03351), malate dehydrogenase (AB_02683), and 50S ribosomal protein L7/L12 (AB_01948) were inferred from homology while AB_01731 was experimentally verified to have the function of a nucleoside diphosphate kinase (**Table 7**). The KeF proteins are shown to be a conglomerate of different types engaged in different biological process starting from carbohydrate, amino acid, and DNA metabolism to even those involved in signal transduction, cell wall synthesis,

TABLE 4 | The candidate proteins of VaC, ViF, KeF, and KFC along with their interactors in *A. baumannii* ATCC19606 strain.

Category	VaCAB		ViFAB		KeFAB		KFC
Protein type	**VaC**	**Interactors**	**ViF**	**Interactors**	**KeF**	**Interactors**	
	AB_01334	AB_00660	AB_03351	AB_00169	AB_03689	AB_01249	AB_02522
	AB_02233	AB_00675	AB_01731	AB_00660	AB_03360	AB_01784	AB_02769
		AB_01226	AB_02683	AB_01226	AB_03276	AB_03688	AB_03233
		AB_01255	AB_01948	AB_01248	AB_03274	AB_03695	AB_00091
		AB_02178		AB_01255	AB_03221	AB_03697	AB_03207
		AB_02683		AB_01359	AB_03191	AB_03371	AB_01641
		AB_02909		AB_01360	AB_02008	AB_03354	AB_03022
		AB_02910		AB_01361	AB_01991	AB_03349	AB_02437
		AB_02914		AB_01641	AB_01903	AB_03269	AB_01350
		AB_03184		AB_01647	AB_01875	AB_03268	AB_01047
		AB_03185		AB_01656	AB_01854	AB_03266	
		AB_03186		AB_01946	AB_01851	AB_03257	
		AB_03351		AB_01947	AB_01810	AB_03245	
				AB_01950	AB_01791	AB_03244	
				AB_01951	AB_01758	AB_03209	
				AB_02069	AB_01757	AB_02016	
				AB_02124	AB_01723	AB_01961	
				AB_02354	AB_01691	AB_01953	
				AB_02522	AB_01682	AB_01952	
				AB_02545		AB_01931	
				AB_02548		AB_01899	
				AB_02626		AB_01885	
				AB_02627		AB_01876	
				AB_02629		AB_01868	
				AB_02909		AB_01834	
				AB_02910		AB_01829	
				AB_03184		AB_01817	
				AB_03186		AB_01816	
				AB_03765		AB_01796	
						AB_01794	
						AB_01793	
						AB_01787	
						AB_01784	
						AB_01755	
						AB_01734	
						AB_01733	
						AB_01725	
						AB_01721	
						AB_01720	
						AB_01711	
						AB_01665	

VaC, ViF, KeF, and KFC denote Vaccine candidates, Virulent Factors, Key Factors, and K-core-Functional modularity-Centrality, respectively. The prefix of the accession numbers for the proteins has been replaced by AB.

ribosomal and translational machineries, as well as some uncharacterized proteins (AB_02008 and AB_01691; **Table 7**). On the contrary, proteins of the KFC class are only concentrated on carbohydrate, amino acid, and fatty acid metabolism with the inclusion of one (AB_01641) having DNA polymerase with 5′-3′ exonuclease activity. Again, proteins of the R6 category are mostly either uncharacterized or belong to the transcriptional regulator family with the inclusion of one CRISPR-associated protein (AB_01430).

All the corresponding candidates from ATCC19606 have been mapped onto ATCC17978 and AYE strains of *A. baumannii* as reflected in **Table 5** for the ease of use by future researchers.

TABLE 5 | Corresponding candidates of *A. baumannii* ATCC19606 in ATCC17978 and AYE strains.

	STRAIN_19606	STRAIN_AYE	STRAIN17978
VaC	HMPREF0010_01334	ABAYE2977	–
	HMPREF0010_02233	ABAYE0990	–
ViF	HMPREF0010_03351	ABAYE3833	–
	HMPREF0010_01731	ABAYE3267	–
	HMPREF0010_02683	ABAYE0465	–
	HMPREF0010_01948	ABAYE3490	–
KeF	HMPREF0010_03689	–	A1S_0003
	HMPREF0010_03360	–	A1S_0028
	HMPREF0010_03276	–	A1S_0061
	HMPREF0010_03274	–	A1S_0063
	HMPREF0010_03221	–	A1S_0114
	HMPREF0010_03191	–	A1S_0147
	HMPREF0010_02008	–	A1S_0217
	HMPREF0010_01991	–	A1S_0236
	HMPREF0010_01903	–	A1S_0334
	HMPREF0010_01875	–	A1S_0364
	HMPREF0010_01854	–	A1S_0388
	HMPREF0010_01851	–	A1S_0391
	HMPREF0010_01810	–	A1S_0428
	HMPREF0010_01791	–	A1S_0447
	HMPREF0010_01758	–	A1S_0469
	HMPREF0010_01757	–	A1S_0470
	HMPREF0010_01723	–	A1S_0506
	HMPREF0010_01691	–	A1S_0561
	HMPREF0010_01682	–	A1S_0571
KFC	HMPREF0010_02522	–	–
	HMPREF0010_02769	–	–
	HMPREF0010_03233	–	–
	HMPREF0010_00091	–	A1S_2232
	HMPREF0010_03207	–	–
	HMPREF0010_01641	–	–
	HMPREF0010_03022	–	–
	HMPREF0010_02437	–	A1S_3280
	HMPREF0010_01350	–	–
	HMPREF0010_01047	–	–

TABLE 6 | The human counterparts of the corresponding VaC, ViF, KeF, KFC, and R6 candidates of *A. baumannii* ATCC19606 strain.

	Modified String_ID	Accession_ID	Query coverage	Identity
VaC	AB_01334	NP_858059.1	29%	26%
	AB_02233	NP_004270.2	25%	23%
ViF	AB_03351	–	–	–
	AB_01731	NP_002504.2	95%	50%
	AB_02683	NP_005908.1	98%	52%
	AB_01948	–	–	–
KeF	AB_03689	XP_016869997.1	13%	35%
	AB_03360	–	–	–
	AB_03276	–	–	–
	AB_03274	–	–	–
	AB_03221	–	–	–
	AB_03191	–	–	–
	AB_02008	–	–	–
	AB_01991	–	–	–
	AB_01903	XP_006712602.1	27%	36%
	AB_01875	NP_008965.2	95%	32%
	AB_01854	–	–	–
	AB_01851	–	–	–
	AB_01810	XP_011511104.1	11%	39%
	AB_01791	–	–	–
	AB_01758	XP_005268046.1	60%	43%
	AB_01757	XP_005273641.1	51%	33%
	AB_01723	NP_001182351.1	27%	26%
	AB_01691	–	–	–
	AB_01682	NP_001177809.1	96%	33%
KFC	AB_02522	XP_024303263.1	3%	43%
	AB_02769	XP_016866853.1	100%	83%
	AB_03233	NP_005580.1	94%	61%
	AB_00091	NP_005580.1	90%	47%
	AB_03207	XP_016862911.1	98%	36%
	AB_01641	NP_861524.2	50%	32%
	AB_03022	NP_001193826.1	97%	38%
	AB_02437	NP_001071.1	95%	53%
	AB_01350	NP_001071.1	98%	50%
	AB_01047	NP_001071.1	98%	55%
R6	AB_00210	–		
	AB_00797	NP_060206.2	10%	41%
	AB_03124	–	–	–
	AB_02872	–	–	–
	AB_02571	–	–	–
	AB_00406	–	–	–
	AB_00641	–	–	–
	AB_01430	NP_005236.2	12%	36%
	AB_01223	–	–	–
	AB_01974	–	–	–

VaC, ViF, KeF, and KFC denote Vaccine candidates, Virulent Factors, Key Factors, K-core-Functional modularity-Centrality and Function-R6, respectively. The prefix of the accession numbers for the proteins has been replaced by AB.

Moreover, to determine the orthologous presence of these proteins in the human host, their pairwise identities (PI) and query coverages (QC) are reflected in **Table 6**. Notably, the VaC proteins have considerably low PI and QC, which is exactly opposite in nature to all but one of the proteins of KFC, having high QC and PI. Intriguingly, ViF, KeF, and R6 comprise a mixture of unique proteins having no human counterpart as well as those having moderate to high PI. Of these, however, only proteins of ViF have very high QC as well (**Table 6**).

DISCUSSION

The sole aim of this study is to look out for plausible vaccine and/or drug candidates among a plethora of proteins from the whole genome of *A. baumannii*. In this context, the work proposed by Moriel et al. (2013) has mentioned an array of proteins as candidates for vaccines to test out in real-life scenario. Besides these, several researchers have proposed

different virulence factors crucial for the XDR *A. baumannii*, probably potential enough to be targeted as drugs. Moreover, the VFDB presents lists of several such factors as well. Considering a filtration to shortlist just few of these would probably be a good idea to save the time and money of future researchers in this field of study. Thus, network analysis is being considered in order to sort and identify, *albeit* theoretically, the most probable candidates among them.

With the target being set out for the pathogenic strains like AYE and ATCC17978, the main hindrance was the lack of protein interaction datasets for these strains in the STRING database. This led to the mapping of the proteins from these strains onto ATCC19606. We started with an initial categorization of the protein sets proposed by Moriel et al. (2013) based on their results. One set, representing the *A. baumannii* antigenic proteins identified through reverse vaccinology approach, was denoted as Vaccine Candidates (VaC). The other set was named Virulence Factors (ViF) and comprised all other proteins listed by the same group including OMVs and secretome, potentially insoluble proteins and periplasmic proteins found in OMV and secretome of *A. baumannii*. Initial filtration through standalone BLAST led to almost the same number of proteins in ATCC19606. A stringent filtering approach with 99–100% identity cutoff threshold, however, was adopted to rule out any ambiguity of the protein functions upon such conversion from the former strains to the latter. Similar strategies were adopted for database- and literature-retrieved proteins searched through keywords and were named Key Factors (KeF).

As the proteins would always be interacting with others to manifest their functions, a PIN construction was the next move for the VaC, ViF, and KeF protein sets to yield the three SPINs namely, VaCAB, ViFAB, and KeFAB. These were analyzed for their importance through the four centrality parameters BC, CC, DC, and EC, often utilized for biological network analysis (Jeong et al., 2001; Lahiri et al., 2014; Pan et al., 2015; Pawar et al., 2017, 2018; Ashraf et al., 2018). Among them, DC reflects the simple network connectedness of any protein, while for a virulent phenotype, CC might bring out the functional proximity of a protein with others. Moreover, BC might help in reflecting the bridging of different functionally important groups of virulent proteins, thereby posing its importance to be targeted for therapeutic purposes. EC, however, might reflect the connectivity of the most important proteins with other important proteins in a virulent network, thereby posing them to be indispensable for therapeutic targets. Notably, there were overlaps between VaC and ViF categories of proteins across different centrality measures (**Table 2**), probably indicating a faint line of difference between them, which actually were set by this study and not by Moriel et al. (2013). Moreover, there were very few overlaps of VaC and ViF proteins with those of KeF sets, probably indicating the uniqueness of the former groups compared to the common KeF proteins already reported in the literature and database searched through keywords (**Table 2**).

The functional aspects of the candidate proteins would be best put forward through the whole genome global scenario for which the GPIN was constructed (**Figure 2D**) and analyzed by the network centrality and other topological parametric measures (**Supplementary Data 4**). The initial characterization of the GPIN was done through the exponential decay of the degree distribution, $P(k)$, of a particular node upon connecting to k other nodes, for large values of k. Such construction at least confirms the non-random (Erds and Rényi, 1960) or non-small-world nature (Watts and Strogatz, 1998) of the GPIN, if not completely following the power law (Albert et al., 2000) and becoming scale free (**Figure 4A**). Hereafter, the constructed GPIN is analyzed with the four centrality measures. Proteins of KeFAB categories occupying most of the EC and DC measures probably indicate the importance of either of these measures in bringing out the top rankers of KeFAB proteins (**Table 2**). Moreover, with no appearance among the top 15 important categories, the VaCAB and ViFAB proteins might not be so essential from the whole genome perspective (**Table 2**).

The actual set of proteins important from the GPIN perspective are probably reflected by the innermost core of the proteins brought about by the k-core/K-shell topological parameters (**Figures 4B,C**). The concept of the importance of the innermost k-core lies in the fact that k-core is a subnetwork with a minimum number of k-links such that the 165th innermost core would have 165 connections of each of those proteins lying in that core (**Figure 4B**; **Supplementary Data 4**). Essentially, the number of proteins in this case is 498 (**Figure 4C**; **Supplementary Data 4**). With such a large inner core member proteins having high connectivity, the core tends to be highly interactive and, thus, robust in nature (Alvarez-Hamelin et al., 2005). This could be indicative of the tight control resistance mechanism of this MDR/XDR species of *A. baumannii*. It is important to note that the relation between a K-shell and k-core is that the former is the part of the latter but not of the $(k + 1)$-core, such that the former is a set of nodes having exactly k-links. This brings out the fact that there are a lower number of proteins (interacting among their partners) with lower k-core that belongs to the outer shell, and thus, the innermost core would have the maximum number of proteins needed to be decomposed to affect the global network of *A. baumannii*. Interestingly, only limited VaC and ViF proteins (2 and 4, respectively) belong to the 165th innermost core identified through all different centrality measures (**Table 3**). These numbers are 12–13% of the total proteins of VaCAB and ViFAB including the VaC and ViF proteins along with their interactors. In comparison to these, ∼32% of the total proteins of KeFAB are important as KeF (**Table 3**). These probably tells us that only few proteins are important from VaC and ViF compared to KeF categories. Thus, considering just these few proteins as either vaccines or drug targets may not be sufficient to break the robust inner core compared to a large number of options available for KeF proteins.

With some preliminary idea about the centrality and k-core measures, we moved on to delve deep into the functional connectivity, R, of the modules formed in the GPIN. Such connectivity of the proteins within and between the functional modules is represented cartographically by P-values and z score, respectively, across the x- and y-axis. This results in the lowest values of P and z for R1 and the highest for R7. The classifications are thus named ultra-peripheral proteins (R1) and peripheral proteins (R2), which can be detached with convenience from

TABLE 7 | Protein analyses of selected VaC, ViF, KeF, and KFC proteins of *A. baumannii* for cellular localization, COG classification, antigenicity, and active site predictions.

String-id	Protein description: function	Localization	COG analysis		Cleavage position		Epitope analysis				Active site analysis
		PSORTb	Accession	Name	SignalP	LipoP	Peptide	Start	End	length	
VaCAB											
AB_01334	Tetratricopeptide repeat protein: DNA binding	Unknown	COG5010	TadD	32,N	31–32	KHANDPQL	547	554	8	511–523
AB_02233	Peptidase M16 inactive domain protein: metal ion binding; metalloendopeptidase activity	Outer Membrane	COG0612	PqqL	24,Y	23–24	KDKPKTLDQTDVKAEPLKDPKVY	454	476	23	404–442
ViFAB AB_03351	Thiol:disulfide interchange protein protein disulfide oxidoreductase activity	Periplasmic	COG1651	DsbG	23,Y	22–23	GKVEVP	38	43	6	67–81
							QGEDGK	184	189	6	
AB_01731	Nucleoside diphosphate kinase: ATP binding; metal ion binding; nucleoside diphosphate kinase activity	Extracellular	COG0105	Ndk	48,N	–	NAAHGSDSVAS	114	124	11	104–128
AB_02683	Malate dehydrogenase: L-malate dehydrogenase activity	Unknown	COG0039	Mdh	28,N	–	GESLKDKINDPAW	204	216	13	225–244
AB_01948	50S ribosomal protein L7/L12: structural constituent of ribosome	Unknown	COG0222	RplL	51,N	–	APAGGAAAAAEEQSE	42	56	15	38–50
KeFAB											
AB_03689	DNA replication and repair protein RecF: ATP binding; single-stranded DNA binding	Cytoplasmic	COG1195	RecF	30,N	–	DPQSTDI	114	120	7	112–165
AB_03360	Alkanesulfonate monooxygenase: alkanesulfonate monooxygenase activity	Cytoplasmic	COG2141	COG2141	37,N	–	TWGEPPAAV	198	206	9	140–172
							ALVGDPETV	306	314	9	356–365
AB_03276	Bacterial sugar transferase: transferase activity, transferring glycosyl groups	Cytoplasmic Membrane	COG2148	WcaJ	30,N	–	FDAQGNPLPDEARI	66	79	14	42–97

(Continued)

TABLE 7 | Continued

String-id	Protein description: function	Localization	COG analysis		Cleavage position		Epitope analysis				Active site analysis
		PSORTb	Accession	Name	SignalP	LipoP	Peptide	Start	End	length	
AB_03274	UDP-glucose 6-dehydrogenase: NAD binding; UDP-glucose 6-dehydrogenase activity	Cytoplasmic	COG1004	Ugd	17,N	–	KENTSSTHN	307	315	9	306–399
AB_03221	Phosphopantetheine attachment domain protein: phosphopantetheine binding	Unknown	COG0236	AcpP	25,N	–	PETIDPDQKF	24	33	10	32–67
AB_03191	ATP synthase F0, I subunit: hydrolase activity	Cytoplasmic Membrane	COG3312	AtpI	31,N	–	AR	67	68	2	68–74
AB_02008	Uncharacterized protein	Unknown	COG2960	COG2960	55,N	–	DEPKKD	14	19	6	32–43
AB_01991	Response regulator receiver domain protein: DNA binding	Cytoplasmic	COG2197	CitB	26,N	–	SDTQQSPFDS	139	148	10	131–174
AB_01903	Ribosome maturation factor RimP: Required for maturation of 30S ribosomal subunits	Cytoplasmic	COG0779	COG0779	21,N	–	PVDENAEPVINEDGEVEQG	46	64	19	60–61
AB_01875	DnaJ domain protein unfolded protein binding+C4	Cytoplasmic	COG0484	DnaJ	62,N	–	GFGGGQQQYQRQ	117	128	12	101–195
AB_01854	S-(Hydroxymethyl)glutathione synthase: carbon-sulfur lyase activity	Unknown	COG3791	COG3791	46,N	–	TPLDQK	104	109	6	72–115
AB_01851	50S ribosomal protein L31 type B: structural constituent of ribosome	Cytoplasmic	COG0254	RpmE	20,N	–	QTKQTKEYQG	30	39	10	40–41
AB_01810	Endonuclease/ exonuclease/ phosphatase family protein: endonuclease activity; exonuclease activity	Cytoplasmic	COG3021	COG3021	23,N	–	PKPPSPTEAKDSTL	208	221	14	245–291
AB_01791	50S ribosomal protein L33: structural constituent of ribosome	Cytoplasmic	COG0267	RpmG	21,N	–	KNKRTM	21	26	6	22–31
AB_01758	Sel1 repeat protein	Unknown	COG0790	COG0790	21,Y	20–21	ASNGDNR	120	126	7	97–129

(Continued)

TABLE 7 | Continued

String-id	Protein description: function	Localization	COG analysis		Cleavage position		Epitope analysis				Active site analysis
		PSORTb	Accession	Name	SignalP	LipoP	Peptide	Start	End	length	
AB_01757	Methionine biosynthesis protein MetW	Cytoplasmic	COG2226	UbiE	31,N	–	IK	12	13	2	8–18
							NQ	72	73	2	81–84
AB_01723	GTPase Der: GTP binding	Cytoplasmic Membrane	COG1160	COG1160	31,N	–	SENPFEGRKSQVDERTA	434	450	17	119–150
AB_01691	Uncharacterized protein	Unknown	NA	NA	21,N	–	TMKPNNHSTETNTPPAI	36	52	17	69–74
AB_01682	AP endonuclease, family 2: endonuclease activity; isomerase activity	Cytoplasmic	COG3622	Hfi	29,N	–	PGRHEPDTAQI	211	221	11	206–242
KFC AB_02522	Glutamate synthase [NADPH], large subunit: glutamate synthase (NADPH) activity	Cytoplasmic	COG0067	GltB	60,N	–	GRSNSGEGGEDPARY	896	910	15	867–996
			COG0069	GltB							
			COG0070	GltB							
AB_02769	Putative fatty acid oxidation complex subunit alpha: 3-hydroxyacyl-CoA dehydrogenase activity; lyase activity	Cytoplasmic	COG1250	FadB	26,N	–	YKIPGGDPKTPA	216	227	12	239–297
AB_03233	Methylmalonate-semialdehyde dehydrogenase (Acylating): methylmalonate-semialdehyde dehydrogenase (acylating) activity	Cytoplasmic	COG1012	PutA	70,N	–	GKTLADAEGD	106	115	10	141–147
AB_00091	Methylmalonate-semialdehyde dehydrogenase (Acylating): methylmalonate-semialdehyde dehydrogenase (acylating) activity	Cytoplasmic	COG1012	PutA	44,N	–	ARKQPVYNPATGEIS	41	55	15	140–145

(Continued)

TABLE 7 | Continued

String-id	Protein description: function	Localization	COG analysis		Cleavage position		Epitope analysis				Active site analysis
		PSORTb	Accession	Name	SignalP	LipoP	Peptide	Start	End	length	
AB_03207	GMP synthase [glutamine-hydrolyzing]: ATP binding; GMP synthase (glutamine-hydrolyzing) activity; pyrophosphatase activity	Cytoplasmic	COG0518	GuaA	23,N	–	GPESVHEEGSPRA	61	73	13	60–92
			COG0519	GuaA							
AB_01641	DNA polymerase I: 3′-5′ exonuclease activity; DNA binding; DNA-directed DNA polymerase activity	Cytoplasmic	COG0749	PolA	20,N	–	VKPAQTIETEDQASLTSQDDQLG	303	325	23	599–687
AB_03022	Putative aminobutyraldehyde dehydrogenase: oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor	Cytoplasmic	COG1012	PutA	36,N	–	EVYAQSPNATEAEV	32	45	14	33–97
AB_02437	Succinate-semialdehyde dehydrogenase [NAD(P)+]: succinate-semialdehyde dehydrogenase [NAD(P)+] activity	Cytoplasmic	COG1012	PutA	61,N	–	DGRQEGSTQGPLI	318	330	13	330–386
AB_01350	Succinate-semialdehyde dehydrogenase [NAD(P)+]: succinate-semialdehyde dehydrogenase [NAD(P)+] activity	Cytoplasmic	COG1012	PutA	38,N	–	NATVPVSNPATGEEIG	26	41	16	59–73

(Continued)

TABLE 7 | Continued

String-id	Protein description: function	Localization	COG analysis		Cleavage position		Epitope analysis				Active site analysis
		PSORTb	Accession	Name	SignalP	LipoP	Peptide	Start	End	length	
AB_01047	Succinate-semialdehyde dehydrogenase [NAD(P)+]: succinate-semialdehyde dehydrogenase [NAD(P)+] activity	Cytoplasmic	COG1012	PutA	16,N	–	GLGREGSKY	459	467	9	410–476
R6											
AB_00210	Uncharacterized protein	Unknown	COG2911	COG2911	21,N	–	VEQQPTSAPSSPK	4	16	13	
AB_00797	Transcriptional regulator, TetR family: DNA binding	Cytoplasmic	COG1309	AcrR	39,N	–	EKA	78	80	3	
AB_03124	Putative phage uncharacterized protein domain protein	Cytoplasmic Membrane	COG5412	COG5412	40,N	–	DPTKPIEPPKPPKLGL GTAPPNPKLGIGTGE KDDKGGSKSSAKS KAEQEAKERQRQAEQ	494	552	59	587–599
AB_02872	Uncharacterized protein	Cytoplasmic	NA	NA	45,N	–	QQHAGESVKKNRKAQSIKS GYDESAEQSVELEED FEQYAAEQQQAR EQAKQQRQQQKREQAEQM	122	185	64	65–89
AB_02571	Uncharacterized protein	Cytoplasmic	NA	NA	42,N	–	SIDPEQVED	118	126	9	
							QQAENPKKG	294	302	9	
AB_00406	Uncharacterized protein	Cytoplasmic Membrane	NA	NA	11,N	–	SGSARPGFS	139	147	9	57–136
AB_00641	Uncharacterized protein	Cytoplasmic	NA	NA	23,N	17–18	AEEKPTEKTEKTS TIKATEQPPKEEN	22	47	26	16–97
AB_01430	CRISPR-associated protein, Csy2 family	Unknown	NA	NA	32,N	–	RQAQDQENTAHA	227	238	12	110–178
AB_01223	Transcriptional regulator, TetR family: DNA binding	Cytoplasmic	COG1309	AcrR	33,N	–	ESNQDDQ	131	137	7	
AB_01974	Transcriptional regulator, TetR family: DNA binding	Cytoplasmic	COG1309	AcrR	11,N	–	EFPANSSERSSVKQ	112	125	14	

the network. Moreover, the non-hub connectors (R3) might be involved in connecting fundamental sets of interactions while the non-hub kinless proteins (R4) connect other proteins evenly distributed across the modules without forming hubs themselves. Furthermore, the provincial hub proteins, R5, have many within-module connections, whereas the connector hubs, R6 proteins connect most of the other modules, and thus are probably most conserved in terms of decomposition as well as evolution. Finally, the kinless hubs, R7 proteins, show the highest connection within and between the members of the GPIN such that they could be the most essential ones to be maintained by the pathogen for its very survival. Taking into account the importance of such functional modules, we have sorted the GPIN as per the KFC method adopted by Ashraf et al. (2018). We found the 165th core getting aligned with the functional module R5 proteins having the highest EC values (**Table 3**; **Supplementary Data 4**: Sheet 2).

With a consolidated set of VaC, ViF, KeF, and KFC proteins, we have set our ultimate goal to determine their antigenic potential and predicted some probable active sites. As cellular location plays an important role in conferring the potential of proteins as vaccines and/or drugs, we have attempted for the same and identified the COG classes for them, keeping in view the uncharacterized proteins taken as reference for the R6 categories. Interestingly, most of the proteins of VAC, ViF, KeF, and KFC could be classified as per COG, suggesting their likelihood to share similar functions. To this end, the antibody epitope site prediction shared 50% similarities with the predicted active sites of the validated homology modeled structures of KeF and KFC proteins, suggesting their plausibility to be used either as vaccine candidates or as drug candidates. On the contrary, VaC, ViF, and R6 proteins were unique enough and would probably have to be experimentally verified for their actual candidature. All such VaC, ViF, KeF, and KFC proteins were mapped back from ATCC19606 strains to the strains of AYE or ATCC17978 for the ease of quick referral for future researchers (**Table 5**). Besides the aforementioned list in **Table 4**, a comparison of the proteins with their *Homo sapiens* counterpart has been made as reflected in **Table 6**. This was done to ensure that the proteins of *A. baumannii* are different from their host, either fully or largely, and thus could be used as targets.

A comparison of the data from **Tables 6**, **7** shows that the protein with the accession ID AB_02233 is a good candidate for vaccine, residing on the outer membrane, having a signal peptide, and bearing much less similarity with its human counterpart. Similarly, the protein AB_03351 is a good candidate for drugs, having periplasmic location, a signal peptide, and no match at all with the human host proteins. A very difficult comparison is posed for the KeF proteins, which largely present themselves as either cytoplasmic or cytoplasmic membrane. The protein with the accession number AB_01758 could have some potential though its similarity with the human counterpart rules out such possibility. Proteins of the KFC category, surprisingly, do not present themselves as good candidates at all, all being cytoplasmic, having no signal peptides, and bearing huge similarity with their human homologs. The protein AB_02522 in this category having much less query coverage (3%), however, does not stand a chance either, owing to its cellular localization in the cytoplasm. Similarly, most cytoplasmic proteins of the R6 category present themselves as poor candidates for vaccines and/or drugs. One uncharacterized protein, viz., AB_00210 of unknown cellular localization in this category could be of some potential due to the absence of any match with human counterpart.

A completely different approach of interactor-free centrality-based ranking of the different classes of the candidates proposed by Moriel et al. (2013) unanimously pulls out AB_00353 from proteins of the outer membrane class, AB_01731, from those of the extracellular region and a set of five proteins from the periplasmic region category (**Supplementary Data 5**). Among these, the protein AB_00353 or BamA has already been found by some *in silico* approach earlier and reported to elicit high IgG antibody titer with the production of opsonizing antibodies against a virulent MDR clinical isolate using a murine pneumonia model (Singh et al., 2017). The other protein, AB_01731, coding for nucleoside diphosphate kinase, has also been reported by another group through reverse vaccinology approach (Chiang et al., 2015). Of the five periplasmic proteins, HMPREF0010_03351 (**Supplementary Data 5**) (reflected as AB_03351 in **Table 7**) has been found out by our detailed interactome-based approach as well. These proteins have the potential to be used as either vaccine candidates, for the outer membrane proteins, or drugs, for other periplasmic or cytoplasmic proteins. Unknown or uncharacterized proteins from different aforementioned categories (**Table 7**), however, can be worked upon by future researchers for more prospective candidates as well. Thus, a mixed-bag result has come out of the analyses done through our two approaches. Of the interactome-based approach, where the interactors have been considered as well, the actual candidates were sorted through network centrality and protein signature analyses and the results need to be further experimentally validated as most of the candidates are novel and not reported earlier. The other, interactor-free approach, considered the candidates from their cellular localization and network centrality analyses unanimously bring out two candidates already reported earlier. The success of future researchers in targeting a protein for vaccine candidates and/or drugs would, thus, depend on further experimental validation for any category of proteins.

CONCLUSION

The study is based on the concept of utilizing the already proclaimed vaccine candidates and virulent and other key pathogenic factors to sort and filter them to a useful list of most probable final candidates. Essentially, the work revolved around a network biological approach of analyzing the conceived networks of the aforementioned proteins and mapping them onto the whole genome perspective to shortlist the candidates. To this end, established methods of antigenicity and active site prediction have been added to produce the final list for further experimental validation.

AUTHOR CONTRIBUTIONS

The analyses and the study were conceptualized, planned, and designed by CL. Data generated by RM and SM were analyzed by CL and supported by SM and RM with tabulation. Artwork was done by RM. CL wrote the manuscript aided by inputs from SP, SM, and DG in general and RM and SM for the Materials and Methods section.

ACKNOWLEDGMENTS

The authors acknowledge the support of Sunway University, Selangor, Malaysia, and IMSc, Chennai, India, for providing the computational facilities. The authors immensely acknowledge the contribution made by Md. Izhar Ashraf in providing some initial network data for further analyses. They also wish to thank Indhuja Thirumudi, Ng Jia Wei, and Vaibhav Kandale for their preliminary initiatives to earlier forms of this work which metamorphosed to the current state.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2019.00203/full#supplementary-material

Supplementary Data 1 | Excel sheets showing the data collection, processing, interactome construction, analysis, and functions of vaccine candidates, VaC of *A. baumannii*.

Supplementary Data 2 | Excel sheets showing the data collection, processing, interactome construction, analysis, and functions of virulent factors, ViF of *A. baumannii*.

Supplementary Data 3 | Excel sheets showing the data collection, processing, interactome construction, analysis, and functions of key factors, KeF of *A. baumannii*.

Supplementary Data 4 | Excel sheets showing the data collection, processing, interactome construction, analysis, and functions of whole genome proteins of *A. baumannii*.

Supplementary Data 5 | Excel sheets showing the strain mapping, cellular localization, and centrality measures along with Venn diagram for the total set of candidate proteins of *A. baumannii*.

REFERENCES

Albert, R., Jeong, H., and Barabasi, A. L. (2000). Error and attack tolerance of complex networks. *Nature* 406:378. doi: 10.1038/35019019

Alvarez-Hamelin, J. I., Dall'Asta, L., Barrat, A., and Vespignani, A. (2005). K-core decomposition of internet graphs: hierarchies, self-similarity and measurement biases. *arXiv*. doi: 10.3934/nhm.2008.3.371

Antunes, L. C., Visca, P., and Towner, K. J. (2014). *Acinetobacter baumannii:* evolution of a global pathogen. *Pathog. Dis.* 71, 292–301. doi: 10.1111/2049-632X.12125

Apweiler, R., Bairoch, A., Wu, C. H., Barker, W. C., Boeckmann, B., Ferro, S., et al. (2004). UniProt: The universal protein knowledgebase. *Nucleic Acids Res.* 32(Suppl.1), D115–D119. doi: 10.1093/nar/gkh131

Ashraf, M. I., Ong, S. K., Mujawar, S., Pawar, S., More, P., Paul, S., et al. (2018). A side-effect free method for identifying cancer drug targets. *Sci. Rep.* 8:25042. doi: 10.1038/s41598-018-25042-2

Assenov, Y., Ramírez, F., Schelhorn, S. E., Lengauer, T., and Albrecht, M. (2007). Computing topological parameters of biological networks. *Bioinformatics* 24, 282–284. doi: 10.1093/bioinformatics/btm554

Bastian, M., Heymann, S., and Jacomy, M. (2009). Gephi: an open source software for exploring and manipulating networks. *Icwsm* 8, 361–362.

Bertot, G. M., Restelli, M. A., Galanternik, L., Urey, R. C. A., Valvano, M. A., and Grinstein, S. (2007). Nasal immunization with Burkholderia multivorans outer membrane proteins and the mucosal adjuvant adamantylamide dipeptide confers efficient protection against experimental lung infections with *B. multivorans* and *B. cenocepacia*. *Infect. Immun.* 75, 2740–2752. doi: 10.1128/IAI.01668-06

Chen, L., Zheng, D., Liu, B., Yang, J., and Jin, Q. (2015). VFDB 2016: hierarchical and refined dataset for big data analysis—10 years on. *Nucleic Acids Res.* 44, D694–D697. doi: 10.1093/nar/gkv1239

Chiang, M. H., Sung, W. C., Lien, S. P., Chen, Y. Z., Lo, A. F., Huang, J. H., et al. (2015). Identification of novel vaccine candidates against *Acinetobacter baumannii* using reverse vaccinology. *Hum. Vaccines Immunother.* 11, 1065–1073. doi: 10.1080/21645515.2015.1010910

Darvishi, M. (2016). Virulence factors profile and antimicrobial resistance of *Acinetobacter baumannii* strains isolated from various infections recovered from immunosuppressive patients. *Biomed. Pharmacol. J.* 9, 1057–1062. doi: 10.13005/bpj/1048

Diestel, R. (2000). *Graph Theory*. Heidelberg: Springer-Verlag.

Dundas, J., Ouyang, Z., Tseng, J., Binkowski, A., Turpaz, Y., and Liang, J. (2006). CASTp: computed atlas of surface topography of proteins with structural and topographical mapping of functionally annotated residues. *Nucleic Acids Res.* 34(Suppl.2), W116–W118. doi: 10.1093/nar/gkl282

Erds, P., and Rényi, A. (1960). On the evolution of random graphs. *Publ. Math. Inst. Hung. Acad. Sci.* 5, 17–61.

Eveillard, M., Kempf, M., Belmonte, O., Pailhoriès, H., and Joly-Guillou, M. L. (2013). Reservoirs of *Acinetobacter baumannii* outside the hospital and potential involvement in emerging human community-acquired infections. *Int. J. Infect. Dis.* 17, e802–e805. doi: 10.1016/j.ijid.2013.03.021

Fournier, P. E., and Richet, H. (2006). The epidemiology and control of *Acinetobacter baumannii* in health care facilities. *Clin. Infect. Dis.* 42, 692–699. doi: 10.1086/500202

Fransen, F., Boog, C. J., van Putten, J. P., and van der Ley, P. (2007). Agonists of Toll-like receptors 3, 4, 7, and 9 are candidates for use as adjuvants in an outer membrane vaccine against *Neisseria meningitidis* serogroup B. *Infect. Immun.* 75, 5939–5946. doi: 10.1128/IAI.00846-07

Guimerà, R., and Amaral, L. A. (2005b). Cartography of complex networks: modules and universal roles. *J. Stat. Mech. Theory Exp.* 2005:P02001. doi: 10.1088/1742-5468/2005/02/P02001

Guimerà, R., and Nunes Amaral, L. A. (2005a). Functional cartography of complex metabolic networks. *Nature* 433:895. doi: 10.1038/nature03288

Jeong, H., Mason, S. P., Barabási, A. L., and Oltvai, Z. N. (2001). Lethality and centrality in protein networks. *Nature* 411:41. doi: 10.1038/35075138

Juncker, A. S., Willenbrock, H., Von Heijne, G., Brunak, S., Nielsen, H., and Krogh, A. (2003). Prediction of lipoprotein signal peptides in gram-negative bacteria. *Protein Sci.* 12, 1652–1662. doi: 10.1110/ps.0303703

Kelley, L. A., Mezulis, S., Yates, C. M., Wass, M. N., and Sternberg, M. J. (2015). The Phyre2 web portal for protein modeling, prediction and analysis. *Nat. Protocols* 10:845. doi: 10.1038/nprot.2015.053

Lahiri, C., Pawar, S., Sabarinathan, R., Ashraf, M. I., Chand, Y., and Chakravortty, D. (2014). Interactome analyses of Salmonella pathogenicity islands reveal SicA indispensable for virulence. *J. Theor. Biol.* 363, 188–197. doi: 10.1016/j.jtbi.2014.08.013

Lee, C. R., Lee, J. H., Park, M., Park, K. S., Bae, I. K., Kim, Y. B., et al. (2017). Biology of *Acinetobacter baumannii*: pathogenesis, antibiotic resistance mechanisms, and prospective treatment options. *Front. Cell. Infect. Microbiol.* 7:55. doi: 10.3389/fcimb.2017.00055

Lin, M. F., and Lan, C. Y. (2014). Antimicrobial resistance in Acinetobacter baumannii: From bench to bedside. *World J. Clin. Cases* 2, 787–814. doi: 10.12998/wjcc.v2.i,12.787

MATLAB and Statistics Toolbox Release (2010). *The MathWorks, Inc.* Natick, MA.

Moriel, D. G., Beatson, S. A., Wurpel, D. J., Lipman, J., Nimmo, G. R., Paterson, D. L., et al. (2013). Identification of novel vaccine candidates against multidrug-resistant *Acinetobacter baumannii*. *PLoS ONE.* 8:e77631. doi: 10.1371/journal.pone.0077631

Oliveros, J. C. (2007–2015). *Venny. An Interactive Tool for Comparing Lists with Venn's Diagrams.* Available online at: http://bioinfogp.cnb.csic.es/tools/venny/index.html (accessed January 31, 2019).

Pan, A., Lahiri, C., Rajendiran, A., and Shanmugham, B. (2015). Computational analysis of protein interaction networks for infectious diseases. *Briefings Bioinf.* 17, 517–526. doi: 10.1093/bib/bbv059

Pavlopoulos, G. A., Secrier, M., Moschopoulos, C. N., Soldatos, T. G., Kossida, S., Aerts, J., et al. (2011). Using graph theory to analyze biological networks. *BioData Mining* 4, 10. doi: 10.1186/1756-0381-4-10

Pawar, S., Ashraf, M. I., Mehata, K. M., and Lahiri, C. (2017). "Computational identification of indispensable virulent proteins of Salmonella Typhi CT18," in *Current Topics in Salmonella and Salmonellosis*, ed M. Mares (InTech Publishers), 21–39.

Pawar, S., Ashraf, M. I., Mujawar, S., Mishra, R., and Lahiri, C. (2018). In silico identification of the indispensable quorum sensing proteins of multidrug resistant Proteus mirabilis. *Front. Cell. Infect. Microbiol.* 8:269. doi: 10.3389/fcimb.2018.00269

Perez, F., Hujer, A. M., Hujer, K. M., Decker, B. K., Rather, P. N., and Bonomo, R. A. (2007). Global challenge of multidrug-resistant Acinetobacter baumannii. *Antimicrob. Agents Chemother.* 51, 3471–3484. doi: 10.1128/AAC.01464-06

Petersen, T. N., Brunak, S., von Heijne, G., and Nielsen, H. (2011). SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat. Methods* 8, 785–786. doi: 10.1038/nmeth.1701

Rosvall, M., and Bergstrom, C. T. (2011). Multilevel compression of random walks on networks reveals hierarchical organization in large integrated systems. *PLoS ONE.* 6:e18209. doi: 10.1371/journal.pone.0018209

Schwede, T., Kopp, J., Guex, N., and Peitsch, M. C. (2003). SWISS-MODEL: an automated protein homology-modeling server. *Nucleic Acids Res.* 31, 3381–3385. doi: 10.1093/nar/gkg520

Seidman, S. B. (1983). Network structure and minimum degree. *Soc. Networks* 5, 269–287. doi: 10.1016/0378-8733(83)90028-X

Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., et al. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* 13, 2498–2504. doi: 10.1101/gr.1239303

Singh, R., Capalash, N., and Sharma, P. (2017). Immunoprotective potential of BamA, the outer membrane protein assembly factor, against MDR Acinetobacter baumannii. *Sci. Rep.* 7:12411. doi: 10.1038/s41598-017-12789-3

Szklarczyk, D., Morris, J. H., Cook, H., Kuhn, M., Wyder, S., Simonovic, M., et al. (2016). The STRING database in 2017: Quality-controlled protein–protein association networks, made broadly accessible. *Nucleic Acids Res.* 2016:gkw937. doi: 10.1093/nar/gkw937

Talbot, G. H., Bradley, J., Edwards, J. E. Jr., Gilbert, D., Scheld, M., and Bartlett, J. G. (2006). Bad bugs need drugs: an update on the development pipeline from the antimicrobial availability task force of the infectious diseases society of America. *Clin. Infect. Dis.* 42, 657–668. doi: 10.1086/499819

Tang, Y., Li, M., Wang, J., Pan, Y., and Wu, F. X. (2015). CytoNCA: a cytoscape plugin for centrality analysis and evaluation of protein interaction networks. *Biosystems* 127, 67–72. doi: 10.1016/j.biosystems.2014.11.005

Vázquez, A. (2003). Growing network with local rules: preferential attachment, clustering hierarchy, and degree correlations. *Phys. Rev. E* 67:056104. doi: 10.1103/PhysRevE.67.056104

Vella, D., Marini, S., Vitali, F., Di Silvestre, D., Mauri, G., and Bellazzi, R. (2018). MTGO: PPI network analysis via topological and functional module identification. *Sci. Rep.* 8:5499. doi: 10.1038/s41598-018-23672-0

Viehman, J. A., Nguyen, M. H., and Doi, Y. (2014). Treatment options for carbapenem-resistant and extensively drug-resistant Acinetobacter baumannii infections. *Drugs* 74, 1315–1333. doi: 10.1007/s40265-014-0267-8

Vincent, J. L., Rello, J., Marshall, J., Silva, E., Anzueto, A., Martin, C. D., et al. (2009). International study of the prevalence and outcomes of infection in intensive care units. *JAMA* 302, 2323–2329. doi: 10.1001/jama.2009.1754

Vita, R., Overton, J. A., Greenbaum, J. A., Ponomarenko, J., Clark, J. D., Cantrell, J. R., et al. (2014). The immune epitope database (IEDB) 3.0. *Nucleic Acids Res.* 43, D405–D412. doi: 10.1093/nar/gku938

Watts, D. J., and Strogatz, S. H. (1998). Collective dynamics of 'small-world' networks. *Nature* 393:440. doi: 10.1038/30918

Wu, S., Zhu, Z., Fu, L., Niu, B., and Li, W. (2011). WebMGA: A customizable web server for fast metagenomic sequence analysis. *BMC Genomics* 12:444. doi: 10.1186/1471-2164-12-444

Yu, N. Y., Wagner, J. R., Laird, M. R., Melli, G., Rey, S., Lo, R., et al. (2010). PSORTb 3.0: improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. *Bioinformatics* 26, 1608–1615. doi: 10.1093/bioinformatics/btq249

Conflict of Interest Statement: 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 Cellular and Infection Microbiology

REVIEW
published: 10 October 2019
doi: 10.3389/fcimb.2019.00344

The Quest for a Truly Universal Influenza Vaccine

Yo Han Jang[1†] *and Baik Lin Seong*[1,2*]

[1] *Molecular Medicine Laboratory, Department of Biotechnology, College of Life Science and Biotechnology, Yonsei University, Seoul, South Korea,* [2] *Vaccine Translational Research Center, Yonsei University, Seoul, South Korea*

There is an unmet public health need for a universal influenza vaccine (UIV) to provide broad and durable protection from influenza virus infections. The identification of broadly protective antibodies and cross-reactive T cells directed to influenza viral targets present a promising prospect for the development of a UIV. Multiple targets for cross-protection have been identified in the stalk and head of hemagglutinin (HA) to develop a UIV. Recently, neuraminidase (NA) has received significant attention as a critical component for increasing the breadth of protection. The HA stalk-based approaches have shown promising results of broader protection in animal studies, and their feasibility in humans are being evaluated in clinical trials. Mucosal immune responses and cross-reactive T cell immunity across influenza A and B viruses intrinsic to live attenuated influenza vaccine (LAIV) have emerged as essential features to be incorporated into a UIV. Complementing the weakness of the stand-alone approaches, prime-boost vaccination combining HA stalk, and LAIV is under clinical evaluation, with the aim to increase the efficacy and broaden the spectrum of protection. Preexisting immunity in humans established by prior exposure to influenza viruses may affect the hierarchy and magnitude of immune responses elicited by an influenza vaccine, limiting the interpretation of preclinical data based on naive animals, necessitating human challenge studies. A consensus is yet to be achieved on the spectrum of protection, efficacy, target population, and duration of protection to define a "universal" vaccine. This review discusses the recent advancements in the development of UIVs, rationales behind cross-protection and vaccine designs, and challenges faced in obtaining balanced protection potency, a wide spectrum of protection, and safety relevant to UIVs.

Keywords: influenza virus, universal influenza vaccine, cross-protection, HA stalk, M2e, T cell, live attenuated influenza vaccine

OPEN ACCESS

Edited by:
Yanmin Wan,
Fudan University, China

Reviewed by:
Peter Palese,
Icahn School of Medicine at Mount Sinai, United States
Florian Krammer,
Icahn School of Medicine at Mount Sinai, United States

***Correspondence:**
Baik Lin Seong
blseong@yonsei.ac.kr

†Present address:
Yo Han Jang,
Department of Biological Sciences and Biotechnology Major in Bio-Vaccine engineering, Andong National University, Andong, South Korea

Specialty section:
This article was submitted to Clinical Microbiology, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *24 May 2019*
Accepted: *24 September 2019*
Published: *10 October 2019*

Citation:
Jang YH and Seong BL (2019) The Quest for a Truly Universal Influenza Vaccine. Front. Cell. Infect. Microbiol. 9:344. doi: 10.3389/fcimb.2019.00344

INTRODUCTION

Influenza viruses present a high level of antigenic diversity and variability due to their segmented RNA genome. These viruses are classified into four major types, A, B, C, and D, based on their nucleoprotein (NP) and matrix (M) genes. Human infecting type A and B viruses are further classified into multiple subtypes or lineages, respectively, depending on the antigenicity of viral surface proteins, hemagglutinin (HA), and neuraminidase (NA) genes (Paules and Subbarao, 2017). Influenza A and B viruses co-circulate in every season and, thus, c represents the primary targets of seasonal influenza vaccines (Sridhar et al., 2015). In addition to seasonal epidemics, influenza viruses have caused pandemics at the intervals

of ∼10–40 years since the 1918 Spanish flu H1N1, the 2009 pandemic H1N1 being the last outbreak (Saunders-Hastings and Krewski, 2016). While vaccination remains the most cost-effective measure to prevent influenza virus infections, antigenic drift in the surface antigens allows these viruses to escape antibody-mediated neutralization (Kim et al., 2018a). In addition, the sudden occurrence of pandemics is often accompanied by zoonotic spillover of the surface genes into the human-infecting viruses, rendering preexisting vaccines ineffective to newly emerging viruses. The variation caused by genetic drift and shift is unpredictable, posing a serious challenge to the management of influenza outbreak. Based on the amino acid sequences of HAs, influenza A viruses (IAVs) are divided into two phylogenetic groups. The IAV HA group 1 viruses include H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, and H18, while the group 2 viruses comprise H3, H4, H7, H10, H14, H15 (**Figure 1**). The NAs of IAVs are also antigenically diverse, presenting two distinct groups. Influenza B viruses (IBVs) are not divided into subtypes but circulate as two distinct Yamagata-like and Victoria-like lineages. Influenza C viruses (ICVs) generally cause a mild respiratory disease in humans and do not cause epidemics (Dykes et al., 1980). Contemporary influenza epidemics are caused by the H1N1 and H3N2 of the IAVs and one or two lineages of the IBVs, dictating trivalent (TIV) or quadrivalent influenza vaccine (QIV) containing two IAV antigens and one or two IBV antigens, respectively (Ambrose and Levin, 2012).

Many strategies have been undertaken on the pursuit of developing a universal influenza vaccine (UIV) (Paules et al., 2017). The induction of cross-protective immune responses directed toward conserved B cell or T cell epitopes is a major principle underlying broad protection (**Figure 2**). The direct binding of antibodies to the viral surface proteins interferes with their functions and results in virus neutralization before cell entry (**Figure 2A**). Alternatively, the antibodies may bind to viral antigens displayed on the surface of virus-infected cells and mediate effector functions to remove the infected cells. A cytotoxic T lymphocyte (CTL) can kill the virus-infected cells in an independent manner (**Figure 2B**). Several conserved viral antigens such as M2 extracellular domain (M2e), HA stalk and receptor binding site, NA, and T cell epitopes in the internal proteins such as polymerase basic protein 1 (PB1), NP, and M1 were defined as targets for eliciting cross-reactive immune response. A variety of vaccine platforms were tested for effective exposure of those antigens to the immune system (Wang et al., 2018a; Estrada and Schultz-Cherry, 2019). While these vaccines showed broader cross-protection than the classical inactivated influenza vaccines (IIVs), their protection potency was weak. They provided partial protection against the antigenically distant viruses, and the protection breadth is usually limited to the same group (subtype-specific or HA group-specific) and not effective in another HA group. Thus, there exists a considerable gap between the current status, and the ultimate goal, of a truly universal vaccine.

Some studies have discovered rare antibodies specific to the conserved HA stalk in animals and humans with prior exposure to influenza viruses. Some of these antibodies show extremely broad specificity, encompassing both HA groups of IAVs (Ekiert et al., 2011) or even both IAVs and IBVs (Dreyfus et al., 2012), representing an optimistic prospect of developing a UIV. However, no studies so far have reported a successful vaccine approach that induces such antibodies to a protective level, reflecting considerable difficulty in selectively inducting particular antibodies in the context of vaccination. While antibody-dependent effector mechanisms and T cell immunity have emerged as potential correlates of broad protection against influenza infections (Krammer and Palese, 2015), the exact molecular mechanisms of their protective action are not completely understood, and immunopathology by T cell responses still remains a challenge (Peiris et al., 2010; Duan and Thomas, 2016; Erbelding et al., 2018). Moreover, understanding how preexisting immunity (immune imprinting or antigenic sin-like phenomenon) shaped by prior exposure to influenza viruses affects the magnitude, hierarchy, and sustainability of antibody response to vaccination is suggested as critical for designing a UIV in humans (Henry et al., 2018). The involvement of multiple factors in eliciting broad protection and the influence of pre-existing immunity on the subsequent vaccination pose a dilemma on establishing the correlate of cross-protection against heterologous or hetero-subtypic viruses, representing a critical obstacle for the licensure of a UIV by the regulatory authorities.

This review updates the recent advances in UIV development and focuses on the critical issues to be addressed in designing a 'truly' UIV. Alternative vaccine antigens and vaccine strategies for durable and broader protection are also discussed in detail. This review is meant to help the readers to acquire general information on the cutting edge of UIV researches and to gain wide perspectives on rational design of a truly UIV with improved potency, breadth, and safety.

CRITERIA FOR A UIV

Definition of Universal Protection

Although there is a clear consensus about the urgent need for a vaccine that provides durable and broad protection against multiple strains of influenza virus, the definition of the "universality" of UIV is still debated (Krammer et al., 2018b). Recently, the National Institute of Allergy and Infectious Diseases (NIAID) held a meeting to identify and develop the criteria to define a UIV. The participants from multiple disciplines agreed that a reasonable UIV should be at least 75% effective against symptomatic influenza virus infection caused by group 1 and group 2 IAVs, with the protection lasting over 1 year for all age groups (Paules et al., 2017; Erbelding et al., 2018) (**Table 1**). Similar criteria have also been suggested as preferred product characteristics (PPC) and target product profiles (TPP), describing the desired characteristics of a UIV, by the World Health Organization (WHO) and the Bill and Melinda Gates Foundation (BMGF), respectively (**Table 1**). Despite the priority given to IAVs in WHO PPC (**Table 1**), the IBVs represent a "low hanging fruit" because of their low antigenic diversity and the lack of animal reservoir, presenting credence to potential eradication from humans (Tan et al., 2018). The proposed consensus to define universal protection

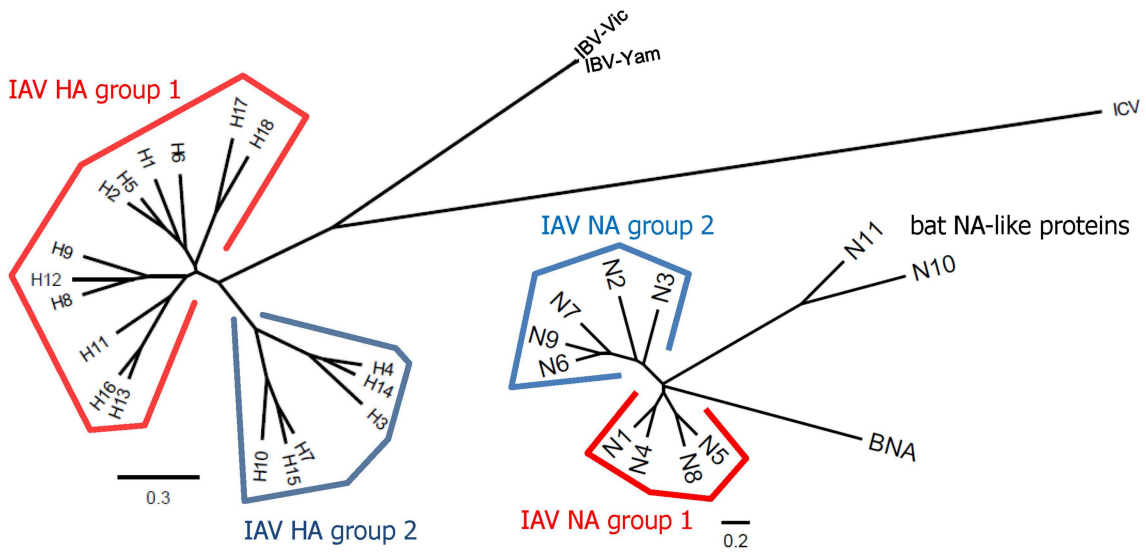


FIGURE 1 | Phylogenetic trees representing HA and NA diversity among influenza viruses. The 18 subtypes of HAs of IAV are divided into two phylogenetic groups according to their amino acid sequences similarities. The HAs of IBVs are divided into Victoria-like and Yamagata-like lineages but they are closer to each other than any of two different subtypes of IAVs. The HAs of ICVs are antigenically distant from those of IAVs and IBVs. The NAs of IAVs also show high levels of antigenic variability and are divided into two groups. Phylogenetic trees were constructed based on amino acid sequence comparisons among influenza viruses. Multiple alignments were carried out using the representative sequence of each HA or NA subtype or lineage. The phylogenetic trees were constructed by the ClustalW algorithm using neighbor joining (N-J) method and visualized by FigTree v1.4.4. The scale bars represent amino acid change (%).

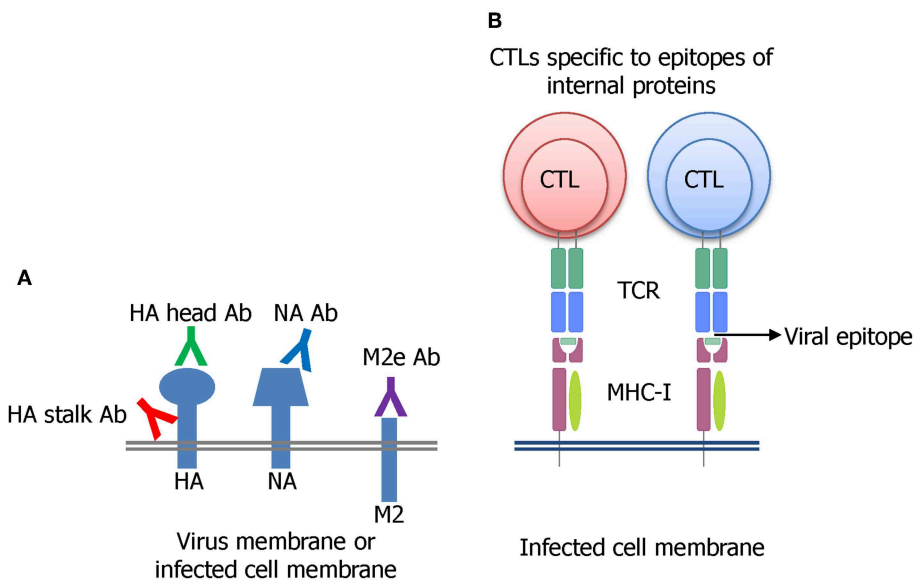


FIGURE 2 | Major principles of developing a UIV. **(A)** Induction of broadly protective antibodies specific to conserved regions in the HA stalk or head, NA, or M2e has been extensively investigated. The antibodies bind to viral surface proteins either on virion or expressed on virus-infected cell membrane. **(B)** Cytotoxic T lymphocyte (CTL) recognizing the conserved epitopes of influenza internal proteins such as NP, M1, or PB1 has been shown to be critical for broad protection through the clearance of the virus-infected cells.

offers a valuable guideline to develop an effective and safe UIV. Ideally, the spectrum of protection may covers both IAVs and IBVs, considering the existence of extremely broadly protective antibodies and T cell epitopes across IAVs and IBVs from animals and humans (Corti et al., 2011; Koutsakos et al., 2019). However, as the antigenic difference between a vaccine and a target virus becomes larger, the number of conserved B cell or T cell epitopes between the two strains decreases. The limited availability of target epitopes may result in compromised protection robustness due to (1) decreased clonal diversity of cross-reactive antibodies

TABLE 1 | Consensus criteria for a UIV.

	NIAID	WHO	BMGF	Consensus
Breadth	All influenza A viruses (influenza B protection would be the second target)	All influenza A viruses	All influenza A and B viruses	All influenza A viruses
Efficacy	At least 75% effective against symptomatic influenza infection	Better than that of current seasonal influenza vaccine	At least 70% effective against symptomatic influenza infection	At least 70% effective
Target population	All age groups	>6 weeks, no upper age limit including high risk groups	>6 weeks, no upper age limit including high risk groups	>6 weeks, no upper age limit
Duration of protection	At least 1 year	At least 5 years	3–5 years	At least 1 year

NIAID, National Institute of Allergy and Infectious Diseases (https://www.niaid.nih.gov/diseases-conditions/universal-influenza-vaccine-research); BMGF, Bill and Melinda Gates Foundation (https://gcgh.grandchallenges.org/challenges). WHO, World Health Organization (https://apps.who.int/iris/bitstream/handle/10665/258767/9789241512466-eng.pdf;jsessionid=37D9E056C3EA58A90EE237432AA7D65F?sequence=1).

and T cells, or (2) occurrence of escape mutants by even small amount of genetic mutations in the epitopes. Therefore, a balanced breadth and robustness of cross-protection should be considered when developing a reliable UIV (**Figure 3**).

The NIAID and the researchers have proposed three research areas to address the knowledge gaps in developing a UIV; (1) understanding of influenza transmission, natural history, and viral pathogenesis, (2) characterization of correlates of protection, and (3) rational design of a UIV to improve potency and breadth of protection (Erbelding et al., 2018). In particular, the correlates of protection elicited by a UIV may vary considerably in quantity and quality, depending on the vaccine type used and the target influenza virus tested. For instance, while the protection potency of HA stalk-based vaccine may easily be evaluated by measuring the neutralizing activity or indirect effector mechanisms by the stalk-reactive antibodies (Jegaskanda et al., 2017b), such correlates of protection cannot be used to evaluate the protective efficacy of a T cell epitope-based vaccine containing non-HA epitopes. Moreover, when using HA stalk-based vaccines, high levels of stalk-reactive antibodies represent a good protective efficacy against the same group IAVs (**Figure 1**). However, the binding affinities of HA stalk antibodies are variable among different viruses, and therefore may require different antibody titers to exhibit sufficient protection against the viruses. This speculation is supported by the observations that broadly reactive HA stalk antibodies show considerably different neutralizing abilities and binding affinities to the viruses within the same HA group (Ekiert et al., 2011, 2012). Furthermore, HA stalk-based vaccine and M2e-based vaccine may demonstrate a different balance of protective abilities to each other, between direct neutralization and indirect effector mechanisms (ADCC, for instance) by the respective antibodies. It has been shown that M2e antibodies provide protection via FcR-dependent effector functions rather than direct virus neutralization (Deng et al., 2015a), whereas HA stalk antibodies exert both direct neutralization and indirect effector functions (Krammer and Palese, 2015). The antibody-mediated inhibition of virus attachment measured by hemagglutinin inhibition assay or microneutralization assay is the gold standard for seasonal influenza vaccines (Ohmit et al., 2011). However, the assay cannot be applied simply to reflect the cross-protection by a UIV against heterologous/heterosubtypic influenza viruses. Therefore, it is essential to develop mechanistically distinct *in vitro* or *in vivo* assays to measure potential correlates of protection in order to evaluate the protection potency and breadth of the vaccine.

Mode of Protection by a UIV

The cornerstone of developing a UIV is the determination of the precise protection mechanisms of immune response against influenza viruses. Influenza HA recognizes sialic acid on the cellular receptors and initiates infection by entering the cell via receptor-mediated endocytosis (**Figure 4**). While HA inhibitory (HAI) antibodies have long been considered as the gold standard for strain-specific protection, very few of them were shown to elicit a broad protection by binding to the conserved receptor-binding site (RBS) of HA, thereby preventing viral entry to the cell (Krause et al., 2011; Ekiert et al., 2012). Recently, multifunctional protection mechanisms have been described for HA stalk-reactive antibodies. It has been shown that HA stalk antibodies may inhibit membrane fusion, the release of viral genome into the cytoplasm of the cell, and maturation of the HA precursor (Krammer and Palese, 2015). Furthermore, HA stalk antibodies can induce antibody-dependent effector functions such as antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and complement-dependent cytolysis (CDC), resulting in clearance of virus-infected cells by the immune cells or the complement system (Jegaskanda et al., 2017b). During viral budding, NA cleaves the sialic acid from HA and supports multiple infection cycles by release of the newly assembled viral particles. NA inhibitory (NAI) antibodies specific to the conserved regions have shown an exceptional breadth, inhibiting divergent influenza viruses (Chen et al., 2018). In addition to the broadly protective antibodies, T cell immunity against conserved viral internal proteins also provides a broad protection. Cross-reactive cytotoxic T lymphocytes (CTLs) recognize the viral epitopes presented on MHC molecules and kill the infected cells. It is noteworthy that the cross-reactivity of T cell immunity has been recently shown to cover both IAVs and IBVs, and even the ICVs (Koutsakos et al., 2019), although its protective role *in vivo* has not been confirmed.

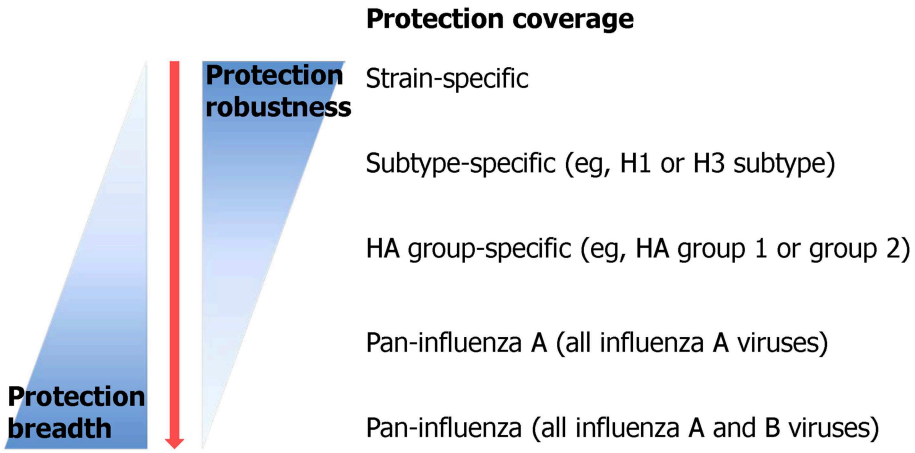


FIGURE 3 | Protection breadth of influenza vaccines ranging from strain-specific protection to pan-influenza universal protection. Currently licensed seasonal influenza vaccine provides only strain-specific protection against well-matched strains. Recently, many efforts have been put in order to improve the protection breadth of a vaccine from subtype-specific protection to, ideally, pan-influenza universal protection [adopted from Erbelding et al. (2018)].

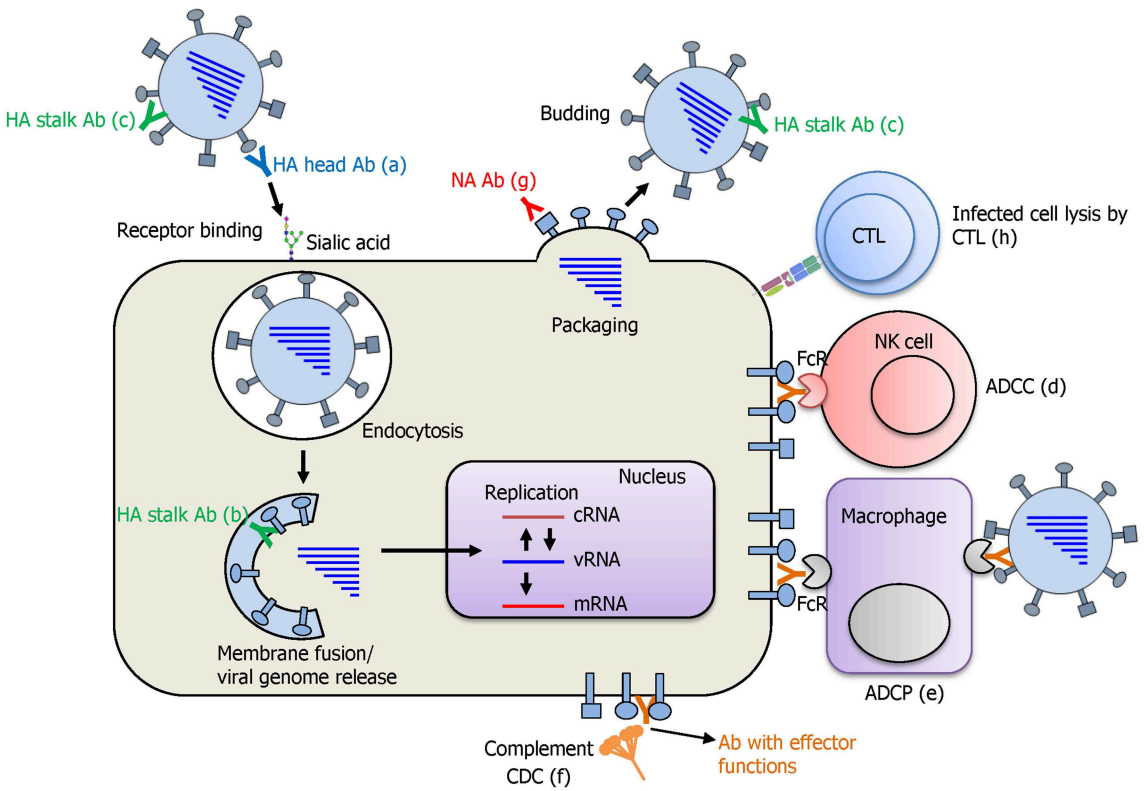


FIGURE 4 | Protection mode of action afforded by a UIV. Antibodies against the HA globular head domain inhibit viral attachment via HA-mediated receptor binding to the sialic acid on cellular receptors (a). HA stalk antibodies have multiple protective functions. As the virus enters the cell, pre-bound stalk antibodies prevent the fusion of viral and endosomal membranes and block the viral genome release into cytoplasm of the cell (b). Binding of stalk antibodies can also limit the access of cellular proteases to the cleavage site located in the stalk domain and inhibit the cleavage and subsequent conformational change of HA that is an essential step for acquiring viral infectivity (c). Different antibodies against HA stalk and also other viral proteins such as NA, M2, and NP are shown to mediate antibody-dependent effector functions such as antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC), leading to the lysis of the virus-infected cells by immune cells or complement system (d–f). NA antibodies inhibit receptor destroying activity of NA and prevent the budding of newly formed viral particles from the cells (g). Cytotoxic T lymphocytes (CTLs) recognize the viral peptide presented on MHC-I molecule and kill the virus infected cell by the secretion of cytotoxic granules and cytokines (h).

CURRENT STATUS OF M2e-BASED UIV APPROACHES

IAVs have two major surface proteins, HA and NA, and one minor surface protein, the M2 ion channel. During the infection cycle, the M2 ion channel is responsible for acidification of the viral interior, facilitating virus uncoating, and unloading of viral ribonucleoproteins (RNPs) into the host cytoplasm (Pinto et al., 1992). The extracellular domain of M2 protein (M2e) consists of 24 amino acids, among which 9 N-terminal amino acids are completely conserved among all IAVs (H1–H18) and minor mutations are observed in the distal portion (Deng et al., 2015a; Tsybalova et al., 2018). Therefore, M2e is considered as a promising target for eliciting broadly reactive antibodies. However, due to poor immunogenicity of the small M2e region, UIV approaches targeting M2e required carriers, vectors, and adjuvants to enhance immune responses (Deng et al., 2015a; Lee et al., 2015). One of the most efficient approaches is to make virus-like particles (VLPs) which display M2e on their surface. It has been shown that the hepatitis B virus core (HBc) protein fused with M2e self-assembled into VLPs that resemble wild type virus particles, expressing M2e on the surface (Neirynck et al., 1999). Following this work, substantial efforts were made to improve the immunogenicity and protective efficacy of M2e-HBc VLP vaccine constructs. Major strategies include the use of multiple copies of M2e by tandem repeats (Ravin et al., 2015; Sun et al., 2015; Tsybalova et al., 2015) and combination with adjuvants such as cholera toxin A1 (De Filette et al., 2006) or B subunit of *Escherichia coli* heat labile enterotoxin (LTB) (Zhang et al., 2009). The M2e-HBc VLP was further harnessed with T cell immunity by combining conserved T cell epitope from NP (Gao et al., 2013). The arginine-rich domain of HBc was shown to induce Th1-biased immune response of M2e-HBc VLP by binding to RNA, leading to improved protection (Ibanez et al., 2013). Besides the HBc, M2e was fused with various coat or capsid proteins derived from other viruses including Malva mosaic virus (Leclerc et al., 2013), tobacco mosaic virus (Petukhova et al., 2013), Papaya mosaic virus (Denis et al., 2008), T7 bacteriophage (Hashemi et al., 2012), and RNA phage Qβ (Bessa et al., 2008). Moreover, the enveloped VLPs displayed M2e in a membrane-anchored manner by combining influenza matrix protein and transmembrane (TM) domain of HA fused into M2e. The Sf9 insect cells infected with baculoviral vectors expressing influenza M1 and M2e-TM of influenza HA produced influenza VLPs displaying much higher levels of M2e than the wild type virions (Kim et al., 2015, 2017). Other vaccine types, including DNA vaccines or recombinant protein vaccines expressing M2e with various carriers to enhance immune responses, have also been advanced (Deng et al., 2015a).

Many studies conducted on animals and humans have elucidated the mechanisms of cross-protection conferred by M2e-based vaccines. A common observation is that antibodies specific to M2e cannot directly neutralize the viruses but confer cross-protection by eliciting several mechanisms of antibody-mediated and cell-mediated immune responses. The most well-characterized protection mechanisms include ADCC, ADCP, and CDC (**Figure 4**). Studies have shown that multiple types of immune cells including the natural killer (NK) cells, neutrophils, dendritic cells, or macrophages have the ability to sense the virus-infected cells by interaction with the Fc receptors (FcR) and the Fc region of M2e antibodies. This results in killing (ADCC) or phagocytosis (ADCP) of the infected cells (Huber et al., 2001; Jegerlehner et al., 2004; Hashimoto et al., 2007; Guilliams et al., 2014). The complement is also known to bind to the Fc of M2e antibodies and triggers the complement cascade, leading to formation of the membrane attack complex and target cell lysis (CDC) (Wang et al., 2008; Kim et al., 2018b). These studies together suggest that M2e-specific non-neutralizing antibodies play a crucial role in broad protection against IAVs through the clearance of the virus-infected cells. Besides non-neutralizing effector functions, it has also been shown that M2e-displaying recombinant bacteriophages induce HAI antibodies in a mouse model (Deng et al., 2015b). In addition, the T cell responses directed to M2e have been shown to contribute to cross-protection. Several studies have shown the presence of CD4 and CD8 T cell epitopes in M2e region in mice and humans (Jameson et al., 1998; Gianfrani et al., 2000; Mozdzanowska et al., 2003; Eliasson et al., 2008). In line with this, it has been demonstrated that CD4 T cell and CD8 T cell-mediated immunity are critical for cross-protection elicited by M2e-based UIVs including VLPs or recombinant peptide vaccines (Lee et al., 2014; Zhang et al., 2016; Arevalo et al., 2017; Pendzialek et al., 2017; Eliasson et al., 2018).

It remains a challenge to overcome the low protective efficacy of M2e-based vaccines, due to intrinsic low immunogenicity of the vaccine antigen and the low abundance of M2 proteins on influenza virions and infected cells. From the clinical and practical standpoints, multiple immunizations with a high dose of vaccine antigens combined with potent adjuvants may pose safety concerns, rendering M2e-based vaccine approaches inadequate as a stand-alone UIV. Consequently, M2e antigen was combined with other influenza viral antigens (mostly HA) or supplemented with strain-specific vaccines, resulting in improved potency, and broader cross-protection. For instance, several studies combined M2e with influenza HA head or stalk domain to elicit broadly protective immunity against heterologous influenza infections with various vaccine platforms including nanoparticles, recombinant proteins, and recombinant influenza viruses (Guo et al., 2017; Bernasconi et al., 2018; Deng et al., 2018; Tsybalova et al., 2018). Supplemented vaccination with M2e was examined to overcome the strain-specific protection or poor immunogenicity of classical inactivated or split vaccines (Music et al., 2016; Song et al., 2016). Besides the approaches described above, a vast amount of research on M2e-based UIVs have accumulated in the last decade, which are described in specialized reviews (Deng et al., 2015a).

HA STALK-BASED UIV APPROACHES

General Principles of HA Stalk-Based Approaches

The HA stalk-based approach currently leads the mainstream of UIV development. Influenza HA is the primary target antigen of the currently licensed seasonal vaccine, and HAI antibodies serve as the "gold standard" when evaluating the protective

efficacy of HA-based vaccines (Ohmit et al., 2011). However, HA-based vaccines provide only strain-specific protection to antigenically homologous viruses, necessitating annual update of the surface antigens of seasonal vaccines to match the circulating viral strains. HA comprises two distinct domains; globular head, and stalk domains. The head domain is highly variable and immunologically dominant and thus the majority of HI antibodies are directed to the head domain that harbors the RBS (**Figure 5**). In contrast, the HA stalk domain is relatively conserved among the viruses, and therefore eliciting antibodies specific to the stalk is considered as a key to developing a UIV. However, the stalk is generally regarded as immunologically subdominant, due to masking effect by bulky head domain and close proximity to the viral membrane (Krammer and Palese, 2013; Crowe, 2018; Krammer, 2019), which necessitates rational design of a HA antigen and novel vaccination strategies to increase stalk-reactive antibodies. The major protective function of HA stalk antibodies is to lock the HA trimer in a prefusion state. This prevents pH-dependent conformational change of HA that triggers the membrane fusion and release of viral genome into cytoplasm of host cell (Ekiert et al., 2009). The membrane fusion inhibitory activity of the stalk antibodies may lead to neutralization of influenza viruses, although the neutralizing potency is weaker than head antibodies that directly prevent the receptor binding. Besides the neutralizing activity, stalk antibodies have multiple indirect mechanisms that contribute to broad protection, including antibody-dependent effector mechanisms (Jegaskanda et al., 2017b) and the inhibition of the NA enzymatic activity (Wohlbold et al., 2016; Chen et al., 2019). To overcome the low immunogenicity of HA stalk, genetic modifications were required for efficient exposure of stalk to the host immune system. Prime-boost vaccination with chimeric HAs or "headless" HAs were designed to boost stalk-reactive antibodies (**Figure 5**). Alternatively, hyperglycosylation in HA head resulted in the redirection of immune response from head to stalk (Krammer and Palese, 2015) (**Figure 5**). These approaches have paved the way to increase the breadth of protection against influenza viruses.

Protection Mechanisms of HA Stalk-Based Vaccine

A primary function of stalk-reactive antibodies is membrane fusion inhibition. Following the influenza virus entry into the cells via endocytosis, a pH-dependent conformational change of HA proteins triggers the membrane fusion between the virus and endosome, leading to release of the viral genome into the cytoplasm. Binding of the antibody to the stalk interrupts the conformational change of HA and prevents subsequent membrane fusion. This leads to the trapping of the virus in the endosome, eventually aborting the infection (Ekiert et al., 2009; Sui et al., 2009). Moreover, binding of the stalk antibody blocks the access of proteases to the cleavage site located in stalk region and prevents protease-dependent cleavage of the HA precursor into HA1 and HA2 subunits, which is a prerequisite for the conformational change of HA (Ekiert et al., 2009; Brandenburg et al., 2013). These two overlapping functions of stalk-reactive antibodies result in inhibition of membrane fusion and direct neutralization of the virus. In addition to these mechanisms that operate at the early stage of infection, the stalk antibody may also bind to the newly expressed HA proteins on the cell surface, and interfere with the viral budding or release, at the later stage of infection (Tan et al., 2014).

In addition to direct neutralization, stalk antibodies have indirect mechanisms that involve diverse innate immune cells to clear the virus-infected cells from the host. The NK cells sense virus-infected cells via interaction between FcR and Fc portion of antibody bound to HA stalk expressed on the cell surface, and kill the infected cells by releasing cytotoxic granules and antiviral cytokines, via ADCC mechanism (DiLillo et al., 2014) (**Figure 4**). The FcR-Fc interaction is also required for ADCP, by which macrophages or neutrophils recognize and engulf antibody-bound influenza viral particles or infected cells (Ana-Sosa-Batiz et al., 2016). In addition, stalk antibodies bound to virus-infected cells are also able to activate the complement system, leading to the lysis of virus-infected cells (CDC) (Terajima et al., 2011). Animal challenge models have demonstrated broad protection by passive immunization with HA stalk monoclonal antibodies (DiLillo et al., 2014, 2016). The stalk-reactive antibodies also inhibit NA enzymatic activity through steric hindrance, limiting NA access to the HA-bound sialic acid, thereby preventing viral egress (Wohlbold et al., 2016; Chen et al., 2019; Kosik et al., 2019). The failure to release newly assembled virions from the infected cells prevents the viruses from entering into multiple cycles of infection. Overall, the stalk antibodies confer a broad protection from influenza virus infection through multiple mechanisms affecting both HA and NA throughout the infection cycle of influenza virus.

HA Stalk-Based Vaccine Constructs

The first reported HA stalk-based UIV construct is the headless HA, a deletion mutant of HA lacking the globular head region (Graves et al., 1983; Sagawa et al., 1996; Steel et al., 2010) (**Figure 5**). While the initial strategies demonstrated broadly protective potentials in animal models, the removal of the head region often led to misfolding of the stalk immunogen, rendering the critical neutralizing epitopes ineffective. Subsequent studies were conducted to enhance the structural integrity of the stalk immunogens by rational designs to mimic the native trimeric conformation (Lu et al., 2014; Mallajosyula et al., 2014; Impagliazzo et al., 2015). Other studies designed and evaluated nanoparticle structures consisting of the stalk antigens for broad protection in mice and ferret models (Yassine et al., 2015; Corbett et al., 2019). The second approach to preferably induce stalk-reactive antibodies is based on chimeric HA that consists of stalk domain derived from the major target strains such as H1, H3, or B viruses fused with head domain from irrelevant avian strains such as H5, H6, or H8 (Krammer and Palese, 2015) (**Figure 5**). Multiple immunization was required to increase the stalk antibodies sufficient to provide protection from viruses within the same HA group (Hai et al., 2012; Krammer et al., 2013, 2014). The chimeric HA approach involves a full-length functional HA protein and, therefore, it may be expressed in genetically engineered viruses of the wild type or live attenuated

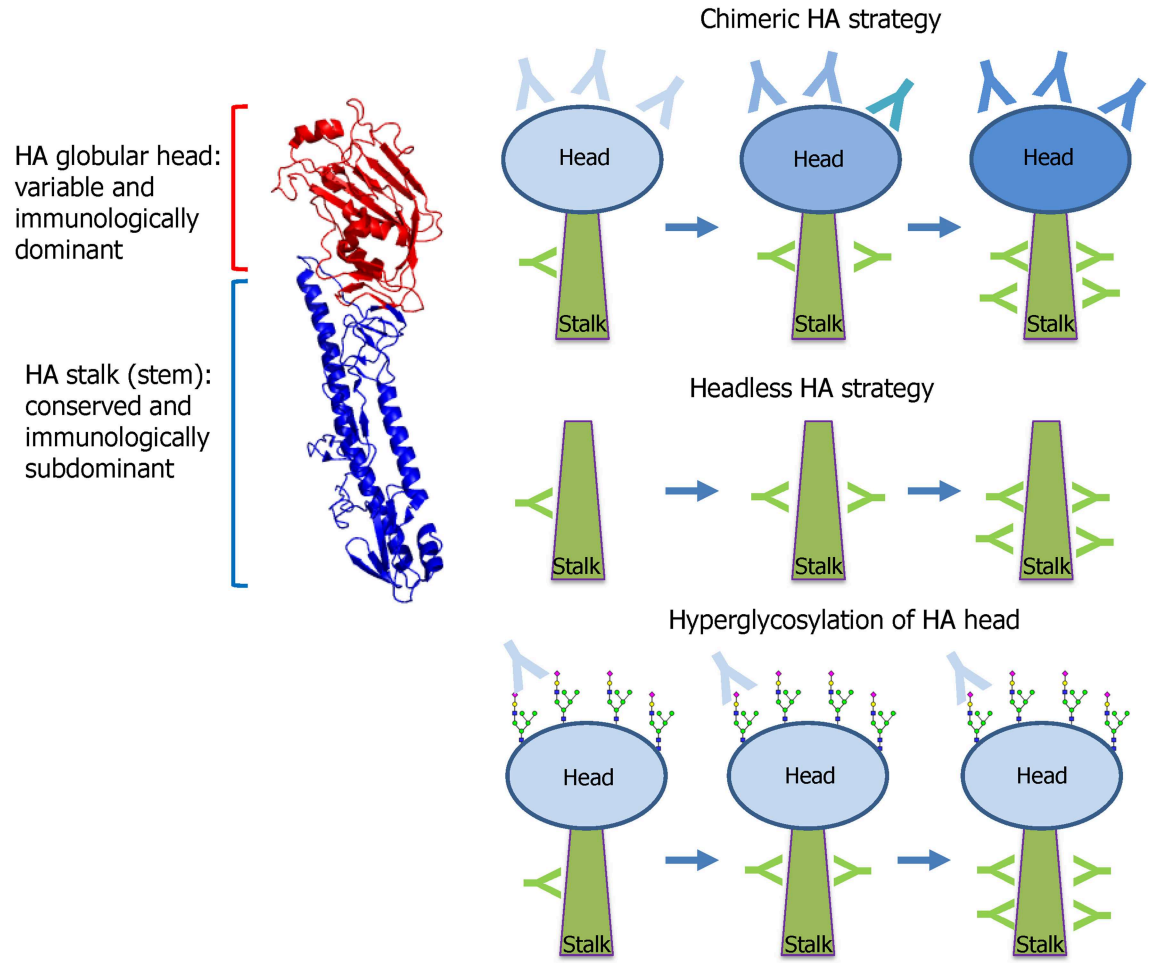


FIGURE 5 | HA stalk-based approaches. Influenza HA comprises two functionally distinct domains; globular head and stalk. The globular head is variable among the viruses and immunologically dominant and, thus, the majority of neutralizing antibodies are directed to the head that harbors the receptor-binding site (RBS). The stalk is conserved and, therefore, stalk antibodies show broad protection against diverse strains within a group. However, the stalk is immunologically subdominant because of the physical shielding by the bulky head and the close proximity to the viral membrane. To preferably induce the broadly protective stalk-reactive antibodies, two vaccine approaches were designed; chimeric HA and headless HA. Chimeric HA consists of stalk domain derived from important target strains such as H1, H3, or B viruses fused with head domain from irrelevant avian strains such as H5, H6, or H8. Multiple immunizations with different chimeric HAs selectively increase stalk antibodies. Headless HA (stalk-only HA) lacking the head domain can induce only stalk antibodies through multiple vaccinations. Hyperglycosylation in antigenic sites in head domain results in the redirection of antibody responses toward stalk domain. The HA structure was downloaded from the Protein Data Bank (HA of A/swine/Iowa/15/1930, PDB ID 1RUY) and the final image was produced by PyMOL program).

influenza viruses (Krammer et al., 2013; Isakova-Sivak et al., 2018). Third approach relies on hyperglycosylation in the HA head domain (**Figure 5**). The introduction of additional N-glycosylation sites into the immunodominant head domain shields the major antigenic sites in the head and redirects the host immune responses toward the immune-subdominant stalk. The hyperglycosylated HA antigens induced higher stalk antibodies and provided better protection than the wild type HA in a mouse model (Eggink et al., 2014). Similar approaches were tested for avian H5N1 vaccines, in which hyperglycosylated HA antigens were delivered in various vaccine formats such as DNA, recombinant protein, VLP, and adenoviral vector (Lin et al., 2012, 2014). A recent study has shown that glycan shielding of HA head resulted in immune focus on a conserved epitope occluded at the head interface, with Fc-dependent protection activity in mice (Bajic et al., 2019a). These results together suggest that hyperglycosylation in the HA head is a promising strategy to find novel target epitopes hiding in the head and stalk in HA.

Current Issues in HA Stalk-Based UIVs

Vaccine Efficacy

As discussed above, the protective efficacy of HA-stalk based vaccines is relatively weak, and multiple boost immunizations are required for efficient protection (Krammer et al., 2013, 2014). In addition, the breadth of protection is within the same HA group rather than both the groups (Krammer et al., 2013). To complement the low efficacy, prime-boost approaches that entail sequential immunization by LAIV followed by inactivated virus

containing chimeric HAs are being evaluated. As examined by a ferret challenge model, this approach provided potent cross protection (Nachbagauer et al., 2017). It was observed that there is a "disconnect" between stalk antibody titers in serum and the protection level (Nachbagauer et al., 2017). The results show that the broad protection observed in the ferret model was primarily mediated by multiple-level immune responses by LAIV and rather than the stalk antibodies (see section Live attenuated influenza vaccine as an alternative strategy below). This observation in preclinical model leads to the question of the relevance of HA stalk antibodies to cross-protection in humans. Studies with human infection model have led to a somewhat different interpretation on the protective role of the stalk antibodies, depending on the study design. A human challenge study showed that the naturally occurring stalk antibody titers were predictive of lowering viral shedding, but demonstrated poor correlation with the reduction of clinical symptoms upon the pandemic A/H1N1 challenge (Park et al., 2018). In contrast, a more recent study suggested the preexisting HA stalk antibodies as an independent correlate of protection against natural infection with the pandemic A/H1N1 virus (Ng et al., 2019). Thus, further studies are required for a conclusive demonstration of the breadth and efficacy of cross-protection offered by HA stalk antibodies in human challenge models.

Potential Vaccine Safety Issues

Although the HA stalk based strategies have shown promising broad protection in animal models, potential safety issues have been raised (Crowe, 2018; Khurana, 2018). It is suggested that the stalk antibodies are responsible for antibody-dependent enhancement of viral infectivity in a swine model. In a study, pigs vaccinated with inactivated H1N2 vaccine developed more severe respiratory diseases upon heterologous H1N1 challenge as compared to the non-vaccinated control, which was ascribed to infectivity-enhancing effects of stalk antibodies (Khurana et al., 2013). Similarly, the HA subunit vaccine resulted in vaccine-associated enhanced respiratory disease (VAERD) in pigs after heterologous challenge (Gauger et al., 2011; Rajao et al., 2014). This effect cannot be generalized as the enhancement was noticed only in the adjuvanted antigen in a swine model, but not seen in similar studies with non-adjuvanted vaccines in a ferret model (Krammer et al., 2014). In addition, it has been recently shown that head-reactive, non-neutralizing monoclonal antibodies increase the stalk flexibility and membrane fusion kinetics, resulting in enhanced respiratory disease in a mouse model (Winarski et al., 2019). These results indicate that head-reactive antibodies may also induce antibody-dependent enhancement. However, similar observations in humans are debatable. For instance, humans immunized with 2008–2009 inactivated TIV exhibited an increase in illness following infection with 2009 H1N1 pandemic virus (Janjua et al., 2010; Skowronski et al., 2010) due to the presence of cross-reactive antibodies (Monsalvo et al., 2011). Similar fatal cases were observed during 1957 H2N2 pandemic, although the relation of HA stalk antibodies to these observations is not known. It is noteworthy that NAI antibodies could reduce VAERD caused by mismatched heterologous HA, suggesting that vaccines which target HA protein alone may be prone to VAERD and cross-protective NAI antibodies may counteract VAERD (Rajao et al., 2016). Although there is no report on VAERD associated with the headless or chimeric HA vaccines, careful monitoring is advised as these approaches rely heavily on stalk antibodies. The inclusion of NA antigens into HA stalk-based vaccine merits further evaluation. Another issue raised by a recent study is the auto-reactivity of stalk antibodies to human proteins, which is significantly higher than the head antibodies, as confirmed by multiple *in vitro* assays (Bajic et al., 2019b). Although biological implications of this observation have not been studied *in vivo*, it was speculated that vaccine strategies focused exclusively on the stalk, or any single conserved epitope may fail to induce adequate antibody titers due to negative selection of the auto-reactive B cell clones (Bajic et al., 2019b). However, clinical observations showed that, despite under potential negative selection, preexisting stalk antibodies conferred protection against the 2009 pandemic A/H1N1 infection in humans (Ng et al., 2019).

Stalk Variability

Although conserved among the influenza viruses, the HA stalk is not antigenically identical, showing sequence variability even within the same HA subtype. In line with this, a study demonstrated that H1 HA stalk could undergo mutations *in vitro* by immune pressure, although the variability was less than the head region (Anderson et al., 2017). On the other hand, another study show that while the stalk domain does evolve over time, this evolution is slow and, historically, is not directed to aid in evading neutralizing antibody responses (Kirkpatrick et al., 2018). Several studies have isolated mutant influenza viruses showing resistance to stalk antibodies (Ekiert et al., 2011; Friesen et al., 2014; Chai et al., 2016). Notably, all isolated resistant viruses were seen to harbor three different mutations (Gln387Lys, Asp391Tyr, and Asp391Gly) in stalk region. While Gln387Lys mutation completely abolished the binding of the antibody to stalk region, the other two mutations rarely affected the antibody binding but enhanced the fusion ability of HA, representing two independent resistance mechanisms of the virus to escape stalk-reactive antibodies (Chai et al., 2016). These reports together show that the stalk may undergo natural or directed antigenic changes, asking important considerations in the context of developing stalk-based UIVs. Especially, the occurrence of mutations that enhance the membrane fusion of HA in presence of stalk antibodies presents a potential concern to vaccine safety issues. It should, however, be noted that the stalk antibody escape mutants tend to lose a viral fitness and are highly attenuated *in vivo* (Henry Dunand et al., 2015; Chai et al., 2016), alleviating the safety concerns.

Rational Design of Stalk

It is well-recognized that the stalk is less immunogenic than head and that the stalk antibodies are less potent in virus neutralization than the head antibodies. Therefore, multiple immunizations (three or four times) with stalk-based vaccines are required for inducing a sufficiently protective level of antibody response. More importantly, most humans have a diverse history of exposure to influenza antigens, by natural

infection, or vaccination. The established immune memory influences the subsequent immune response to a UIV, posing a great challenge to rendering qualitatively uniform and protective immune responses in humans. As discussed earlier, the HA stalk-based vaccines elicit broad-spectrum protection within the same HA group, but usually fail to provide protection to strains belonging to different HA group (Krammer et al., 2013; Margine et al., 2013). These results apparently dissent from the finding that some of the stalk monoclonal antibodies isolated from humans recognize all IAV subtypes, neutralizing both group 1 and group 2 viruses, presenting a promising prospect of developing pan-influenza A therapeutic solution (Corti et al., 2011). Isolation of rare antibodies with extremely broad neutralization potency from humans with prior vaccinations or infections (Corti et al., 2011) indicates that our immune system is able to find the cryptic and conserved epitopes and generate specific antibodies to the regions. However, inducing such antibodies to a protective level by vaccination remains a big challenge. Some recent reports encourage other options such as the activation of the conserved "cryptic" epitopes via antigen processing mechanisms (Lee et al., 2018b) or deliberately down-regulating surface antigens (Yang et al., 2013). Moreover, Fc-engineering technologies developed to enhance the therapeutic efficacy of antibodies may be harnessed to modulate FcR-Fc mediated effector functions (DiLillo et al., 2014) to achieve broad protection. A comprehensive understanding of immune response to broadly neutralizing epitopes and structure-based antigen design is required for the rational design of a pan-influenza A vaccine covering both group 1 and group 2 IAVs, as a consensus criterion of UIV (**Table 1**).

CONSERVED TARGETS IN HA OTHER THAN STALK

The inability to induce a complete protection by HA stalk-based approach led to a search for alternative targets for UIVs. Human monoclonal antibody CH65, mimicking the interaction with sialic acid, was shown to bind to the RBS in HA and neutralize multiple H1N1 influenza strains (Whittle et al., 2011). A caveat is that this antibody demonstrated stringent structural requirements for neutralization and mutation at the binding region led to generation of escape mutants. A panel of head-reactive monoclonal antibodies was also isolated and shown to recognize conserved region in the RBS and neutralize multiple influenza viruses (Krause et al., 2011, 2012; Ekiert et al., 2012; Benjamin et al., 2014).

The HA head domain was also reported to contain conserved epitopes outside the RBS. Different broadly neutralizing antibodies recognized the conserved regions located in HA head domain and neutralized multiple influenza viruses without detectable HI activity (Iba et al., 2014; Raymond et al., 2018). Generally, neutralization breadth of head antibodies was considerably variable to each other, ranging from pan-subtype (covering the same subtype) to pan-type (covering both group 1 and 2) coverage, indicating the presence of variably conserved regions. Additionally, a novel class of cross-reactive antibodies was discovered in humans vaccinated with seasonal TIV (Lee et al., 2016). These antibodies were shown to bind to a highly conserved region located on the HA RBS that was occluded in the HA trimer, conferring protective immunity against H1N1 and H3N2 strains *in vivo*, without neutralizing activity *in vitro*. Furthermore, a novel class of antibodies targeting vestigial esterase (VE) domain in HA has been characterized. The VE domain consists of two non-continuous sequences in HA head domain, which together forms a structurally distinct subdomain from the RBS and HA stalk domain (Zheng et al., 2018). The VE domain of the HEF protein of ICV is responsible for cleaving the host receptor to facilitate viral release, whereas in IAVs and IBVs the same receptor cleaving function is provided by a separate NA. The VE domains are found in both IAVs and IBVs, although their functions are not well-defined. The VE domains are highly conserved within a subtype of IAV but are variable among different subtypes (Ha et al., 2002). Monoclonal antibodies to the VE domain of H5N1 virus demonstrated broad neutralization against multiple clades of H5N1 subtype by preventing viral entry into cells (Oh et al., 2010). To date, different monoclonal antibodies have been isolated and they bind to different epitopes in the VE domain of H5N1 viruses, suggesting the presence of multiple neutralization epitopes in the VE domain (Paul et al., 2017). The VE-binding antibodies are reported to mediate ADCC for *in vivo* protection via FcR-Fc interaction (Wang et al., 2017). Besides H5N1, several monoclonal antibodies neutralizing H3 or H7 of IAVs or IBVs have also been recently characterized (Tan et al., 2016; Chai et al., 2017; Bangaru et al., 2018). Taken together, possibility remains to identify conserved neutralizing epitopes in the head domain in HA, in addition to extensively characterized HA stalk. Activation of these epitopes via antigen processing machineries (Lee et al., 2018b) may offer an option for enhancing the potency of cross-protection.

Despite the presence of conserved epitopes, the head domain is not a feasible vaccine antigen because of the immunodominance of the surrounding variable regions in the head that compete and prevent effective induction of antibodies toward the conserved regions. Therefore, several strategies were designed to enhance the breadth of protection by HA-based vaccines. The centralized HA was reconstructed such that whole HA contained consensus amino acids derived from diverse strains within a subtype (Weaver et al., 2011). This engineered HA antigen induced stronger immune response and provided better protection against heterologous influenza viruses, as compared to natural wild type HA antigen. Another approach, conceptually similar to the centralized HA, is based on a computationally optimized broadly reactive antigen (COBRA), in which the HA was designed to carry consensus sequences. The COBRA strategy was tested against H1N1, H3N2, and H5N1 influenza viruses, and demonstrated broad protection within a subtype (Giles and Ross, 2011; Crevar et al., 2015; Carter et al., 2016). A third strategy is the use of ancestral sequences as vaccine antigens to widen the window of cross-protection against diversified lineages or clades. Through phylogenetic tree analysis, putative ancestral HA and NA sequences have been determined and used as vaccine antigens, showing broadened cross-protection against multiple clades of H5N1 viruses in

animal models (Ducatez et al., 2011, 2013). Collectively, the UIV candidates using HA head or full-length antigen are based on reconstructed HA containing consensus sequences. Although the protection breadth of those strategies appears to be restricted to a subtype or a specific clade, similar concepts may be applied to other antigens such as HA stalk or NA to substantially improve the protection breadth.

NA AS A NOVEL TARGET FOR A UIV

Multiple Function of NA in Infection Cycle

NA is a tetrameric type II transmembrane glycoprotein and the second major surface protein of influenza viruses. The role of NA in influenza infection cycle is classically known as an expedited release of virus particle from infected cells by cleaving off the sialic acid residue present in host cell membrane, thus enabling multiple rounds of infection by the newly generated viral progeny. In addition to the canonical role that operates at later stage of infection, other functions of NA relevant to the infection cycle are being recognized. For instance, the sialidase activity of NA is critical for viral entry into a host cell at early stage of infection. At the entry site in the mucus, influenza virus meets mucosal defense proteins such as mucins that are highly glycosylated and capture viral HA. NA is able to release the captured viral particles via sialidase activity, allowing them to reach the host cells successfully (Cohen et al., 2013; Yang et al., 2014). Furthermore, with the same sialidase activity, NA facilitates HA-dependent membrane fusion and enhances the viral infectivity by removing the sialic acid residues from the virion-expressed HAs (Su et al., 2010). Additionally, NA cooperates with HA to enable crawling and gliding motions of influenza virus on cell surface to enhance viral entry into a cell (Sakai et al., 2017). More interestingly, some of the H3N2 viruses use their NA for receptor binding instead of HA, suggesting a novel role of NA other than receptor-destroying activity (Lin et al., 2010; Mogling et al., 2017). These observations show that NA performs multiple functions in the entire infection cycle, suggesting that NA antibodies may represent an important means of protection against influenza viruses.

NA Antibodies as Important Correlate of Protection

The 1968 H3N2 pandemic gave us important lessons pertaining to NA-mediated protection. The antigenic drift of NA is independent of HA; the pandemic involved a shift in HA, but NA remained close to the previous influenza A/H2N2 viruses (Schulman and Kilbourne, 1969). Notably, it has been shown that individuals with higher N2 antibody titers are less likely to be infected with the H3N2 pandemic, depicting the contribution of NA antibodies to broad protection (Schulman, 1969; Murphy et al., 1972; Monto and Kendal, 1973). However, NA has been largely ignored in the formulation of influenza vaccines due to the general beliefs that NA antibodies do not inhibit viral entry and that NA is less abundant than HA on a virion. Furthermore, the lack of a convenient assay to measure functional NA antibodies has rendered the NA forgotten antigens in influenza vaccines for decades (Eichelberger and Monto, 2019). Most of the current vaccine approaches focus on the induction of HA antibodies, both in trivalent/quadrivalent seasonal influenza vaccines and in the recent UIV candidates. However, it has been increasingly acknowledged that NA antibodies are important and independent correlates of protection and that NA immunity should be considered when evaluating vaccine potency. Clinical studies have shown a correlation between vaccination-induced or preexisting NAI antibody levels and decreased frequency of influenza infection and illness (Couch et al., 2013; Monto et al., 2015; Park et al., 2018). Further, a human challenge model depicted that NAI titers correlated more significantly with protection and disease severity than HAI titers (Memoli et al., 2016), or even HA stalk antibodies (Park et al., 2018). The observations in the human challenge models suggest that NA should be given full consideration as a vaccine antigen for better protection.

Several animal studies have identified NAI monoclonal antibodies that show protective effects against heterologous influenza infection. The breadth of NAI antibodies varied from subtype-specific to pan-influenza, depending on the conserved epitopes (Doyle et al., 2013a,b; Wan et al., 2013). Recently, it was reported that influenza infection in humans induces a variety of broadly reactive antibodies directed to the NA (Chen et al., 2018). In this study, it was shown that among the total influenza-specific antibodies induced by infection, the NA-reactive antibodies accounted for 23% and HA-reactive antibodies 35%. By contrast, the subunit or split vaccine resulted in antibody response directed predominantly to HA (87%), with only 1% for NA. The poor ability of the seasonal vaccine to induce NA antibodies was apparently due to insufficient content or structural integrity of NA antigen used in current vaccine formulation. This research suggests that correctly folded and immunologically relevant NA antigen is capable of inducing broadly protective antibody responses.

NA-Based Vaccine as Low-Hanging Fruit for a UIV?

Although the importance of NA-immunity in protection against homologous and heterologous influenza infections is clearly established, only a few literatures have demonstrated the cross-protection of NA-only vaccine constructs. One recent study in a mouse model has reported that computationally engineered recombinant NA proteins containing consensus sequences show broad protection within the H1N1 subtype (Job et al., 2018). Some other studies reported that VLPs expressing NA provided cross-protection against heterologous challenge in mice and ferrets (Easterbrook et al., 2012; Walz et al., 2018; Kim et al., 2019), and recombinant NA in a mouse model (Liu et al., 2015; Wohlbold et al., 2015). However, co-administration of H7 HA and N3 NA in modified vaccinia virus Ankara (MVA) vectors did not demonstrate enhanced protection efficacy as compared to the efficacy of MVA-HA or MVA-NA vaccine alone (Meseda et al., 2018). A predominant immune response in favor of HA over NA, when presented in an influenza virion, is already well-documented (Johansson et al., 1987), and the dissociation of HA and NA eliminates this antigenic competition (Johansson

and Kilbourne, 1993, 1996). These observations together suggest that NA-specific immunity may be dwarfed by competition with highly immunogenic HA in the final vaccine formulation. It could be argued that if the controlled ratio of HA and NA (dwarfing NA) is the strategy adopted by successful virus infection to minimize the host immune surveillance, then a deliberate perturbation of their ratio (increasing NA) in the vaccine formulation may be a promising strategy for effective protection. It was shown that the ratio of HA/NA could vary widely (up to 50 fold) without affecting viral fitness by a single mutation in the viral promoter (Lee and Seong, 1998). It remains to be seen if such a reverse-genetic approach could be harnessed to enhance the potency of NA-based vaccines.

Currently, we have very limited knowledge about anti-NA immunity. To develop a broadly protective vaccine based on NA, there are several critical questions that need to be answered. Firstly, although the NAI antibodies have been determined as an independent correlate of protection in humans (Couch et al., 2013; Monto et al., 2015), this needs to be further validated by the NA-only vaccine constructs in animal and human models. Secondly, very little is known about the breadth of NA immunity. The literature discussed earlier has consistently demonstrated a subtype-specific protection (e.g., within N1 or N2) of NA-based vaccines in animal models. Considering the repertoire of influenza viruses infecting humans and animals (including N1, N2, N3, N7, and N9 encompassing both NA group 1 and 2) (**Figure 1**), a successful NA-based vaccine should be designed to elicit broad protection covering both NA groups. Hence, the determination of conserved regions or epitopes hidden in NA is urgently needed. Thirdly, the mechanisms by which NA antibodies contribute to protection are not completely understood. Many NAI antibodies inhibit its enzymatic activity and thus prevent the release of newly formed viral particles. However, the extent to which NAI antibody titers may be considered protective has not been determined yet. Evaluation of cross-protection against mismatched or heterologous strains may be even more complicated. While ADCC was shown to be involved in protection by non-neutralizing NA antibodies (Jegaskanda et al., 2014, 2017a; Wohlbold et al., 2017), other protective mechanisms are yet to be further elucidated. Further isolation and characterization of broadly protective NA antibodies is required for better design of NA-based vaccines. Comprehensive reviews on NA-based immunity and the perspectives on current knowledge gaps to be addressed may be found in specialized reviews (Wohlbold and Krammer, 2014; Krammer et al., 2018a).

UIV AGAINST INFLUENZA B VIRUSES

Besides IAVs, ∼25% of all human influenza virus infections in each epidemic season is caused by IBVs that are classified into two distinct lineages, Victoria-like and Yamagata-like lineages (Ambrose and Levin, 2012). The current seasonal influenza vaccine is prepared in a trivalent or quadrivalent formulation, depending on the inclusion of one or two lineages of IBV antigens. Although priority is given to IAVs owing to a greater impact, IBVs may be more vulnerable targets against which to develop a UIV (**Table 1**) because of their low antigenic diversity and lack of animal reservoir (**Figure 1**) (Tan et al., 2018). Indirect evidence is being accumulated by the isolation of cross-protective antibodies against IBVs. Several broadly protective antibodies binding to head or stalk domain of influenza B HA have been isolated in humans. Overall, these monoclonal antibodies show lineage-specific neutralizing activity *in vitro*. Further, *in vivo* protection against both lineages was also demonstrated in mice by passive transfer, through non-neutralizing antibody-dependent effector functions (Shen et al., 2017; Hirano et al., 2018; Vigil et al., 2018; Asthagiri Arunkumar et al., 2019; Liu et al., 2019b). Notably, some of B HA stalk antibodies demonstrated extremely broad binding ability (Hirano et al., 2018) or protection against both IAVs and IBVs (Dreyfus et al., 2012). Influenza B NA-reactive broadly neutralizing antibodies were also isolated in animals and humans. Seasonal QIV induced NA antibodies with broad and potent antiviral activity against both lineages in humans (Piepenbrink et al., 2019). Additionally, murine NA antibodies also showed broad protection against both lineages of IBV (Wohlbold et al., 2017).

In line with these observations, chimeric HA strategy has also been tested for a UIV against IBVs. Chimeric HAs consisting of HA head domain from IAV and stalk domain from IBV, delivered as a DNA vaccine (prime), followed by two boosting immunizations with protein vaccines into mice, provided broad protection against both the lineages as well as an ancestral strain of IBV (Ermler et al., 2017). Mosaic HA is an advanced version, in which major antigenic sites of type B HA head domain were replaced by those of type A HA head so that antibodies directed to conserved regions both in the head and stalk domains of type B HA could be induced (Sun et al., 2019). The mosaic B HA provided broad protection against both lineages of IBV, through non-neutralizing ADCC-mediating antibody responses. There are only a few studies reporting B NA-based vaccine offering cross-lineage protection. A study showed that mice immunized with recombinant B NA protein of B/Yamagata/16/88 were protected from homologous Yamagata-like and Victoria-like lineages (Wohlbold et al., 2015). Another study demonstrated that a B NA-based vaccine inhibited the transmission of both homologous and heterologous IBVs in Guinea pig model (McMahon et al., 2019). As compared to IAVs, very little effort has been made so far to develop a UIV against IBVs. However, considering the limited diversity and variability (**Figure 1**) compared to IAVs, further identifications of broadly protective B cell and T cell epitopes would make it possible to develop a pan-influenza B vaccine in the near future (Tan et al., 2018).

T CELL IMMUNITY AS AN ESSENTIAL FACTOR FOR TRULY UNIVERSAL INFLUENZA PROTECTION

A vast majority of current efforts to develop a UIV are focused on inducing antibody response toward surface glycoproteins, M2e, HA, and NA. However, T cell immunity has been acknowledged

as a potential immune correlate of broad protection against influenza infections (Sridhar, 2016; Clemens et al., 2018). T cell immunity may not provide sterilizing or neutralizing immunity against influenza viruses but improves the standard of care by reducing the disease severity and duration of infection, facilitating recovery from illness (Sridhar, 2016). It, therefore, seems that multiple immune arms including both antibodies and T cell immunity are critical to provide a truly universal protection against highly variable influenza viruses. The influenza-specific T cell immunity is known to be highly cross-reactive by recognition of conserved peptides between different subtypes of IAV (Assarsson et al., 2008; Kreijtz et al., 2008; Lee et al., 2008; van de Sandt et al., 2014). Extensive studies have proven the protective role of vaccination or infection-induced cross-reactive CD8+ T cells in various animal models (Kreijtz et al., 2007, 2009; Bodewes et al., 2011; Hillaire et al., 2011). Additionally, in humans, CD8+ T cells offered cross-protection across seasonal, pandemic, avian IAVs, and both lineages of IBVs (Gras et al., 2010; Hayward et al., 2015; van de Sandt et al., 2015; Wang et al., 2018b). The majority of cross-reactive T cell epitopes of IAVs are derived from internal proteins; among >70 T cell epitopes identified between H5N1 and H3N2 viruses, only one derived from HA and other from internal proteins (Lee et al., 2008). This is not surprising given that the conservation rate of internal proteins is >90%, whereas that of surface HA and NA is only 40–70% among IAVs (Lee et al., 2008), which shows that inducing T cell immunity directed to internal proteins of influenza virus may provide a basis of developing T cell-based UIVs.

Despite poor sequence homology between the HAs of IAVs and IBVs, HA stalk harbors not only extremely broad B cell epitopes but also T cell epitopes encompassing both types of influenza viruses. The fusion peptide in HA stalk contains a cross-reactive CD4+ T cell epitope conserved in both IAVs and IBVs, although its protective role has not been examined *in vivo* (Babon et al., 2012). A number of CD4+ and CD8+ T cell epitopes are highly conserved in internal proteins (Terajima et al., 2013). A recent study has discovered a universal human CD8+ T cell epitope in PB1 (NMLSTVLGV $PB1_{413–412}$) that is identical across IAVs, IBVs, and ICVs (Koutsakos et al., 2019). The preexisting memory $PB1_{413–412}$+CD8+ T cells were detected in the blood and lung tissues of healthy donors and clonally expanded upon infection with IAV or IBV. This report not only showed the existence of heterotypic memory CD8+ T cells in humans that could be induced by exposure to influenza viruses, but also presents the prospect of designing a T cell-based UIV. However, these cross-reactive T cells were not induced in HHD-A2 mouse model despite multiple influenza infections or vaccinations and the protective role of the T cells could not be assessed in the study. Nonetheless, the existence of a number of cross-reactive T cell epitopes between IAVs and IBVs provides an avenue to address to a UIV.

Several T cell-based vaccine candidates are in different stages of clinical development, the major underlying strategy of which is to deliver multiple T cell epitopes derived from different viral antigens including internal as well as surface antigens (Sridhar, 2016). Delivery platforms include replicating or non-replicating viral vectors derived from vaccinia or adenovirus, recombinant VLPs, recombinant protein or peptide vaccines, and DNA vaccines (Sridhar, 2016; Clemens et al., 2018). Modified vaccinia Ankara (MVA) vector encoding NP and M1 was shown to induce robust T cell responses and provide cross-protection against multiple subtypes in animals and humans (Antrobus et al., 2012; Powell et al., 2013; Hessel et al., 2014; Folegatti et al., 2019). The baculovirus VLPs carrying influenza HA/NA and M1 offered cross-protection where T cells played a significant role in protection in mice (Hemann et al., 2013; Keshavarz et al., 2019). Synthetic peptides or fusion proteins harboring multiple conserved T cell epitopes have also been evaluated for immunogenicity and protective efficacy in animal models (Adar et al., 2009; Rosendahl Huber et al., 2015). While the vaccine approaches described above deliver exogenous antigens and induce CD4+ T cells as well as CD8+ T cells by cross-presentation, DNA vaccines are designed to predominantly activate cytotoxic CD8+ T cells to recognize endogenously expressed antigens presented on MHC class I molecules. In fact, the first report on DNA vaccines was targeted to influenza virus (Cohen, 1993; Ulmer et al., 1993), but initial success in a mouse model did not well-translate into higher animal models due to poor performance (Porter and Raviprakash, 2017). To date, much progress has been made to improve the efficacy of DNA vaccine against influenza virus, encompassing rational design of antigens and expression vectors and the development of novel adjuvants and delivery methods (Lee et al., 2018a). Candidate universal DNA vaccines encoding NP, matrix proteins, or PB1 were shown to decrease viral load and cross-protect against heterologous challenges in diverse animal models including mice, pigs, ferrets, and macaques (Ulmer et al., 1993; Tompkins et al., 2007; Price et al., 2009; Bragstad et al., 2013; Wang et al., 2015; Koday et al., 2017). Further studies are required for to refine DNA vaccine approaches as a stand-alone UIV. Recent studies have indicated that DNA vaccines may serve an attractive component of prime-boost strategy, considering it as a very effective means to priming immune system when preexisting immunity is low (Ledgerwood et al., 2013; DeZure et al., 2017). Despite the potential for broad protection, the safety issues need to be monitored closely, especially because of the documented rise in pathological consequences associated with CTL responses (Peiris et al., 2010; Duan and Thomas, 2016).

LIVE ATTENUATED INFLUENZA VACCINE AS AN ALTERNATIVE STRATEGY

Cross-Protective Immunogenicity of LAIV

LAIV has been approved for clinical use in humans since 2003 and is proven effective in preventing influenza infections (Mohn et al., 2018). The protection of LAIV is superior to IIVs due to multifaceted immune arms including antibody responses and cell-mediated responses that operate systemically and locally (Jang and Seong, 2013a,b; Sridhar et al., 2015). Further, displaying a whole set of viral antigens in a native conformation into the host immune system presents a definite advantage of LAIV to generate better protective immunity than the other strategies relying on a limited set of antigens. As discussed above, T cell

immunity directed to the conserved viral epitopes constitutes the cornerstone of cross-protection. A large number of researches have shown that T cell responses induced by LAIV are critical for broad protection against heterologous and heterosubtypic influenza infections in animal models (Cheng et al., 2013; Jang and Seong, 2013a; Rekstin et al., 2017). In young children, only LAIVs were shown to induce durable and potent T cell responses, while developing similar levels of antibody response as compared to IIVs (Belshe et al., 2000; Hoft et al., 2011; Mohn et al., 2015, 2017). Despite well-documented cross-protection, LAIV has received little attention to develop a UIV. This may be attributed to the general belief that LAIV is not effective at inducing broadly neutralizing antibodies against conserved domains in surface antigens (e.g., M2e or HA stalk). However, close attention is recently being given to LAIV as an alternative platform as a potent and cross-protective vehicle than previously thought, through inducing multifaceted immune correlates including T cell response and antibody-mediated effector functions (Jang and Seong, 2013a, 2014).

UIV Approaches Using LAIV

In the UIV approaches reported so far, LAIV was used either as a component in prime-boost regimens with other different vaccine formats such as IIV, DNA vaccine, or recombinant protein vaccine. Alternatively, LAIVs were also studies as a stand-alone vaccine given in single or multiple doses. A reassortant LAIV expressing a chimeric HA was constructed under the genetic background of Russian strain (A/Leningrad/134/17/57 cold-adapted virus) and used as a boosting vaccine in a ferret model (Nachbagauer et al., 2017). Notably, the LAIV-IIV regimen showed greater protective efficacy against the pandemic H1N1 challenge than the IIV-IIV regimen in terms of viral loads in the respiratory tissues, despite 32-fold lower stalk antibody titers in serum. Several factors were presumed to account for this disconnect between stalk antibody titers and protection efficacy, including anti-NA immunity, mucosal IgA antibodies, cell-mediated immunity, and non-specific innate immune responses offered by the LAIV. Similar results were obtained when using a different LAIV strain (A/Ann Arbor/6/60 cold-adapted strain) (Nachbagauer et al., 2018). In a subsequent study performed by the same group, chimeric HA-containing the LAIV-LAIV (A/Leningrad/134/17/57 cold-adapted strain) vaccine regimen was compared with the LAIV-IIV combination in terms of protection efficacy in a ferret model, in which the LAIV-LAIV vaccine regimen conferred superior protection against pandemic H1N1 and H6N1 challenges than the LAIV-IIV (Liu et al., 2019a). Another group tested a vaccination regimen comprising only LAIV (A/Leningrad/134/17/57 cold-adapted strain) as prime-boost vaccination in a mouse model (Isakova-Sivak et al., 2018). To enhance the breadth of protection, an internal gene of cold-adapted virus was replaced with the wild type. This study compared the immunogenicity and cross-protection between chimeric HA-containing LAIVs and natural HA-containing LAIVs. The chimeric HA-containing LAIVs induced higher HA stalk antibody titers and showed better cross-protection against heterologous infection with various group 1 IAVs. Thus, a cooperative role of cell-mediated immunity and HA stalk antibodies was suggested, although their individual contribution to protection were not assessed directly. It would be interesting to investigate if the cross-protection could be extended to group 2 influenza viruses such as H3 or H7 strains.

Besides the Leningrad and Ann Arbor strains, an independent cold-adapted vaccine strain (X-31ca) that was previously used for trivalent seasonal vaccine (Jang et al., 2014), H5N1 pre-pandemic vaccine (Jang et al., 2013c), and 2009 pdmH1N1 vaccine (Jang et al., 2012, 2013a), was recently tested as a UIV in a mouse model (Jang et al., 2018). Mice vaccinated with single or two doses of X-31 ca LAIVs were completely protected against lethal challenge of heterosubtypic strains encompassing both HA group 1 and 2 IAVs. Interestingly, boosting with heterosubtypic LAIVs carrying different HA and NA surface antigens showed more potent cross-protection than homologous boosting. T cell immunity and NK cell-mediated ADCC activity was demonstrated to contribute significantly to the observed cross-protection. As the first report of pan-influenza A protection covering both HA groups, these results merit further studies in a ferret model for clinical relevance. Hence, the LAIVs appear to be a powerful tool to develop a UIV that confers broad and potent cross-protection as a stand-alone vaccine or in combination with other strategies.

Future Prospects of LAIV-Based UIVs

While the LAIV presents a remarkable prospect for a broadly protective influenza vaccine, several important issues need to be addressed for it to serve as a reliable vaccine modality. The protection efficacy of a LAIV substantially differs with age. The estimated protection efficacy of a LAIV is 80% in young children and only 40% in adults, to the matched strains (Jefferson et al., 2008, 2010). As for T cell immune responses, clinical studies indicate that LAIVs induce better T cell response than IIVs in both children and adults (He et al., 2006; Subbramanian et al., 2010; Hoft et al., 2011). However, clinical studies have reported that LAIVs are not effective at inducing T cell responses in adults and the elderly, perhaps due to preexisting "vector" immunity, which limits the replication of LAIVs (He et al., 2006; Forrest et al., 2008; Hoft et al., 2011). Considering that both humoral and cell-mediated immunity contribute to broad protection, it will be important to elucidate how preexisting immunity or immunologic imprinting affects B cell and T cell immune response induced by LAIVs in humans (Gostic et al., 2016; Henry et al., 2018). For this purpose, animal models mimicking preexisting immunity and controlled human challenge studies will be needed. Considering that LAIVs mimic natural infection, a fundamental question remains to be answered: if infection (or vaccination with LAIV) is effective for conferring protection, why are humans vulnerable to repeated infections with homologous or heterologous strains? At the population level, currently used cold-adapted LAIVs provide a relatively low protection rates (∼40% in adults) even against matched strains (Jefferson et al., 2010). However, little is known whether individuals who successfully acquired protective immunity by a cold-adapted LAIV were protected against other heterologous strain(s) in the next epidemic. This may be directly addressed by a well-controlled longitudinal cohort study using human challenge models. Some studies showed that LAIVs were able to generate

cross-reactive T cell responses in children for up to 1 year after vaccination, as a basis of long-term cross-protection in humans (Mohn et al., 2015, 2017).

Most of the LAIV-based approaches are based on cold-adapted attenuated strains (Nachbagauer et al., 2017, 2018; Jang et al., 2018; Liu et al., 2019a). These strains acquire multiple attenuation mutations in the internal genes during the cold-adaptation process, which contribute to genetic stability to overall attenuation (Jang et al., 2016). A common concern for live vaccines is safety issues, especially those associated with potential reversion of attenuating mutation(s) into virulence during vaccination. LAIVs acquired multiple attenuation mutations among various internal genes and proven safe as seasonal influenza vaccines (especially for A/Ann Arbor strain as the master strain for FluMist). However, genetically engineered LAIVs with a limited set of attenuation mutations, e.g., NS1-deletion or elastase-dependent HA cleavage (Talon et al., 2000; Stech et al., 2005), may require additional monitoring on safety.

Defining the precise correlates of protection represents the most challenging step in the development of a UIV (Erbelding et al., 2018). Significant efforts were made to identify the protection mechanisms of HA stalk-based vaccines, suggesting that direct neutralization in combination with Fc-dependent indirect effector mechanisms mediated by stalk antibodies were the primary correlates of protection. In contrast to HA stalk-based vaccines, LAIVs elicit multiple immunological factors including serum IgG antibodies and mucosal IgA antibodies to surface antigens (HA, NA, and M2) and cell-mediated immunity to internal antigens, synergistically contributing to protection. However, their quantitative relationship to protection has not been determined, not even for homologous protection (Sridhar et al., 2015; Mohn et al., 2018), let alone for cross-protection against heterologous infection. The development of standardized assays to quantitatively measure T cell-mediated protection is particularly challenging, as the magnitude and the subset of T cells critical for protection is likely to differ according to strains of LAIV and challenge viruses. Further, mucosal IgA antibodies are believed to correlate with cross-protection, but it is still challenging to measure the neutralizing activity or effector functions of mucosal IgA antibodies. The complicated nature of LAIV-induced immunity, including non-neutralizing antibodies and diverse subsets of T cells, present a bottleneck to identifying precise correlates of protection.

Another important aspect of LAIV-based strategies lies on the LAIV strains. During the past century, H1N1 and H3N2 subtypes of influenza A viruses were the most prevalent strains in humans, causing annual epidemics as well as occasional pandemics, except for the temporal circulation of H2N2 during 1957–1968 (Kilbourne, 2006) (**Figure 6**). Accordingly, seasonal influenza vaccines are recommended to include H1N1 and H3N2 vaccine antigens for more than 40 years since 1977. Therefore, it is likely that most contemporary population has preexisting T cell immunity to H1N1 and H3N2 strains through natural infections or vaccinations. It should be remembered that currently licensed LAIVs (A/Ann Arbor/6/60 ca and A/Leningrad/134/17/57 ca) are of H2N2 subtypes. Probably, the nature of strain itself does not really matter for seasonal influenza vaccine, for which strain-specific immunity is focused on the surface HA antigen. However, for eliciting cross-protection, the role of conserved region become important (see section HA stalk-based UIV approaches, Conserved targets in HA other than stalk, NA as a novel target for a UIV for HA/NA and section T cell immunity as an essential factor for truly universal influenza protection for internal proteins). It is likely that human populations under 50 years of age (born after 1968 when H2N2 became extinct) has little preexisting immunity against H2N2, but predominantly against H1N1 and H3N2 viruses. It will therefore be worthwhile to examine whether cold-adapted LAIVs of H1N1 (Jang et al., 2018) or H3N2 origin (non-existent, to our knowledge) offer a beneficial effects on boosting the preexisting cross-reactive T cell immunity and antibody effector functions (section Mode of protection by a UIV; **Figure 3**).

As for IBVs, two distinct lineages diverged and circulated after 1985, which necessitates the incorporation of Victoria-like and Yamagata-like lineages in seasonal influenza vaccines. This leads us to raise a possibility of using an ancestral influenza B strain (such as B/Lee/40) before divergence into two different lineages as a UIV candidate for IBVs. Establishing new cold-adapted LAIV strains is a time-consuming and laborious. However, recent advances in reverse genetics and rational approaches to attenuate viral virulence have enabled the rapid conversion of a wild type virus into a novel LAIV strain. These approaches include NS1 truncation, elastase-dependent HA cleavage, caspase-dependent NP and NS1 cleavage, microRNA-mediated silencing, and codon deoptimization (Talon et al., 2000; Stech et al., 2005; Coleman et al., 2008; Perez et al., 2009; Jang et al., 2013b).

The redirection of host immune responses from surface proteins toward internal proteins may be achieved by rational vaccination strategies with LAIVs. For example, the down regulation of expression levels of HA or NA in a LAIV (Yang et al., 2013) is likely to result in preferable induction of T cell immunity to internal proteins. Alternatively, vaccination with LAIV carrying HA from non-human influenza viruses such as H5 or H9 may be effective at boosting preexisting T cell immunity to internal proteins in humans. Given the cross-reactivity of T cell immunity between IAVs and IBVs, A type LAIVs and B type LAIVs may be administered as a bivalent formulation or by sequential vaccination to induce improved protection.

OTHER STRATEGIES FOR UIVs

A number of alternative strategies are being investigated for their potential to serve as a UIV platform. First approach is to use infection-competent but replication-defective viruses. For instance, the M2 knock-out influenza virus (M2SR) rescued from M2-producing cells is able to infect cells but does not produce progeny virus due to the lack of functional M2 protein. The M2SR was shown to induce strong cell-mediated and humoral immunity and to provide broad protection against both homologous and heterologous influenza virus challenge in mice and ferrets (Sarawar et al., 2016; Hatta et al., 2017, 2018). A conceptually similar to M2SR, PB2 knock-out influenza virus could be produced in PB2-expressing cells as a vaccine.

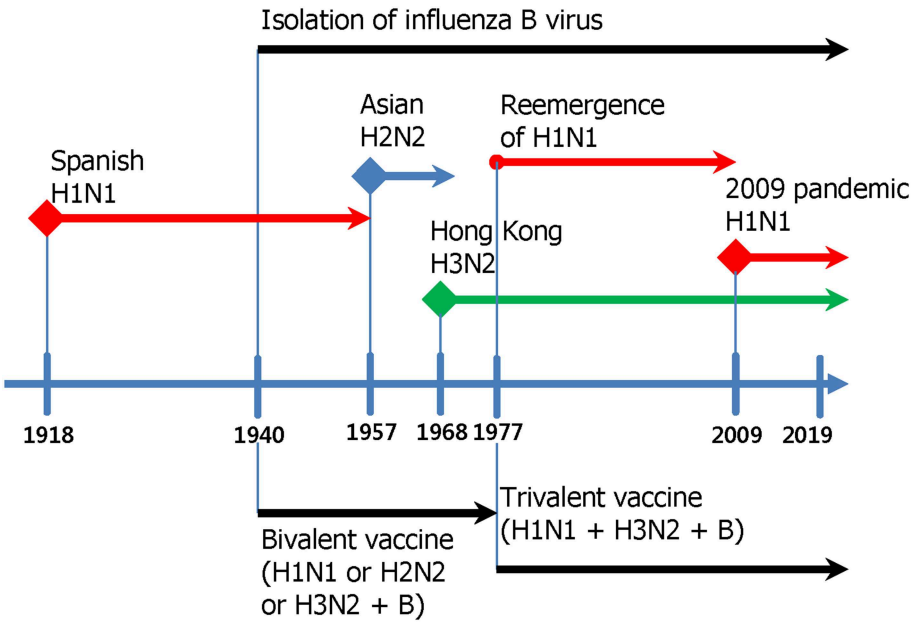


FIGURE 6 | Co-evolution of influenza viruses and influenza vaccines. Within the past century, there were four influenza pandemics; 1918 Spanish flu (H1N1), 1957 Asian flu (H2N2), 1968 Hong Kong flu (H3N2), and 2009 swine flu (H1N1) (Saunders-Hastings and Krewski, 2016). The 1918 Spanish flu (H1N1) evolved into seasonal influenza strain and had circulated for ∼40 years until the next pandemic by 1957 Asian flu (H2N2), which after ∼10 years of circulation was replaced by 1968 Hong Kong flu (H3N2). The 1968 Hong Kong flu (H3N2) has circulated until now as seasonal influenza strains. In 1977, H1N1 strain reemerged and was replaced by the 2009 swine flu (H1N1), which evolved into seasonal influenza strains circulating until now. Thus, H1N1 and H3N2 strains began to co-circulate from 1977. After the reemergence of H1N1 in 1977, World Health Organization has issued recommendations for trivalent vaccine composition containing A/H1N1, A/H3N2, and B strains (Hannoun, 2013).

The protection efficacy of PB2-KO vaccine was tested against diverse influenza strains in mice (Victor et al., 2012; Uraki et al., 2013; Ui et al., 2017). Pseudotyped influenza A virus also presents a similar replication-incompetent virus approach. The pseudotyped influenza virus lacking HA produced in HA-expressing cells can infect cells and express all the viral proteins except for HA. Studies have shown that the vaccination of mice and ferrets with the pseudotyped influenza virus generated a vigorous T cell response and reduced lung viral loads and weight loss after challenge with homologous and heterologous influenza viruses (Powell et al., 2012; Baz et al., 2015). These approaches based on replication-defective viruses should be rigorously evaluated for safety, considering potential reversion into virulence by acquiring the wild type gene by reassortment with circulating viruses (Lowen, 2017).

Secondly, mRNA vaccines have emerged as a promising alternative to conventional vaccine platforms against various infectious diseases including the influenza virus (Pardi et al., 2018a). Initially, the mRNA vaccines successfully induced protective B cell and T cell immune response in mice, ferrets, and pigs (Petsch et al., 2012). In subsequent studies, mRNA vaccines encoding HA induced neutralizing antibodies and T cell immunity essential for heterologous protection in mice and ferrets (Brazzoli et al., 2016). Notably, mRNA vaccines encoding HA elicited HA stalk antibodies in mice, rabbits, and ferrets, and the stalk antibody responses were associated with protection against homologous and heterologous influenza virus infection in mice (Pardi et al., 2018b), depicting promising potential as a UIV platform.

CONCLUSIONS AND PROSPECTS

During the past few years, several critically important issues have emerged in developing a UIV. While identification of a number of broadly protective antibodies presents an optimistic prospect for pan-influenza therapeutics, induction of such antibodies at a sufficiently protective level by vaccination has not been accomplished yet. A variety of viral targets have been identified to induce broad protection, including M2e and the conserved regions in the HA, such as stalk, RBS, and VE, and the long-neglected NA has emerged as an essential target for durable and broad protection. In virtually all cases, the rationale for broad protection is to redirect the immune responses from the variable immunodominant regions to conserved immunosubdominant regions. While effective in eliciting cross-protection, concerns were raised to non-neutralizing antibodies that potentially trigger the enhancement of disease. T-cell immunity has also been considered as an important correlate of cross-protection. LAIVs are able to induce multifaceted immune response and thus have increasingly received close attention as a promising vaccine platform, either alone, or in boosting format. Preexisting immunity or immunologic imprinting, established by prior exposure to influenza by infection or vaccination, may influence

or thwart the desired breadth of immune response by UIVs. Therefore, disparities may exist between pre-clinical evaluation of a UIV without due recognition of immune imprinting and human challenge studies. Lastly, the correlates of protection and their precise molecular mechanisms have not been determined yet, which remains a significant gap between development and licensing of a UIV. Given the limited antigenic diversity of IBVs among humans and the lack of animal reservoir, pan-influenza B virus vaccine may be attainable in the near future. On the other hand, IAVs continually change their antigenicity via antigenic drift and shift and zoonotic spillover from animal reservoirs, requiring multi-faceted immune arms to increase the breadth of protection. Judicious choice of antigens and their efficient designing to confer a broad protection for a prolonged period are required to counterfeit the immune evasion and provide a truly universal protection against the ever-changing influenza viruses.

AUTHOR CONTRIBUTIONS

BS designed and conceived the review. YJ and BS investigated the literature and wrote the manuscript.

FUNDING

This work was supported by grants from National Research Foundation of Korea (NRF-2018R1D1A1B07048881; NRF-2018M3A9H4079358) and also supported by grant from the Ministry of Food and Drug Safety of the Republic of Korea (19172MFDS255).

REFERENCES

Adar, Y., Singer, Y., Levi, R., Tzehoval, E., Perk, S., Banet-Noach, C., et al. (2009). A universal epitope-based influenza vaccine and its efficacy against H5N1. *Vaccine* 27, 2099–2107. doi: 10.1016/j.vaccine.2009.02.011

Ambrose, C. S., and Levin, M. J. (2012). The rationale for quadrivalent influenza vaccines. *Hum. Vaccin Immunother.* 8, 81–88. doi: 10.4161/hv.8.1.17623

Ana-Sosa-Batiz, F., Vanderven, H., Jegaskanda, S., Johnston, A., Rockman, S., Laurie, K., et al. (2016). Influenza-specific antibody-dependent phagocytosis. *PLoS ONE* 11:e0154461. doi: 10.1371/journal.pone.0154461

Anderson, C. S., Ortega, S., Chaves, F. A., Clark, A. M., Yang, H., Topham, D. J., et al. (2017). Natural and directed antigenic drift of the H1 influenza virus hemagglutinin stalk domain. *Sci. Rep.* 7:14614. doi: 10.1038/s41598-017-14931-7

Antrobus, R. D., Lillie, P. J., Berthoud, T. K., Spencer, A. J., McLaren, J. E., Ladell, K., et al. (2012). A T cell-inducing influenza vaccine for the elderly: safety and immunogenicity of MVA-NP+M1 in adults aged over 50 years. *PLoS ONE* 7:e48322. doi: 10.1371/journal.pone.0048322

Arevalo, M. T., Li, J., Diaz-Arevalo, D., Chen, Y., Navarro, A., Wu, L., et al. (2017). A dual purpose universal influenza vaccine candidate confers protective immunity against anthrax. *Immunology* 150, 276–289. doi: 10.1111/imm.12683

Assarsson, E., Bui, H. H., Sidney, J., Zhang, Q., Glenn, J., Oseroff, C., et al. (2008). Immunomic analysis of the repertoire of T-cell specificities for influenza A virus in humans. *J. Virol.* 82, 12241–12251. doi: 10.1128/JVI.01563-08

Asthagiri Arunkumar, G., Ioannou, A., Wohlbold, T. J., Meade, P., Aslam, S., Amanat, F., et al. (2019). Broadly cross-reactive, nonneutralizing antibodies against influenza B virus hemagglutinin demonstrate effector function-dependent protection against lethal viral challenge in mice. *J. Virol.* 93:e01696–18. doi: 10.1128/JVI.01696-18

Babon, J. A., Cruz, J., Ennis, F. A., Yin, L., and Terajima, M. (2012). A human CD4+ T cell epitope in the influenza hemagglutinin is cross-reactive to influenza A virus subtypes and to influenza B virus. *J. Virol.* 86, 9233–9243. doi: 10.1128/JVI.06325-11

Bajic, G., Maron, M. J., Adachi, Y., Onodera, T., McCarthy, K. R., McGee, C. E., et al. (2019a). Influenza antigen engineering focuses immune responses to a subdominant but broadly protective viral epitope. *Cell Host Microbe* 25, 827–835.e826. doi: 10.1016/j.chom.2019.04.003

Bajic, G., van der Poel, C. E., Kuraoka, M., Schmidt, A. G., Carroll, M. C., Kelsoe, G., et al. (2019b). Autoreactivity profiles of influenza hemagglutinin broadly neutralizing antibodies. *Sci. Rep.* 9:3492. doi: 10.1038/s41598-019-40175-8

Bangaru, S., Zhang, H., Gilchuk, I. M., Voss, T. G., Irving, R. P., Gilchuk, P., et al. (2018). A multifunctional human monoclonal neutralizing antibody that targets a unique conserved epitope on influenza HA. *Nat. Commun.* 9:2669. doi: 10.1038/s41467-018-04704-9

Baz, M., Boonnak, K., Paskel, M., Santos, C., Powell, T., Townsend, A., et al. (2015). Nonreplicating influenza A virus vaccines confer broad protection against lethal challenge. *MBio* 6:e01487–e01415. doi: 10.1128/mBio.01487-15

Belshe, R. B., Gruber, W. C., Mendelman, P. M., Cho, I., Reisinger, K., Block, S. L., et al. (2000). Efficacy of vaccination with live attenuated, cold-adapted, trivalent, intranasal influenza virus vaccine against a variant (A/Sydney) not contained in the vaccine. *J. Pediatr.* 136, 168–175. doi: 10.1016/S0022–3476(00)70097–7

Benjamin, E., Wang, W., McAuliffe, J. M., Palmer-Hill, F. J., Kallewaard, N. L., Chen, Z., et al. (2014). A broadly neutralizing human monoclonal antibody directed against a novel conserved epitope on the influenza virus H3 hemagglutinin globular head. *J. Virol.* 88, 6743–6750. doi: 10.1128/JVI.03562-13

Bernasconi, V., Bernocchi, B., Ye, L., Le, M. Q., Omokanye, A., Carpentier, R., et al. (2018). Porous nanoparticles with self-adjuvanting M2e-fusion protein and recombinant hemagglutinin provide strong and broadly protective immunity against influenza virus infections. *Front. Immunol.* 9:2060. doi: 10.3389/fimmu.2018.02060

Bessa, J., Schmitz, N., Hinton, H. J., Schwarz, K., Jegerlehner, A., and Bachmann, M. F. (2008). Efficient induction of mucosal and systemic immune responses by virus-like particles administered intranasally: implications for vaccine design. *Eur. J. Immunol.* 38, 114–126. doi: 10.1002/eji.200636959

Bodewes, R., Kreijtz, J. H., Geelhoed-Mieras, M. M., van Amerongen, G., Verburgh, R. J., van Trierum, S. E., et al. (2011). Vaccination against seasonal influenza A/H3N2 virus reduces the induction of heterosubtypic immunity against influenza A/H5N1 virus infection in ferrets. *J. Virol.* 85, 2695–2702. doi: 10.1128/JVI.02371-10

Bragstad, K., Vinner, L., Hansen, M. S., Nielsen, J., and Fomsgaard, A. (2013). A polyvalent influenza A DNA vaccine induces heterologous immunity and protects pigs against pandemic A(H1N1)pdm09 virus infection. *Vaccine* 31, 2281–2288. doi: 10.1016/j.vaccine.2013.02.061

Brandenburg, B., Koudstaal, W., Goudsmit, J., Klaren, V., Tang, C., Bujny, M. V., et al. (2013). Mechanisms of hemagglutinin targeted influenza virus neutralization. *PLoS ONE* 8:e80034. doi: 10.1371/journal.pone.0080034

Brazzoli, M., Magini, D., Bonci, A., Buccato, S., Giovani, C., Kratzer, R., et al. (2016). Induction of broad-based immunity and protective efficacy by self-amplifying mRNA vaccines encoding influenza virus hemagglutinin. *J. Virol.* 90, 332–344. doi: 10.1128/JVI.01786-15

Carter, D. M., Darby, C. A., Lefoley, B. C., Crevar, C. J., Alefantis, T., Oomen, R., et al. (2016). Design and characterization of a computationally optimized broadly reactive hemagglutinin vaccine for H1N1 Influenza Viruses. *J. Virol.* 90, 4720–4734. doi: 10.1128/JVI.03152-15

Chai, N., Swem, L. R., Park, S., Nakamura, G., Chiang, N., Estevez, A., et al. (2017). A broadly protective therapeutic antibody against influenza B virus with two mechanisms of action. *Nat. Commun.* 8:14234. doi: 10.1038/ncomms14234

Chai, N., Swem, L. R., Reichelt, M., Chen-Harris, H., Luis, E., Park, S., et al. (2016). Two escape mechanisms of influenza A virus to a broadly neutralizing stalk-binding antibody. *PLoS Pathog.* 12:e1005702. doi: 10.1371/journal.ppat.1005702

Chen, Y. Q., Lan, L. Y., Huang, M., Henry, C., and Wilson, P. C. (2019). Hemagglutinin stalk-reactive antibodies interfere with influenza virus neuraminidase activity by steric hindrance. *J. Virol.* 93:e01526–18. doi: 10.1128/JVI.01526-18

Chen, Y. Q., Wohlbold, T. J., Zheng, N. Y., Huang, M., Huang, Y., Neu, K. E., et al. (2018). Influenza infection in humans induces broadly cross-reactive and protective neuraminidase-reactive antibodies. *Cell* 173, 417–429.e410. doi: 10.1016/j.cell.2018.03.030

Cheng, X., Zengel, J. R., Suguitan, A. L. Jr., Xu, Q., Wang, W., Lin, J., et al. (2013). Evaluation of the humoral and cellular immune responses elicited by the live attenuated and inactivated influenza vaccines and their roles in heterologous protection in ferrets. *J. Infect. Dis.* 208, 594–602. doi: 10.1093/infdis/jit207

Clemens, E. B., van de Sandt, C., Wong, S. S., Wakim, L. M., and Valkenburg, S. A. (2018). Harnessing the power of T cells: the promising hope for a universal influenza vaccine. *Vaccines* 6:18. doi: 10.3390/vaccines6020018

Cohen, J. (1993). Naked DNA points way to vaccines. *Science* 259, 1691–1692. doi: 10.1126/science.8456293

Cohen, M., Zhang, X. Q., Senaati, H. P., Chen, H. W., Varki, N. M., Schooley, R. T., et al. (2013). Influenza A penetrates host mucus by cleaving sialic acids with neuraminidase. *Virol. J.* 10:321. doi: 10.1186/1743-422X-10-321

Coleman, J. R., Papamichail, D., Skiena, S., Futcher, B., Wimmer, E., and Mueller, S. (2008). Virus attenuation by genome-scale changes in codon pair bias. *Science* 320, 1784–1787. doi: 10.1126/science.1155761

Corbett, K. S., Moin, S. M., Yassine, H. M., Cagigi, A., Kanekiyo, M., Boyoglu-Barnum, S., et al. (2019). Design of nanoparticulate group 2 influenza virus hemagglutinin stem antigens that activate unmutated ancestor B cell receptors of broadly neutralizing antibody lineages. *MBio* 10:e02810–18. doi: 10.1128/mBio.02810-18

Corti, D., Voss, J., Gamblin, S. J., Codoni, G., Macagno, A., Jarrossay, D., et al. (2011). A neutralizing antibody selected from plasma cells that binds to group 1 and group 2 influenza A hemagglutinins. *Science* 333, 850–856. doi: 10.1126/science.1205669

Couch, R. B., Atmar, R. L., Franco, L. M., Quarles, J. M., Wells, J., Arden, N., et al. (2013). Antibody correlates and predictors of immunity to naturally occurring influenza in humans and the importance of antibody to the neuraminidase. *J. Infect. Dis.* 207, 974–981. doi: 10.1093/infdis/jis935

Crevar, C. J., Carter, D. M., Lee, K. Y., and Ross, T. M. (2015). Cocktail of H5N1 COBRA HA vaccines elicit protective antibodies against H5N1 viruses from multiple clades. *Hum. Vaccin Immunother.* 11, 572–583. doi: 10.1080/21645515.2015.1012013

Crowe, J. E. Jr. (2018). Is it possible to develop a "universal" influenza virus vaccine? potential for a universal influenza vaccine. *Cold Spring Harb. Perspect. Biol.* 10:a029496. doi: 10.1101/cshperspect.a029496

De Filette, M., Fiers, W., Martens, W., Birkett, A., Ramne, A., Lowenadler, B., et al. (2006). Improved design and intranasal delivery of an M2e-based human influenza A vaccine. *Vaccine* 24, 6597–6601. doi: 10.1016/j.vaccine.2006.05.082

Deng, L., Cho, K. J., Fiers, W., and Saelens, X. (2015a). M2e-based universal influenza a vaccines. *Vaccines* 3, 105–136. doi: 10.3390/vaccines3010105

Deng, L., Ibanez, L. I., Van den Bossche, V., Roose, K., Youssef, S. A., de Bruin, A., et al. (2015b). Protection against influenza A virus challenge with M2e-displaying filamentous Escherichia coli phages. *PLoS ONE* 10:e0126650. doi: 10.1371/journal.pone.0126650

Deng, L., Mohan, T., Chang, T. Z., Gonzalez, G. X., Wang, Y., Kwon, Y. M., et al. (2018). Double-layered protein nanoparticles induce broad protection against divergent influenza A viruses. *Nat. Commun.* 9:359. doi: 10.1038/s41467-017-02725-4

Denis, J., Acosta-Ramirez, E., Zhao, Y., Hamelin, M. E., Koukavica, I., Baz, M., et al. (2008). Development of a universal influenza A vaccine based on the M2e peptide fused to the papaya mosaic virus (PapMV) vaccine platform. *Vaccine* 26, 3395–3403. doi: 10.1016/j.vaccine.2008.04.052

DeZure, A. D., Coates, E. E., Hu, Z., Yamshchikov, G. V., Zephir, K. L., Enama, M. E., et al. (2017). An avian influenza H7 DNA priming vaccine is safe and immunogenic in a randomized phase I clinical trial. *NPJ Vaccines* 2:15. doi: 10.1038/s41541-017-0016-6

DiLillo, D. J., Palese, P., Wilson, P. C., and Ravetch, J. V. (2016). Broadly neutralizing anti-influenza antibodies require Fc receptor engagement for in vivo protection. *J. Clin. Invest.* 126, 605–610. doi: 10.1172/JCI84428

DiLillo, D. J., Tan, G. S., Palese, P., and Ravetch, J. V. (2014). Broadly neutralizing hemagglutinin stalk-specific antibodies require FcγR interactions for protection against influenza virus *in vivo*. *Nat. Med.* 20, 143–151. doi: 10.1038/nm.3443

Doyle, T. M., Hashem, A. M., Li, C., Van Domselaar, G., Larocque, L., Wang, J., et al. (2013a). Universal anti-neuraminidase antibody inhibiting all influenza A subtypes. *Antiviral Res.* 100, 567–574. doi: 10.1016/j.antiviral.2013.09.018

Doyle, T. M., Li, C., Bucher, D. J., Hashem, A. M., Van Domselaar, G., Wang, J., et al. (2013b). A monoclonal antibody targeting a highly conserved epitope in influenza B neuraminidase provides protection against drug resistant strains. *Biochem. Biophys. Res. Commun.* 441, 226–229. doi: 10.1016/j.bbrc.2013.10.041

Dreyfus, C., Laursen, N. S., Kwaks, T., Zuijdgeest, D., Khayat, R., Ekiert, D. C., et al. (2012). Highly conserved protective epitopes on influenza B viruses. *Science* 337, 1343–1348. doi: 10.1126/science.1222908

Duan, S., and Thomas, P. G. (2016). Balancing immune protection and immune pathology by CD8(+) T-cell responses to influenza infection. *Front. Immunol.* 7:25. doi: 10.3389/fimmu.2016.00025

Ducatez, M. F., Bahl, J., Griffin, Y., Stigger-Rosser, E., Franks, J., Barman, S., et al. (2011). Feasibility of reconstructed ancestral H5N1 influenza viruses for cross-clade protective vaccine development. *Proc. Natl. Acad. Sci. U.S.A.* 108, 349–354. doi: 10.1073/pnas.1012457108

Ducatez, M. F., Webb, A., Crumpton, J. C., and Webby, R. J. (2013). Long-term vaccine-induced heterologous protection against H5N1 influenza viruses in the ferret model. *Influenza Other Respir. Viruses* 7, 506–512. doi: 10.1111/j.1750-2659.2012.00423.x

Dykes, A. C., Cherry, J. D., and Nolan, C. E. (1980). A clinical, epidemiologic, serologic, and virologic study of influenza C virus infection. *Arch. Intern. Med.* 140, 1295–1298. doi: 10.1001/archinte.1980.00330210043021

Easterbrook, J. D., Schwartzman, L. M., Gao, J., Kash, J. C., Morens, D. M., Couzens, L., et al. (2012). Protection against a lethal H5N1 influenza challenge by intranasal immunization with virus-like particles containing 2009 pandemic H1N1 neuraminidase in mice. *Virology* 432, 39–44. doi: 10.1016/j.virol.2012.06.003

Eggink, D., Goff, P. H., and Palese, P. (2014). Guiding the immune response against influenza virus hemagglutinin toward the conserved stalk domain by hyperglycosylation of the globular head domain. *J. Virol.* 88, 699–704. doi: 10.1128/JVI.02608-13

Eichelberger, M. C., and Monto, A. S. (2019). Neuraminidase, the forgotten surface antigen, emerges as an influenza vaccine target for broadened protection. *J. Infect. Dis.* 219(Suppl 1), S75–80. doi: 10.1093/infdis/jiz017

Ekiert, D. C., Bhabha, G., Elsliger, M. A., Friesen, R. H., Jongeneelen, M., Throsby, M., et al. (2009). Antibody recognition of a highly conserved influenza virus epitope. *Science* 324, 246–251. doi: 10.1126/science.1171491

Ekiert, D. C., Friesen, R. H., Bhabha, G., Kwaks, T., Jongeneelen, M., Yu, W., et al. (2011). A highly conserved neutralizing epitope on group 2 influenza A viruses. *Science* 333, 843–850. doi: 10.1126/science.1204839

Ekiert, D. C., Kashyap, A. K., Steel, J., Rubrum, A., Bhabha, G., Khayat, R., et al. (2012). Cross-neutralization of influenza A viruses mediated by a single antibody loop. *Nature* 489, 526–532. doi: 10.1038/nature11414

Eliasson, D. G., El Bakkouri, K., Schon, K., Ramne, A., Festjens, E., Lowenadler, B., et al. (2008). CTA1-M2e-DD: a novel mucosal adjuvant targeted influenza vaccine. *Vaccine* 26, 1243–1252. doi: 10.1016/j.vaccine.2007.12.027

Eliasson, D. G., Omokanye, A., Schon, K., Wenzel, U. A., Bernasconi, V., Bemark, M., et al. (2018). M2e-tetramer-specific memory CD4 T cells are broadly protective against influenza infection. *Mucosal Immunol.* 11, 273–289. doi: 10.1038/mi.2017.14

Erbelding, E. J., Post, D. J., Stemmy, E. J., Roberts, P. C., Augustine, A. D., Ferguson, S., et al. (2018). A universal influenza vaccine: the strategic plan for the National Institute of Allergy and Infectious Diseases. *J. Infect. Dis* 218, 347–354. doi: 10.1093/infdis/jiy103

Ermler, M. E., Kirkpatrick, E., Sun, W., Hai, R., Amanat, F., Chromikova, V., et al. (2017). Chimeric hemagglutinin constructs induce broad protection against influenza B virus challenge in the mouse model. *J. Virol.* 91:e00286-17. doi: 10.1128/JVI.00286-17

Estrada, L. D., and Schultz-Cherry, S. (2019). Development of a universal influenza vaccine. *J. Immunol.* 202, 392–398. doi: 10.4049/jimmunol.1801054

Folegatti, P. M., Bellamy, D., Flaxman, A., Mair, C., Ellis, C., Ramon, R. L., et al. (2019). Safety and immunogenicity of the heterosubtypic influenza A vaccine

MVA-NP+M1 mnufactured on the AGE1.CR.pIX avian cell line. *Vaccines* 7:33. doi: 10.3390/vaccines7010033

Forrest, B. D., Pride, M. W., Dunning, A. J., Capeding, M. R., Chotpitayasunondh, T., Tam, J. S., et al. (2008). Correlation of cellular immune responses with protection against culture-confirmed influenza virus in young children. *Clin. Vaccine Immunol.* 15, 1042–1053. doi: 10.1128/CVI.00397-07

Friesen, R. H., Lee, P. S., Stoop, E. J., Hoffman, R. M., Ekiert, D. C., Bhabha, G., et al. (2014). A common solution to group 2 influenza virus neutralization. *Proc. Natl. Acad. Sci. U.S.A.* 111, 445–450. doi: 10.1073/pnas.1319058110

Gao, X., Wang, W., Li, Y., Zhang, S., Duan, Y., Xing, L., et al. (2013). Enhanced influenza VLP vaccines comprising matrix-2 ectodomain and nucleoprotein epitopes protects mice from lethal challenge. *Antiviral Res.* 98, 4–11. doi: 10.1016/j.antiviral.2013.01.010

Gauger, P. C., Vincent, A. L., Loving, C. L., Lager, K. M., Janke, B. H., Kehrli, M. E. Jr., et al. (2011). Enhanced pneumonia and disease in pigs vaccinated with an inactivated human-like (delta-cluster) H1N2 vaccine and challenged with pandemic 2009 H1N1 influenza virus. *Vaccine* 29, 2712–2719. doi: 10.1016/j.vaccine.2011.01.082

Gianfrani, C., Oseroff, C., Sidney, J., Chesnut, R. W., and Sette, A. (2000). Human memory CTL response specific for influenza A virus is broad and multispecific. *Hum. Immunol.* 61, 438–452. doi: 10.1016/S0198-8859(00)00105-1

Giles, B. M., and Ross, T. M. (2011). A computationally optimized broadly reactive antigen (COBRA) based H5N1 VLP vaccine elicits broadly reactive antibodies in mice and ferrets. *Vaccine* 29, 3043–3054. doi: 10.1016/j.vaccine.2011.01.100

Gostic, K. M., Ambrose, M., Worobey, M., and Lloyd-Smith, J. O. (2016). Potent protection against H5N1 and H7N9 influenza via childhood hemagglutinin imprinting. *Science* 354, 722–726. doi: 10.1126/science.aag1322

Gras, S., Kedzierski, L., Valkenburg, S. A., Laurie, K., Liu, Y. C., Denholm, J. T., et al. (2010). Cross-reactive CD8 T-cell immunity between the pandemic H1N1–2009 and H1N1–1918 influenza A viruses. *Proc. Natl. Acad. Sci. U.S.A.* 107, 12599–12604. doi: 10.1073/pnas.1007270107

Graves, P. N., Schulman, J. L., Young, J. F., and Palese, P. (1983). Preparation of influenza virus subviral particles lacking the HA1 subunit of hemagglutinin: Unmasking of cross-reactive HA2 determinants. *Virology* 126, 106–116. doi: 10.1016/0042-6822(83)90465-8

Guilliams, M., Bruhns, P., Saeys, Y., Hammad, H., and Lambrecht, B. N. (2014). The function of Fcgamma receptors in dendritic cells and macrophages. *Nat. Rev. Immunol.* 14, 94–108. doi: 10.1038/nri3582

Guo, Y., He, L., Song, N., Li, P., Sun, S., Zhao, G., et al. (2017). Highly conserved M2e and hemagglutinin epitope-based recombinant proteins induce protection against influenza virus infection. *Microbes Infect.* 19, 641–647. doi: 10.1016/j.micinf.2017.08.010

Ha, Y., Stevens, D. J., Skehel, J. J., and Wiley, D. C. (2002). H5 avian and H9 swine influenza virus haemagglutinin structures: possible origin of influenza subtypes. *EMBO J.* 21, 865–875. doi: 10.1093/emboj/21.5.865

Hai, R., Krammer, F., Tan, G. S., Pica, N., Eggink, D., Maamary, J., et al. (2012). Influenza viruses expressing chimeric hemagglutinins: globular head and stalk domains derived from different subtypes. *J. Virol.* 86, 5774–5781. doi: 10.1128/JVI.00137-12

Hannoun, C. (2013). The evolving history of influenza viruses and influenza vaccines. *Exp. Rev. Vaccines* 12, 1085–1094. doi: 10.1586/14760584.2013.824709

Hashemi, H., Pouyanfard, S., Bandehpour, M., Noroozbabaei, Z., Kazemi, B., Saelens, X., et al. (2012). Immunization with M2e-displaying T7 bacteriophage nanoparticles protects against influenza A virus challenge. *PLoS ONE* 7:e45765. doi: 10.1371/journal.pone.0045765

Hashimoto, Y., Moki, T., Takizawa, T., Shiratsuchi, A., and Nakanishi, Y. (2007). Evidence for phagocytosis of influenza virus-infected, apoptotic cells by neutrophils and macrophages in mice. *J. Immunol.* 178, 2448–2457. doi: 10.4049/jimmunol.178.4.2448

Hatta, Y., Boltz, D., Sarawar, S., Kawaoka, Y., Neumann, G., and Bilsel, P. (2017). M2SR, a novel live influenza vaccine, protects mice and ferrets against highly pathogenic avian influenza. *Vaccine* 35, 4177–4183. doi: 10.1016/j.vaccine.2017.06.039

Hatta, Y., Boltz, D., Sarawar, S., Kawaoka, Y., Neumann, G., and Bilsel, P. (2018). Novel influenza vaccine M2SR protects against drifted H1N1 and H3N2 influenza virus challenge in ferrets with pre-existing immunity. *Vaccine* 36, 5097–5103. doi: 10.1016/j.vaccine.2018.06.053

Hayward, A. C., Wang, L., Goonetilleke, N., Fragaszy, E. B., Bermingham, A., Copas, A., et al. (2015). Natural T cell–mediated protection against seasonal and pandemic influenza. Results of the Flu watch cohort study. *Am. J. Respir. Crit. Care Med.* 191, 1422–1431. doi: 10.1164/rccm.201411-1988OC

He, X. S., Holmes, T. H., Zhang, C., Mahmood, K., Kemble, G. W., Lewis, D. B., et al. (2006). Cellular immune responses in children and adults receiving inactivated or live attenuated influenza vaccines. *J. Virol.* 80, 11756–11766. doi: 10.1128/JVI.01460-06

Hemann, E. A., Kang, S. M., and Legge, K. L. (2013). Protective CD8 T cell-mediated immunity against influenza A virus infection following influenza virus-like particle vaccination. *J. Immunol.* 191, 2486–2494. doi: 10.4049/jimmunol.1300954

Henry Dunand, C. J., Leon, P. E., Kaur, K., Tan, G. S., Zheng, N. Y., Andrews, S., et al. (2015). Preexisting human antibodies neutralize recently emerged H7N9 influenza strains. *J. Clin. Invest.* 125, 1255–1268. doi: 10.1172/JCI74374

Henry, C., Palm, A. E., Krammer, F., and Wilson, P. C. (2018). From original antigenic sin to the universal influenza virus vaccine. *Trends Immunol.* 39, 70–79. doi: 10.1016/j.it.2017.08.003

Hessel, A., Savidis-Dacho, H., Coulibaly, S., Portsmouth, D., Kreil, T. R., Crowe, B. A., et al. (2014). MVA vectors expressing conserved influenza proteins protect mice against lethal challenge with H5N1, H9N2 and H7N1 viruses. *PLoS ONE* 9:e88340. doi: 10.1371/journal.pone.0088340

Hillaire, M. L., van Trierum, S. E., Kreijtz, J. H., Bodewes, R., Geelhoed-Mieras, M. M., Nieuwkoop, N. J., et al. (2011). Cross-protective immunity against influenza pH1N1 2009 viruses induced by seasonal influenza A (H3N2) virus is mediated by virus-specific T-cells. *J. Gen Virol.* 92(Pt 10), 2339–2349. doi: 10.1099/vir.0.033076-0

Hirano, D., Ohshima, N., Kubota-Koketsu, R., Yamasaki, A., Kurosawa, G., Okuno, Y., et al. (2018). Three types of broadly reacting antibodies against influenza B viruses induced by vaccination with seasonal influenza viruses. *J. Immunol. Res.* 2018:7251793. doi: 10.1155/2018/7251793

Hoft, D. F., Babusis, E., Worku, S., Spencer, C. T., Lottenbach, K., Truscott, S. M., et al. (2011). Live and inactivated influenza vaccines induce similar humoral responses, but only live vaccines induce diverse T-cell responses in young children. *J. Infect. Dis.* 204, 845–853. doi: 10.1093/infdis/jir436

Huber, V. C., Lynch, J. M., Bucher, D. J., Le, J., and Metzger, D. W. (2001). Fc receptor-mediated phagocytosis makes a significant contribution to clearance of influenza virus infections. *J. Immunol.* 166, 7381–7388. doi: 10.4049/jimmunol.166.12.7381

Iba, Y., Fujii, Y., Ohshima, N., Sumida, T., Kubota-Koketsu, R., Ikeda, M., et al. (2014). Conserved neutralizing epitope at globular head of hemagglutinin in H3N2 influenza viruses. *J. Virol.* 88, 7130–7144. doi: 10.1128/JVI.00420-14

Ibanez, L. I., Roose, K., De Filette, M., Schotsaert, M., De Sloovere, J., Roels, S., et al. (2013). M2e-displaying virus-like particles with associated RNA promote T helper 1 type adaptive immunity against influenza A. *PLoS ONE* 8:e59081. doi: 10.1371/journal.pone.0059081

Impagliazzo, A., Milder, F., Kuipers, H., Wagner, M. V., Zhu, X., Hoffman, R. M., et al. (2015). A stable trimeric influenza hemagglutinin stem as a broadly protective immunogen. *Science* 349, 1301–1306. doi: 10.1126/science.aac7263

Isakova-Sivak, I., Korenkov, D., Smolonogina, T., Kotomina, T., Donina, S., Matyushenko, V., et al. (2018). Broadly protective anti-hemagglutinin stalk antibodies induced by live attenuated influenza vaccine expressing chimeric hemagglutinin. *Virology* 518, 313–323. doi: 10.1016/j.virol.2018.03.013

Jameson, J., Cruz, J., and Ennis, F. A. (1998). Human cytotoxic T-lymphocyte repertoire to influenza A viruses. *J. Virol.* 72, 8682–8689.

Jang, Y. H., Byun, Y. H., Lee, D. H., Lee, K. H., Lee, Y. J., Lee, Y. H., et al. (2013a). Cold-adapted X-31 live attenuated 2009 pandemic H1N1 influenza vaccine elicits protective immune responses in mice and ferrets. *Vaccine* 31, 1320–1327. doi: 10.1016/j.vaccine.2012.12.072

Jang, Y. H., Byun, Y. H., Lee, K. H., Park, E. S., Lee, Y. H., Lee, Y. J., et al. (2013b). Host defense mechanism-based rational design of live vaccine. *PLoS ONE* 8:e75043. doi: 10.1371/journal.pone.0075043

Jang, Y. H., Byun, Y. H., Lee, Y. J., Lee, Y. H., Lee, K. H., and Seong, B. L. (2012). Cold-adapted pandemic 2009 H1N1 influenza virus live vaccine elicits cross-reactive immune responses against seasonal and H5 influenza A viruses. *J. Virol.* 86, 5953–5958. doi: 10.1128/JVI.07149-11

Jang, Y. H., Jung, E. J., Byun, Y. H., Lee, K. H., Lee, E. Y., Lee, Y. J., et al. (2013c). Immunogenicity and protective efficacy of cold-adapted X-31 live

attenuated pre-pandemic H5N1 influenza vaccines. *Vaccine* 31, 3339–3346. doi: 10.1016/j.vaccine.2013.05.080

Jang, Y. H., Jung, E. J., Lee, K. H., Byun, Y. H., Yang, S. W., and Seong, B. L. (2016). Genetic analysis of attenuation markers of cold-adapted X-31 influenza live vaccine donor strain. *Vaccine* 34, 1343–1349. doi: 10.1016/j.vaccine.2016.01.053

Jang, Y. H., Kim, J. Y., Byun, Y. H., Son, A., Lee, J. Y., Lee, Y. J., et al. (2018). Pan-influenza A protection by prime-boost vaccination with cold-adapted live-attenuated influenza vaccine in a mouse model. *Front. Immunol.* 9:116. doi: 10.3389/fimmu.2018.00116

Jang, Y. H., Lee, E. Y., Byun, Y. H., Jung, E. J., Lee, Y. J., Lee, Y. H., et al. (2014). Protective efficacy in mice of monovalent and trivalent live attenuated influenza vaccines in the background of cold-adapted A/X-31 and B/Lee/40 donor strains. *Vaccine* 32, 535–543. doi: 10.1016/j.vaccine.2013.12.002

Jang, Y. H., and Seong, B. L. (2013a). Cross-protective immune responses elicited by live attenuated influenza vaccines. *Yonsei Med. J.* 54, 271–282. doi: 10.3349/ymj.2013.54.2.271

Jang, Y. H., and Seong, B. L. (2013b). Toward a universal influenza vaccine: from the perspective of protective efficacy. *Clin. Exp. Vaccine Res.* 2, 71–73. doi: 10.7774/cevr.2013.2.2.71

Jang, Y. H., and Seong, B. L. (2014). Options and obstacles for designing a universal influenza vaccine. *Viruses* 6, 3159–3180. doi: 10.3390/v6083159

Janjua, N. Z., Skowronski, D. M., Hottes, T. S., Osei, W., Adams, E., Petric, M., et al. (2010). Seasonal influenza vaccine and increased risk of pandemic A/H1N1-related illness: first detection of the association in British Columbia, Canada. *Clin. Infect. Dis.* 51, 1017–1027. doi: 10.1086/656586

Jefferson, T., Di Pietrantonj, C., Rivetti, A., Bawazeer, G. A., Al-Ansary, L. A., and Ferroni, E. (2010). Vaccines for preventing influenza in healthy adults. *Cochrane Database Syst. Rev.* 7:Cd001269. doi: 10.1002/14651858.CD001269.pub4

Jefferson, T., Rivetti, A., Harnden, A., Di Pietrantonj, C., and Demicheli, V. (2008). Vaccines for preventing influenza in healthy children. *Cochrane Database Syst. Rev.* 16:Cd004879. doi: 10.1002/14651858.CD004879.pub3

Jegaskanda, S., Co, M. D. T., Cruz, J., Subbarao, K., Ennis, F. A., and Terajima, M. (2017a). Induction of H7N9-cross-reactive antibody-dependent cellular cytotoxicity antibodies by human seasonal influenza A viruses that are directed toward the nucleoprotein. *J. Infect. Dis.* 215, 818–823. doi: 10.1093/infdis/jiw629

Jegaskanda, S., Vandenberg, K., Laurie, K. L., Loh, L., Kramski, M., Winnall, W. R., et al. (2014). Cross-reactive influenza-specific antibody-dependent cellular cytotoxicity in intravenous immunoglobulin as a potential therapeutic against emerging influenza viruses. *J. Infect. Dis.* 210, 1811–1822. doi: 10.1093/infdis/jiu334

Jegaskanda, S., Vanderven, H. A., Wheatley, A. K., and Kent, S. J. (2017b). Fc or not Fc; that is the question: antibody Fc-receptor interactions are key to universal influenza vaccine design. *Hum. Vaccin Immunother.* 13, 1–9. doi: 10.1080/21645515.2017.1290018

Jegerlehner, A., Schmitz, N., Storni, T., and Bachmann, M. F. (2004). Influenza A vaccine based on the extracellular domain of M2: weak protection mediated via antibody-dependent NK cell activity. *J. Immunol.* 172, 5598–5605. doi: 10.4049/jimmunol.172.9.5598

Job, E. R., Ysenbaert, T., Smet, A., Christopoulou, I., Strugnell, T., Oloo, E. O., et al. (2018). Broadened immunity against influenza by vaccination with computationally designed influenza virus N1 neuraminidase constructs. *NPJ Vaccines* 3:55. doi: 10.1038/s41541-018-0093-1

Johansson, B. E., and Kilbourne, E. D. (1993). Dissociation of influenza virus hemagglutinin and neuraminidase eliminates their intravirionic antigenic competition. *J. Virol.* 67, 5721–5723.

Johansson, B. E., and Kilbourne, E. D. (1996). Immunization with dissociated neuraminidase, matrix, and nucleoproteins from influenza A virus eliminates cognate help and antigenic competition. *Virology* 225, 136–144. doi: 10.1006/viro.1996.0581

Johansson, B. E., Moran, T. M., and Kilbourne, E. D. (1987). Antigen-presenting B cells and helper T cells cooperatively mediate intravirionic antigenic competition between influenza A virus surface glycoproteins. *Proc. Natl. Acad. Sci. U.S.A.* 84, 6869–6873. doi: 10.1073/pnas.84.19.6869

Keshavarz, M., Namdari, H., Arjeini, Y., Mirzaei, H., Salimi, V., Sadeghi, A., et al. (2019). Induction of protective immune response to intranasal administration of influenza virus-like particles in a mouse model. *J. Cell Physiol.* 226, 1110–1117. doi: 10.1002/jcp.28339

Khurana, S. (2018). Development and regulation of novel influenza virus vaccines: a United States young scientist perspective. *Vaccines* 6:24. doi: 10.3390/vaccines6020024

Khurana, S., Loving, C. L., Manischewitz, J., King, L. R., Gauger, P. C., Henningson, J., et al. (2013). Vaccine-induced anti-HA2 antibodies promote virus fusion and enhance influenza virus respiratory disease. *Sci. Transl. Med.* 5:200ra114. doi: 10.1126/scitranslmed.3006366

Kilbourne, E. D. (2006). Influenza pandemics of the 20th century. *Emerg Infect. Dis.* 12, 9–14. doi: 10.3201/eid1201.051254

Kim, H., Webster, R. G., and Webby, R. J. (2018a). Influenza virus: dealing with a drifting and shifting pathogen. *Viral. Immunol.* 31, 174–183. doi: 10.1089/vim.2017.0141

Kim, K. H., Lee, Y. T., Park, S., Jung, Y. J., Lee, Y., Ko, E. J., et al. (2019). Neuraminidase expressing virus-like particle vaccine provides effective cross protection against influenza virus. *Virology* 535, 179–188. doi: 10.1016/j.virol.2019.07.008

Kim, M. C., Lee, J. W., Choi, H. J., Lee, Y. N., Hwang, H. S., Lee, J., et al. (2015). Microneedle patch delivery to the skin of virus-like particles containing heterologous M2e extracellular domains of influenza virus induces broad heterosubtypic cross-protection. *J. Control Release* 210, 208–216. doi: 10.1016/j.jconrel.2015.05.278

Kim, Y. J., Kim, K. H., Ko, E. J., Kim, M. C., Lee, Y. N., Jung, Y. J., et al. (2018b). Complement C3 plays a key role in inducing humoral and cellular immune responses to influenza virus strain-specific hemagglutinin-based or cross-protective M2 extracellular domain-based vaccination. *J. Virol.* 92:e00969–18. doi: 10.1128/JVI.00969-18

Kim, Y. J., Lee, Y. T., Kim, M. C., Lee, Y. N., Kim, K. H., Ko, E. J., et al. (2017). Cross-protective efficacy of influenza virus M2e containing virus-like particles is superior to hemagglutinin vaccines and variable depending on the genetic backgrounds of mice. *Front. Immunol.* 8:1730. doi: 10.3389/fimmu.2017.01730

Kirkpatrick, E., Qiu, X., Wilson, P. C., Bahl, J., and Krammer, F. (2018). The influenza virus hemagglutinin head evolves faster than the stalk domain. *Sci. Rep.* 8:10432. doi: 10.1038/s41598-018-28706-1

Koday, M. T., Leonard, J. A., Munson, P., Forero, A., Koday, M., Bratt, D. L., et al. (2017). Multigenic DNA vaccine induces protective cross-reactive T cell responses against heterologous influenza virus in nonhuman primates. *PLoS ONE* 12:e0189780. doi: 10.1371/journal.pone.0189780

Kosik, I., Angeletti, D., Gibbs, J. S., Angel, M., Takeda, K., Kosikova, M., et al. (2019). Neuraminidase inhibition contributes to influenza A virus neutralization by anti-hemagglutinin stem antibodies. *J. Exp. Med.* 216, 304–316. doi: 10.1084/jem.20181624

Koutsakos, M., Illing, P. T., Nguyen, T. H. O., Mifsud, N. A., Crawford, J. C., Rizzetto, S., et al. (2019). Human CD8(+) T cell cross-reactivity across influenza A, B and C viruses. *Nat. Immunol.* 20, 613–625. doi: 10.1038/s41590-019-0320-6

Krammer, F. (2019). The human antibody response to influenza A virus infection and vaccination. *Nat. Rev. Immunol.* 19, 383–397. doi: 10.1038/s41577-019-0143-6

Krammer, F., Fouchier, R. A. M., Eichelberger, M. C., Webby, R. J., Shaw-Saliba, K., Wan, H., et al. (2018a). NAction! How can neuraminidase-based immunity contribute to better influenza virus vaccines? *MBio* 9:e02332–e02317. doi: 10.1128/mBio.02332-17

Krammer, F., Garcia-Sastre, A., and Palese, P. (2018b). Is it possible to develop a "universal" influenza virus vaccine? Potential target antigens and critical aspects for a universal influenza vaccine. *Cold Spring Harb Perspect. Biol.* 10:a028845. doi: 10.1101/cshperspect.a028845

Krammer, F., Hai, R., Yondola, M., Tan, G. S., Leyva-Grado, V. H., Ryder, A. B., et al. (2014). Assessment of influenza virus hemagglutinin stalk-based immunity in ferrets. *J. Virol.* 88, 3432–3442. doi: 10.1128/JVI.03004-13

Krammer, F., and Palese, P. (2013). Influenza virus hemagglutinin stalk-based antibodies and vaccines. *Curr. Opin. Virol.* 3, 521–530. doi: 10.1016/j.coviro.2013.07.007

Krammer, F., and Palese, P. (2015). Advances in the development of influenza virus vaccines. *Nat. Rev. Drug Discov.* 14, 167–182. doi: 10.1038/nrd4529

Krammer, F., Pica, N., Hai, R., Margine, I., and Palese, P. (2013). Chimeric hemagglutinin influenza virus vaccine constructs elicit broadly protective stalk-specific antibodies. *J. Virol.* 87, 6542–6550. doi: 10.1128/JVI.00641-13

Krause, J. C., Tsibane, T., Tumpey, T. M., Huffman, C. J., Albrecht, R., Blum, D. L., et al. (2012). Human monoclonal antibodies to pandemic 1957 H2N2 and pandemic 1968 H3N2 influenza viruses. *J. Virol.* 86, 6334–6340. doi: 10.1128/JVI.07158-11

Krause, J. C., Tsibane, T., Tumpey, T. M., Huffman, C. J., Basler, C. F., and Crowe, J. E. Jr. (2011). A broadly neutralizing human monoclonal antibody that recognizes a conserved, novel epitope on the globular head of the influenza H1N1 virus hemagglutinin. *J. Virol.* 85, 10905–10908. doi: 10.1128/JVI.00700-11

Kreijtz, J. H., Bodewes, R., van Amerongen, G., Kuiken, T., Fouchier, R. A., Osterhaus, A. D., et al. (2007). Primary influenza A virus infection induces cross-protective immunity against a lethal infection with a heterosubtypic virus strain in mice. *Vaccine* 25, 612–620. doi: 10.1016/j.vaccine.2006.08.036

Kreijtz, J. H., Bodewes, R., van den Brand, J. M., de Mutsert, G., Baas, C., van Amerongen, G., et al. (2009). Infection of mice with a human influenza A/H3N2 virus induces protective immunity against lethal infection with influenza A/H5N1 virus. *Vaccine* 27, 4983–4989. doi: 10.1016/j.vaccine.2009.05.079

Kreijtz, J. H., de Mutsert, G., van Baalen, C. A., Fouchier, R. A., Osterhaus, A. D., and Rimmelzwaan, G. F. (2008). Cross-recognition of avian H5N1 influenza virus by human cytotoxic T-lymphocyte populations directed to human influenza A virus. *J. Virol.* 82, 5161–5166. doi: 10.1128/JVI.02694-07

Leclerc, D., Rivest, M., Babin, C., Lopez-Macias, C., and Savard, P. (2013). A novel M2e based flu vaccine formulation for dogs. *PLoS ONE* 8:e77084. doi: 10.1371/journal.pone.0077084

Ledgerwood, J. E., Zephir, K., Hu, Z., Wei, C. J., Chang, L., Enama, M. E., et al. (2013). Prime-boost interval matters: a randomized phase 1 study to identify the minimum interval necessary to observe the H5 DNA influenza vaccine priming effect. *J. Infect. Dis.* 208, 418–422. doi: 10.1093/infdis/jit180

Lee, J., Boutz, D. R., Chromikova, V., Joyce, M. G., Vollmers, C., Leung, K., et al. (2016). Molecular-level analysis of the serum antibody repertoire in young adults before and after seasonal influenza vaccination. *Nat. Med.* 22, 1456–1464. doi: 10.1038/nm.4224

Lee, K. H., and Seong, B. L. (1998). The position 4 nucleotide at the 3' end of the influenza virus neuraminidase vRNA is involved in temporal regulation of transcription and replication of neuraminidase RNAs and affects the repertoire of influenza virus surface antigens. *J. Gen Virol.* 79 (Pt 8), 1923–1934. doi: 10.1099/0022-1317-79-8-1923

Lee, L. Y., Ha do, L. A., Simmons, C., de Jong, M. D., Chau, N. V., Schumacher, R., et al. (2008). Memory T cells established by seasonal human influenza A infection cross-react with avian influenza A (H5N1) in healthy individuals. *J. Clin. Invest.* 118, 3478–3490. doi: 10.1172/JCI32460

Lee, L. Y. Y., Izzard, L., and Hurt, A. C. (2018a). A review of DNA vaccines against influenza. *Front. Immunol.* 9:1568. doi: 10.3389/fimmu.2018.01568

Lee, Y. J., Yu, J. E., Kim, P., Lee, J. Y., Cheong, Y. C., Lee, Y. J., et al. (2018b). Eliciting unnatural immune responses by activating cryptic epitopes in viral antigens. *FASEB J.* 32, 4658–4669. doi: 10.1096/fj.201701024RRR

Lee, Y. N., Kim, M. C., Lee, Y. T., Kim, Y. J., and Kang, S. M. (2015). Mechanisms of cross-protection by influenza virus M2-based vaccines. *Immune Netw.* 15, 213–221. doi: 10.4110/in.2015.15.5.213

Lee, Y. N., Lee, Y. T., Kim, M. C., Hwang, H. S., Lee, J. S., Kim, K. H., et al. (2014). Fc receptor is not required for inducing antibodies but plays a critical role in conferring protection after influenza M2 vaccination. *Immunology* 143, 300–309. doi: 10.1111/imm.12310

Lin, S. C., Lin, Y. F., Chong, P., and Wu, S. C. (2012). Broader neutralizing antibodies against H5N1 viruses using prime-boost immunization of hyperglycosylated hemagglutinin DNA and virus-like particles. *PLoS ONE* 7:e39075. doi: 10.1371/journal.pone.0039075

Lin, S. C., Liu, W. C., Jan, J. T., and Wu, S. C. (2014). Glycan masking of hemagglutinin for adenovirus vector and recombinant protein immunizations elicits broadly neutralizing antibodies against H5N1 avian influenza viruses. *PLoS ONE* 9:e92822. doi: 10.1371/journal.pone.0092822

Lin, Y. P., Gregory, V., Collins, P., Kloess, J., Wharton, S., Cattle, N., et al. (2010). Neuraminidase receptor binding variants of human influenza A(H3N2) viruses resulting from substitution of aspartic acid 151 in the catalytic site: a role in virus attachment? *J. Virol.* 84, 6769–6781. doi: 10.1128/JVI.00458-10

Liu, W.-C., Nachbagauer, R., Stadlbauer, D., Solórzano, A., Berlanda-Scorza, F., García-Sastre, A., et al. (2019a). Sequential immunization with live-attenuated chimeric hemagglutinin-based vaccines confers heterosubtypic immunity against influenza A viruses in a preclinical ferret model. *Front. Immunol.* 10:756. doi: 10.3389/fimmu.2019.00756

Liu, W. C., Lin, C. Y., Tsou, Y. T., Jan, J. T., and Wu, S. C. (2015). Cross-reactive neuraminidase-inhibiting antibodies elicited by immunization with recombinant neuraminidase proteins of H5N1 and pandemic H1N1 influenza A viruses. *J. Virol.* 89, 7224–7234. doi: 10.1128/JVI.00585-15

Liu, Y., Tan, H. X., Koutsakos, M., Jegaskanda, S., Esterbauer, R., Tilmanis, D., et al. (2019b). Cross-lineage protection by human antibodies binding the influenza B hemagglutinin. *Nat. Commun.* 10:324. doi: 10.1038/s41467-018-08165-y

Lowen, A. C. (2017). Constraints, drivers, and implications of influenza A virus reassortment. *Annu. Rev. Virol.* 4, 105–121. doi: 10.1146/annurev-virology-101416-041726

Lu, Y., Welsh, J. P., and Swartz, J. R. (2014). Production and stabilization of the trimeric influenza hemagglutinin stem domain for potentially broadly protective influenza vaccines. *Proc. Natl. Acad. Sci. U.S.A.* 111, 125–130. doi: 10.1073/pnas.1308701110

Mallajosyula, V. V., Citron, M., Ferrara, F., Lu, X., Callahan, C., Heidecker, G. J., et al. (2014). Influenza hemagglutinin stem-fragment immunogen elicits broadly neutralizing antibodies and confers heterologous protection. *Proc. Natl. Acad. Sci. U.S.A.* 111, E2514–2523. doi: 10.1073/pnas.1402766111

Margine, I., Krammer, F., Hai, R., Heaton, N. S., Tan, G. S., Andrews, S. A., et al. (2013). Hemagglutinin stalk-based universal vaccine constructs protect against group 2 influenza A viruses. *J. Virol.* 87, 10435–10446. doi: 10.1128/JVI.01715-13

McMahon, M., Kirkpatrick, E., Stadlbauer, D., Strohmeier, S., Bouvier, N. M., and Krammer, F. (2019). Mucosal immunity against neuraminidase prevents influenza B virus transmission in Guinea Pigs. *MBio* 10:e00560–19. doi: 10.1128/mBio.00560-19

Memoli, M. J., Shaw, P. A., Han, A., Czajkowski, L., Reed, S., Athota, R., et al. (2016). Evaluation of antihemagglutinin and antineuraminidase antibodies as correlates of protection in an influenza A/H1N1 virus healthy human challenge model. *MBio* 7:e00417–e00416. doi: 10.1128/mBio.00417-16

Meseda, C. A., Atukorale, V., Soto, J., Eichelberger, M. C., Gao, J., Wang, W., et al. (2018). Immunogenicity and protection against influenza H7N3 in mice by modified vaccinia virus Ankara vectors expressing influenza virus hemagglutinin or neuraminidase. *Sci. Rep.* 8:5364. doi: 10.1038/s41598-018-23712-9

Mogling, R., Richard, M. J., Vliet, S. V., Beek, R. V., Schrauwen, E. J. A., Spronken, M. I., et al. (2017). Neuraminidase-mediated haemagglutination of recent human influenza A(H3N2) viruses is determined by arginine 150 flanking the neuraminidase catalytic site. *J. Gen Virol.* 98, 1274–1281. doi: 10.1099/jgv.0.000809

Mohn, K. G., Bredholt, G., Brokstad, K. A., Pathirana, R. D., Aarstad, H. J., Tondel, C., et al. (2015). Longevity of B-cell and T-cell responses after live attenuated influenza vaccination in children. *J. Infect. Dis.* 211, 1541–1549. doi: 10.1093/infdis/jiu654

Mohn, K. G., Smith, I., Sjursen, H., and Cox, R. J. (2018). Immune responses after live attenuated influenza vaccination. *Hum. Vaccin Immunother.* 14, 571–578. doi: 10.1080/21645515.2017.1377376

Mohn, K. G. I., Zhou, F., Brokstad, K. A., Sridhar, S., and Cox, R. J. (2017). Boosting of cross-reactive and protection-associated T cells in children after live attenuated influenza vaccination. *J. Infect. Dis.* 215, 1527–1535. doi: 10.1093/infdis/jix165

Monsalvo, A. C., Batalle, J. P., Lopez, M. F., Krause, J. C., Klemenc, J., Hernandez, J. Z., et al. (2011). Severe pandemic 2009 H1N1 influenza disease due to pathogenic immune complexes. *Nat. Med.* 17, 195–199. doi: 10.1038/nm.2262

Monto, A. S., and Kendal, A. P. (1973). Effect of neuraminidase antibody on Hong Kong influenza. *Lancet* 1, 623–625. doi: 10.1016/S0140-6736(73)92196-X

Monto, A. S., Petrie, J. G., Cross, R. T., Johnson, E., Liu, M., Zhong, W., et al. (2015). Antibody to influenza virus neuraminidase: an independent correlate of protection. *J. Infect. Dis.* 212, 1191–1199. doi: 10.1093/infdis/jiv195

Mozdzanowska, K., Feng, J., Eid, M., Kragol, G., Cudic, M., Otvos, L. Jr., et al. (2003). Induction of influenza type A virus-specific resistance by immunization of mice with a synthetic multiple antigenic peptide vaccine

that contains ectodomains of matrix protein 2. *Vaccine* 21, 2616–2626. doi: 10.1016/S0264-410X(03)00040-9

Murphy, B. R., Kasel, J. A., and Chanock, R. M. (1972). Association of serum anti-neuraminidase antibody with resistance to influenza in man. *N. Engl. J. Med.* 286, 1329–1332. doi: 10.1056/NEJM197206222862502

Music, N., Reber, A. J., Kim, M. C., York, I. A., and Kang, S. M. (2016). Supplementation of H1N1pdm09 split vaccine with heterologous tandem repeat M2e5x virus-like particles confers improved cross-protection in ferrets. *Vaccine* 34, 466–473. doi: 10.1016/j.vaccine.2015.12.023

Nachbagauer, R., Krammer, F., and Albrecht, R. A. (2018). A live-attenuated prime, inactivated boost vaccination strategy with chimeric hemagglutinin-based universal influenza virus vaccines provides protection in ferrets: a confirmatory study. *Vaccines* 6:47. doi: 10.3390/vaccines6030047

Nachbagauer, R., Liu, W. C., Choi, A., Wohlbold, T. J., Atlas, T., Rajendran, M., et al. (2017). A universal influenza virus vaccine candidate confers protection against pandemic H1N1 infection in preclinical ferret studies. *NPJ Vaccines* 2:26. doi: 10.1038/s41541-017-0026-4

Neirynck, S., Deroo, T., Saelens, X., Vanlandschoot, P., Jou, W. M., and Fiers, W. (1999). A universal influenza A vaccine based on the extracellular domain of the M2 protein. *Nat. Med.* 5, 1157–1163. doi: 10.1038/13484

Ng, S., Nachbagauer, R., Balmaseda, A., Stadlbauer, D., Ojeda, S., Patel, M., et al. (2019). Novel correlates of protection against pandemic H1N1 influenza A virus infection. *Nat. Med.* 25, 962–967. doi: 10.1038/s41591-019-0463-x

Oh, H. L., Akerstrom, S., Shen, S., Bereczky, S., Karlberg, H., Klingstrom, J., et al. (2010). An antibody against a novel and conserved epitope in the hemagglutinin 1 subunit neutralizes numerous H5N1 influenza viruses. *J. Virol.* 84, 8275–8286. doi: 10.1128/JVI.02593-09

Ohmit, S. E., Petrie, J. G., Cross, R. T., Johnson, E., and Monto, A. S. (2011). Influenza hemagglutination-inhibition antibody titer as a correlate of vaccine-induced protection. *J. Infect. Dis.* 204, 1879–1885. doi: 10.1093/infdis/jir661

Pardi, N., Hogan, M. J., Porter, F. W., and Weissman, D. (2018a). mRNA vaccines - a new era in vaccinology. *Nat. Rev. Drug Discov.* 17, 261–279. doi: 10.1038/nrd.2017.243

Pardi, N., Parkhouse, K., Kirkpatrick, E., McMahon, M., Zost, S. J., Mui, B. L., et al. (2018b). Nucleoside-modified mRNA immunization elicits influenza virus hemagglutinin stalk-specific antibodies. *Nat. Commun.* 9:3361. doi: 10.1038/s41467-018-05482-0

Park, J. K., Han, A., Czajkowski, L., Reed, S., Athota, R., Bristol, T., et al. (2018). Evaluation of preexisting anti-hemagglutinin stalk antibody as a correlate of protection in a healthy volunteer challenge with influenza A/H1N1pdm Virus. *MBio* 9:e02284–17. doi: 10.1128/mBio.02284-17

Paul, S. S., Mok, C. K., Mak, T. M., Ng, O. W., Aboagye, J. O., Wohlbold, T. J., et al. (2017). A cross-clade H5N1 influenza A virus neutralizing monoclonal antibody binds to a novel epitope within the vestigial esterase domain of hemagglutinin. *Antiviral Res.* 144, 299–310. doi: 10.1016/j.antiviral.2017.06.012

Paules, C., and Subbarao, K. (2017). Influenza. *Lancet* 390, 697–708. doi: 10.1016/S0140-6736(17)30129-0

Paules, C. I., Marston, H. D., Eisinger, R. W., Baltimore, D., and Fauci, A. S. (2017). The pathway to a universal influenza vaccine. *Immunity* 47, 599–603. doi: 10.1016/j.immuni.2017.09.007

Peiris, J. S., Hui, K. P., and Yen, H. L. (2010). Host response to influenza virus: protection versus immunopathology. *Curr. Opin. Immunol.* 22, 475–481. doi: 10.1016/j.coi.2010.06.003

Pendzialek, J., Roose, K., Smet, A., Schepens, B., Kufer, P., Raum, T., et al. (2017). Bispecific T cell engaging antibody constructs targeting a universally conserved part of the viral M2 ectodomain cure and prevent influenza A virus infection. *Antiviral Res.* 141, 155–164. doi: 10.1016/j.antiviral.2017.02.016

Perez, J. T., Pham, A. M., Lorini, M. H., Chua, M. A., Steel, J., and tenOever, B. R. (2009). MicroRNA-mediated species-specific attenuation of influenza A virus. *Nat. Biotechnol.* 27, 572–576. doi: 10.1038/nbt.1542

Petsch, B., Schnee, M., Vogel, A. B., Lange, E., Hoffmann, B., Voss, D., et al. (2012). Protective efficacy of in vitro synthesized, specific mRNA vaccines against influenza A virus infection. *Nat. Biotechnol.* 30, 1210–1216. doi: 10.1038/nbt.2436

Petukhova, N. V., Gasanova, T. V., Stepanova, L. A., Rusova, O. A., Potapchuk, M. V., Korotkov, A. V., et al. (2013). Immunogenicity and protective efficacy of candidate universal influenza A nanovaccines produced in plants by Tobacco mosaic virus-based vectors. *Curr. Pharm. Des.* 19, 5587–5600. doi: 10.2174/13816128113199990337

Piepenbrink, M. S., Nogales, A., Basu, M., Fucile, C. F., Liesveld, J. L., Keefer, M. C., et al. (2019). Broad and protective influenza B virus neuraminidase antibodies in humans after vaccination and their clonal persistence as plasma cells. *MBio* 10:e00066-19. doi: 10.1128/mBio.00066-19

Pinto, L. H., Holsinger, L. J., and Lamb, R. A. (1992). Influenza virus M2 protein has ion channel activity. *Cell* 69, 517–528. doi: 10.1016/0092-8674(92)90452-I

Porter, K. R., and Raviprakash, K. (2017). DNA vaccine delivery and improved immunogenicity. *Curr. Issues Mol. Biol.* 22, 129–138. doi: 10.21775/cimb.022.129

Powell, T. J., Peng, Y., Berthoud, T. K., Blais, M. E., Lillie, P. J., Hill, A. V., et al. (2013). Examination of influenza specific T cell responses after influenza virus challenge in individuals vaccinated with MVA-NP+M1 vaccine. *PLoS ONE* 8:e62778. doi: 10.1371/journal.pone.0062778

Powell, T. J., Silk, J. D., Sharps, J., Fodor, E., and Townsend, A. R. (2012). Pseudotyped influenza A virus as a vaccine for the induction of heterotypic immunity. *J. Virol.* 86, 13397–13406. doi: 10.1128/JVI.01820-12

Price, G. E., Soboleski, M. R., Lo, C. Y., Misplon, J. A., Pappas, C., Houser, K. V., et al. (2009). Vaccination focusing immunity on conserved antigens protects mice and ferrets against virulent H1N1 and H5N1 influenza A viruses. *Vaccine* 27, 6512–6521. doi: 10.1016/j.vaccine.2009.08.053

Rajao, D. S., Chen, H., Perez, D. R., Sandbulte, M. R., Gauger, P. C., Loving, C. L., et al. (2016). Vaccine-associated enhanced respiratory disease is influenced by haemagglutinin and neuraminidase in whole inactivated influenza virus vaccines. *J. Gen. Virol.* 97, 1489–1499. doi: 10.1099/jgv.0.000468

Rajao, D. S., Loving, C. L., Gauger, P. C., Kitikoon, P., and Vincent, A. L. (2014). Influenza A virus hemagglutinin protein subunit vaccine elicits vaccine-associated enhanced respiratory disease in pigs. *Vaccine* 32, 5170–5176. doi: 10.1016/j.vaccine.2014.07.059

Ravin, N. V., Blokhina, E. A., Kuprianov, V. V., Stepanova, L. A., Shaldjan, A. A., Kovaleva, A. A., et al. (2015). Development of a candidate influenza vaccine based on virus-like particles displaying influenza M2e peptide into the immunodominant loop region of hepatitis B core antigen: Insertion of multiple copies of M2e increases immunogenicity and protective efficiency. *Vaccine* 33, 3392–3397. doi: 10.1016/j.vaccine.2015.04.066

Raymond, D. D., Bajic, G., Ferdman, J., Suphaphiphat, P., Settembre, E. C., Moody, M. A., et al. (2018). Conserved epitope on influenza-virus hemagglutinin head defined by a vaccine-induced antibody. *Proc. Natl. Acad. Sci. U.S.A.* 115, 168–173. doi: 10.1073/pnas.1715471115

Rekstin, A., Isakova-Sivak, I., Petukhova, G., Korenkov, D., Losev, I., Smolonogina, T., et al. (2017). Immunogenicity and cross protection in mice afforded by pandemic H1N1 live attenuated influenza vaccine containing wild-type nucleoprotein. *Biomed. Res. Int.* 2017:9359276. doi: 10.1155/2017/9359276

Rosendahl Huber, S. K., Camps, M. G., Jacobi, R. H., Mouthaan, J., van Dijken, H., van Beek, J., et al. (2015). Synthetic long peptide influenza vaccine containing conserved T and B cell epitopes reduces viral load in lungs of mice and ferrets. *PLoS ONE* 10:e0127969. doi: 10.1371/journal.pone.0127969

Sagawa, H., Ohshima, A., Kato, I., Okuno, Y., and Isegawa, Y. (1996). The immunological activity of a deletion mutant of influenza virus haemagglutinin lacking the globular region. *J. Gen Virol.* 77 (Pt 7), 1483–1487. doi: 10.1099/0022-1317-77-7-1483

Sakai, T., Nishimura, S. I., Naito, T., and Saito, M. (2017). Influenza A virus hemagglutinin and neuraminidase act as novel motile machinery. *Sci. Rep.* 7:45043. doi: 10.1038/srep45043

Sarawar, S., Hatta, Y., Watanabe, S., Dias, P., Neumann, G., Kawaoka, Y., et al. (2016). M2SR, a novel live single replication influenza virus vaccine, provides effective heterosubtypic protection in mice. *Vaccine* 34, 5090–5098. doi: 10.1016/j.vaccine.2016.08.061

Saunders-Hastings, P. R., and Krewski, D. (2016). Reviewing the history of pandemic influenza: understanding patterns of emergence and transmission. *Pathogens* 5:66. doi: 10.3390/pathogens5040066

Schulman, J. L. (1969). The role of antineuraminidase antibody in immunity to influenza virus infection. *Bull World Health Organ.* 41, 647–650.

Schulman, J. L., and Kilbourne, E. D. (1969). Independent variation in nature of hemagglutinin and neuraminidase antigens of influenza virus: distinctiveness of hemagglutinin antigen of Hong Kong-68 virus. *Proc. Natl. Acad. Sci. U.S.A.* 63, 326–333. doi: 10.1073/pnas.63.2.326

Shen, C., Chen, J., Li, R., Zhang, M., Wang, G., Stegalkina, S., et al. (2017). A multimechanistic antibody targeting the receptor binding site potently cross-protects against influenza B viruses. *Sci. Transl. Med.* 9:eaam5752. doi: 10.1126/scitranslmed.aam5752

Skowronski, D. M., De Serres, G., Crowcroft, N. S., Janjua, N. Z., Boulianne, N., Hottes, T. S., et al. (2010). Association between the 2008–09 seasonal influenza vaccine and pandemic H1N1 illness during Spring-Summer 2009: four observational studies from Canada. *PLoS Med.* 7:e1000258. doi: 10.1371/journal.pmed.1000258

Song, B. M., Kang, H. M., Lee, E. K., Jung, S. C., Kim, M. C., Lee, Y. N., et al. (2016). Supplemented vaccination with tandem repeat M2e virus-like particles enhances protection against homologous and heterologous HPAI H5 viruses in chickens. *Vaccine* 34, 678–686. doi: 10.1016/j.vaccine.2015.11.074

Sridhar, S. (2016). Heterosubtypic T-cell immunity to influenza in humans: challenges for universal T-cell influenza vaccines. *Front. Immunol.* 7:195. doi: 10.3389/fimmu.2016.00195

Sridhar, S., Brokstad, K. A., and Cox, R. J. (2015). Influenza vaccination strategies: comparing inactivated and live attenuated influenza vaccines. *Vaccines* 3, 373–389. doi: 10.3390/vaccines3020373

Stech, J., Garn, H., Wegmann, M., Wagner, R., and Klenk, H. D. (2005). A new approach to an influenza live vaccine: modification of the cleavage site of hemagglutinin. *Nat. Med.* 11, 683–689. doi: 10.1038/nm1256

Steel, J., Lowen, A. C., Wang, T. T., Yondola, M., Gao, Q., Haye, K., et al. (2010). Influenza virus vaccine based on the conserved hemagglutinin stalk domain. *MBio* 1:e00018–10. doi: 10.1128/mBio.00018-10

Su, B., Wurtzer, S., Rameix-Welti, M.-A., Dwyer, D., van der Werf, S., Naffakh, N., et al. (2010). Enhancement of the influenza A hemagglutinin (HA)-mediated cell-cell fusion and virus entry by the viral neuraminidase (NA). *PLoS ONE* 4:e8495. doi: 10.1371/journal.pone.0008495

Subbramanian, R. A., Basha, S., Shata, M. T., Brady, R. C., and Bernstein, D. I. (2010). Pandemic and seasonal H1N1 influenza hemagglutinin-specific T cell responses elicited by seasonal influenza vaccination. *Vaccine* 28, 8258–8267. doi: 10.1016/j.vaccine.2010.10.077

Sui, J., Hwang, W. C., Perez, S., Wei, G., Aird, D., Chen, L. M., et al. (2009). Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. *Nat. Struct. Mol. Biol.* 16, 265–273. doi: 10.1038/nsmb.1566

Sun, W., Kirkpatrick, E., Ermler, M., Nachbagauer, R., Broecker, F., Krammer, F., et al. (2019). Development of influenza B universal vaccine candidates using the "mosaic" hemagglutinin approach. *J. Virol.* 93:e00333-19. doi: 10.1128/JVI.00333-19

Sun, X., Wang, Y., Dong, C., Hu, J., and Yang, L. (2015). High copy numbers and N terminal insertion position of influenza A M2E fused with hepatitis B core antigen enhanced immunogenicity. *Biosci. Trends* 9, 221–227. doi: 10.5582/bst.2015.01060

Talon, J., Salvatore, M., O'Neill, R. E., Nakaya, Y., Zheng, H., Muster, T., et al. (2000). Influenza A and B viruses expressing altered NS1 proteins: a vaccine approach. *Proc. Natl. Acad. Sci. U.S.A.* 97, 4309–4314. doi: 10.1073/pnas.070525997

Tan, G. S., Lee, P. S., Hoffman, R. M., Mazel-Sanchez, B., Krammer, F., Leon, P. E., et al. (2014). Characterization of a broadly neutralizing monoclonal antibody that targets the fusion domain of group 2 influenza A virus hemagglutinin. *J. Virol.* 88, 13580–13592. doi: 10.1128/JVI.02289-14

Tan, G. S., Leon, P. E., Albrecht, R. A., Margine, I., Hirsh, A., Bahl, J., et al. (2016). Broadly-reactive neutralizing and non-neutralizing antibodies directed against the H7 influenza virus hemagglutinin reveal divergent mechanisms of protection. *PLoS Pathog.* 12:e1005578. doi: 10.1371/journal.ppat.1005578

Tan, J., Asthagiri Arunkumar, G., and Krammer, F. (2018). Universal influenza virus vaccines and therapeutics: where do we stand with influenza B virus? *Curr. Opin. Immunol.* 53, 45–50. doi: 10.1016/j.coi.2018.04.002

Terajima, M., Babon, J. A., Co, M. D., and Ennis, F. A. (2013). Cross-reactive human B cell and T cell epitopes between influenza A and B viruses. *Virol. J.* 10:244. doi: 10.1186/1743-422X-10-244

Terajima, M., Cruz, J., Co, M. D., Lee, J. H., Kaur, K., Wrammert, J., et al. (2011). Complement-dependent lysis of influenza a virus-infected cells by broadly cross-reactive human monoclonal antibodies. *J. Virol.* 85, 13463–13467. doi: 10.1128/JVI.05193-11

Tompkins, S. M., Zhao, Z. S., Lo, C. Y., Misplon, J. A., Liu, T., Ye, Z., et al. (2007). Matrix protein 2 vaccination and protection against influenza viruses, including subtype H5N1. *Emerg. Infect. Dis.* 13, 426–435. doi: 10.3201/eid1303.061125

Tsybalova, L. M., Stepanova, L. A., Kuprianov, V. V., Blokhina, E. A., Potapchuk, M. V., Korotkov, A. V., et al. (2015). Development of a candidate influenza vaccine based on virus-like particles displaying influenza M2e peptide into the immunodominant region of hepatitis B core antigen: broad protective efficacy of particles carrying four copies of M2e. *Vaccine* 33, 3398–3406. doi: 10.1016/j.vaccine.2015.04.073

Tsybalova, L. M., Stepanova, L. A., Shuklina, M. A., Mardanova, E. S., Kotlyarov, R. Y., Potapchuk, M. V., et al. (2018). Combination of M2e peptide with stalk HA epitopes of influenza A virus enhances protective properties of recombinant vaccine. *PLoS ONE* 13:e0201429. doi: 10.1371/journal.pone.0201429

Ui, H., Yamayoshi, S., Uraki, R., Kiso, M., Oishi, K., Murakami, S., et al. (2017). Evaluation of seasonal influenza vaccines for H1N1pdm09 and type B viruses based on a replication-incompetent PB2-KO virus. *Vaccine* 35, 1892–1897. doi: 10.1016/j.vaccine.2017.02.041

Ulmer, J. B., Donnelly, J. J., Parker, S. E., Rhodes, G. H., Felgner, P. L., Dwarki, V. J., et al. (1993). Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* 259, 1745–1749. doi: 10.1126/science.8456302

Uraki, R., Kiso, M., Iwatsuki-Horimoto, K., Fukuyama, S., Takashita, E., Ozawa, M., et al. (2013). A novel bivalent vaccine based on a PB2-knockout influenza virus protects mice from pandemic H1N1 and highly pathogenic H5N1 virus challenges. *J. Virol.* 87, 7874–7881. doi: 10.1128/JVI.00076-13

van de Sandt, C. E., Dou, Y., Vogelzang-van Trierum, S. E., Westgeest, K. B., Pronk, M. R., Osterhaus, A. D. M. E., et al. (2015). Influenza B virus-specific CD8+ T-lymphocytes strongly cross-react with viruses of the opposing influenza B lineage. *J. Gen Virol.* 96, 2061–2073. doi: 10.1099/vir.0.000156

van de Sandt, C. E., Kreijtz, J. H., de Mutsert, G., Geelhoed-Mieras, M. M., Hillaire, M. L., Vogelzang-van Trierum, S. E., et al. (2014). Human cytotoxic T lymphocytes directed to seasonal influenza A viruses cross-react with the newly emerging H7N9 virus. *J. Virol.* 88, 1684–1693. doi: 10.1128/JVI.02843-13

Victor, S. T., Watanabe, S., Katsura, H., Ozawa, M., and Kawaoka, Y. (2012). A replication-incompetent PB2-knockout influenza A virus vaccine vector. *J. Virol.* 86, 4123–4128. doi: 10.1128/JVI.06232-11

Vigil, A., Estelles, A., Kauvar, L. M., Johnson, S. K., Tripp, R. A., and Wittekind, M. (2018). Native human monoclonal antibodies with potent cross-lineage neutralization of influenza B viruses. *Antimicrob. Agents Chemother.* 62:e02269–e02217. doi: 10.1128/AAC.02269-17

Walz, L., Kays, S. K., Zimmer, G., and von Messling, V. (2018). Neuraminidase-inhibiting antibody titers correlate with protection from heterologous influenza virus strains of the same neuraminidase subtype. *J. Virol.* 92:e01006–e01018. doi: 10.1128/JVI.01006-18

Wan, H., Gao, J., Xu, K., Chen, H., Couzens, L. K., Rivers, K. H., et al. (2013). Molecular basis for broad neuraminidase immunity: conserved epitopes in seasonal and pandemic H1N1 as well as H5N1 influenza viruses. *J. Virol.* 87, 9290–9300. doi: 10.1128/JVI.01203-13

Wang, R., Song, A., Levin, J., Dennis, D., Zhang, N. J., Yoshida, H., et al. (2008). Therapeutic potential of a fully human monoclonal antibody against influenza A virus M2 protein. *Antiviral Res.* 80, 168–177. doi: 10.1016/j.antiviral.2008.06.002

Wang, S., Ren, H., Jiang, W., Chen, H., Hu, H., Chen, Z., et al. (2017). Divergent requirement of Fc-Fcγ receptor interactions for *in vivo* protection against influenza viruses by two pan-H5 hemagglutinin antibodies. *J. Virol.* 91, e02065–e02016. doi: 10.1128/JVI.02065-16

Wang, W., Li, R., Deng, Y., Lu, N., Chen, H., Meng, X., et al. (2015). Protective efficacy of the conserved NP, PB1, and M1 proteins as immunogens in DNA- and vaccinia virus-based universal influenza A virus vaccines in mice. *Clin. Vaccine Immunol.* 22, 618–630. doi: 10.1128/CVI.00091-15

Wang, Y., Deng, L., Kang, S. M., and Wang, B. Z. (2018a). Universal influenza vaccines: from viruses to nanoparticles. *Exp. Rev. Vaccines* 17, 967–976. doi: 10.1080/14760584.2018.1541408

Wang, Z., Zhu, L., Nguyen, T. H. O., Wan, Y., Sant, S., Quiñones-Parra, S. M., et al. (2018b). Clonally diverse CD38+HLA-DR+CD8+ T cells persist during fatal H7N9 disease. *Nat. Commun.* 9:824. doi: 10.1038/s41467-018-03243-7

Weaver, E. A., Rubrum, A. M., Webby, R. J., and Barry, M. A. (2011). Protection against divergent influenza H1N1 virus by a centralized influenza hemagglutinin. *PLoS ONE* 6:e18314. doi: 10.1371/journal.pone.0018314

Whittle, J. R., Zhang, R., Khurana, S., King, L. R., Manischewitz, J., Golding, H., et al. (2011). Broadly neutralizing human antibody that recognizes the receptor-binding pocket of influenza virus hemagglutinin. *Proc. Natl. Acad. Sci. U.S.A.* 108, 14216–14221. doi: 10.1073/pnas.1111497108

Winarski, K. L., Tang, J., Klenow, L., Lee, J., Coyle, E. M., Manischewitz, J., et al. (2019). Antibody-dependent enhancement of influenza disease promoted by increase in hemagglutinin stem flexibility and virus fusion kinetics. *Proc. Natl. Acad. Sci. U.S.A.* (2019) 116, 15194–15199. doi: 10.1073/pnas.1821317116

Wohlbold, T. J., Chromikova, V., Tan, G. S., Meade, P., Amanat, F., Comella, P., et al. (2016). Hemagglutinin stalk- and neuraminidase-specific monoclonal antibodies protect against lethal H10N8 influenza virus infection in mice. *J. Virol.* 90, 851–861. doi: 10.1128/JVI.02275-15

Wohlbold, T. J., and Krammer, F. (2014). In the shadow of hemagglutinin: a growing interest in influenza viral neuraminidase and its role as a vaccine antigen. *Viruses* 6, 2465–2494. doi: 10.3390/v6062465

Wohlbold, T. J., Nachbagauer, R., Xu, H., Tan, G. S., Hirsh, A., Brokstad, K. A., et al. (2015). Vaccination with adjuvanted recombinant neuraminidase induces broad heterologous, but not heterosubtypic, cross-protection against influenza virus infection in mice. *MBio* 6:e02556. doi: 10.1128/mBio.02556-14

Wohlbold, T. J., Podolsky, K. A., Chromikova, V., Kirkpatrick, E., Falconieri, V., Meade, P., et al. (2017). Broadly protective murine monoclonal antibodies against influenza B virus target highly conserved neuraminidase epitopes. *Nat. Microbiol.* 2, 1415–1424. doi: 10.1038/s41564-017-0011-8

Yang, C., Skiena, S., Futcher, B., Mueller, S., and Wimmer, E. (2013). Deliberate reduction of hemagglutinin and neuraminidase expression of influenza virus leads to an ultraprotective live vaccine in mice. *Proc. Natl. Acad. Sci. U.S.A.* 110, 9481–9486. doi: 10.1073/pnas.1307473110

Yang, X., Steukers, L., Forier, K., Xiong, R., Braeckmans, K., Van Reeth, K., et al. (2014). A beneficiary role for neuraminidase in influenza virus penetration through the respiratory mucus. *PLoS ONE* 9:e110026. doi: 10.1371/journal.pone.0110026

Yassine, H. M., Boyington, J. C., McTamney, P. M., Wei, C. J., Kanekiyo, M., Kong, W. P., et al. (2015). Hemagglutinin-stem nanoparticles generate heterosubtypic influenza protection. *Nat. Med.* 21, 1065–1070. doi: 10.1038/nm.3927

Zhang, G. G., Li, D. X., Zhang, H. H., Zeng, Y. M., and Chen, L. (2009). Enhancement of mucosal immune response against the M2eHBc+ antigen in mice with the fusion expression products of LTB and M2eHBc+ through mucosal immunization route. *Vet. Res. Commun.* 33, 735–747. doi: 10.1007/s11259-009-9222-7

Zhang, J., Fan, H. Y., Zhang, Z., Zhang, J., Zhang, J., Huang, J. N., et al. (2016). Recombinant baculovirus vaccine containing multiple M2e and adjuvant LTB induces T cell dependent, cross-clade protection against H5N1 influenza virus in mice. *Vaccine* 34, 622–629. doi: 10.1016/j.vaccine.2015.12.039

Zheng, Z., Paul, S. S., Mo, X., Yuan, Y. A., and Tan, Y. J. (2018). The vestigial esterase domain of haemagglutinin of H5N1 avian influenza A virus: antigenicity and contribution to viral pathogenesis. *Vaccines* 6:53. doi: 10.3390/vaccines6030053

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.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: info@frontiersin.org | +41 21 510 17 00

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership