WHY VACCINES TO HIV, HCV AND MALARIA HAVE SO FAR FAILED — CHALLENGES TO DEVELOPING VACCINES AGAINST IMMUNOREGULATING PATHOGENS

EDITED BY: Shuo Li, Magdalena Plebanski, Peter Smooker and Eric J. Gowans

PUBLISHED IN: Frontiers in Microbiology and Frontiers in Immunology

frontiers Research Topics

Frontiers Copyright Statement

© Copyright 2007-2016 Frontiers Media SA. All rights reserved.

All content included on this site, such as text, graphics, logos, button icons, images, video/audio clips, downloads, data compilations and software, is the property of or is licensed to Frontiers Media SA ("Frontiers") or its licensees and/or subcontractors. The copyright in the text of individual articles is the property of their respective authors, subject to a license granted to Frontiers.

The compilation of articles constituting this e-book, wherever published, as well as the compilation of all other content on this site, is the exclusive property of Frontiers. For the conditions for downloading and copying of e-books from Frontiers' website, please see the Terms for Website Use. If purchasing Frontiers e-books from other websites or sources, the conditions of the website concerned apply.

Images and graphics not forming part of user-contributed materials may not be downloaded or copied without permission.

Individual articles may be downloaded and reproduced in accordance with the principles of the CC-BY licence subject to any copyright or other notices. They may not be re-sold as an e-book.

As author or other contributor you grant a CC-BY licence to others to reproduce your articles, including any graphics and third-party materials supplied by you, in accordance with the Conditions for Website Use and subject to any copyright notices which you include in connection with your articles and materials.

All copyright, and all rights therein, are protected by national and international copyright laws.

The above represents a summary only. For the full conditions see the Conditions for Authors and the Conditions for Website Use.

ISSN 1664-8714
ISBN 978-2-88919-966-2
DOI 10.3389/978-2-88919-966-2

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**

WHY VACCINES TO HIV, HCV AND MALARIA HAVE SO FAR FAILED – CHALLENGES TO DEVELOPING VACCINES AGAINST IMMUNOREGULATING PATHOGENS

Topic Editors:
Shuo Li, Monash University, Australia & Swiss Institute of Allergy and Asthma Research, Switzerland
Magdalena Plebanski, Monash University, Australia
Peter Smooker, RMIT University, Australia
Eric J. Gowans, University of Adelaide, Australia

Despite continuous progress in the development of anti-viral and anti-bacterial/parasite drugs, the high cost of medicines and the potential for re-infection, especially in high risk groups, suggest that protective vaccines to some of the most dangerous persistent infections are still highly desirable. There are no vaccines available for HIV, HCV and Malaria, and all attempts to make a broadly effective vaccine have failed so far. In this Research Topic we look into why vaccines have failed over the years, and what we have learn from these attempts.

Rather than only showing positive results, this issue aims to reflect on failed efforts in vaccine development. Coming to understand our limitations will have theoretical and practical implications for the future development of vaccines to these major global disease burdens.

Citation: Li, S., Plebanski, M., Smooker, P., Gowans, E. J., eds. (2016). Why Vaccines to HIV, HCV and Malaria Have So Far Failed — Challenges to Developing Vaccines against Immunoregulating Pathogens. Lausanne: Frontiers Media. doi: 10.3389/978-2-88919-966-2

Table of Contents

frontiers in Microbiology

EDITORIAL
published: 27 November 2015
doi: 10.3389/fmicb.2015.01318

Editorial: Why Vaccines to HIV, HCV, and Malaria Have So Far Failed—Challenges to Developing Vaccines Against Immunoregulating Pathogens

Shuo Li[1,2]*, *Magdalena Plebanski*[2], *Peter Smooker*[3] and *Eric J. Gowans*[4]

[1] Department of Microbiology, Monash University, Melbourne, VIC, Australia, [2] Swiss Institute of Allergy and Asthma Research, Davos, Switzerland, [3] School of Applied Sciences, Health Innovations Research Institute, RMIT University, Melbourne, VIC, Australia, [4] Discipline of Surgery, University of Adelaide, Adelaide, SA, Australia

Keywords: HIV, HCV, malaria, vaccine, immunity

Together with sanitation, vaccination is one of the most effective life-saving interventions available in the fight against infectious diseases. As you read this issue of Frontier in Microbiology, scientists around the globe are working toward developing vaccines against diverse infectious diseases, allergies, cancer and autoimmune diseases. We believe that every major disease will eventually have its vaccine. However, if we consider major infectious agents, such as human immunodeficiency virus (HIV), hepatitis C virus (HCV), and Malaria, despite many years of effort, billions of dollars spent and countless animal lives sacrificed, no vaccine is available to protect against these infections. How did this happen? What prevents us from being victorious? In this issue of Frontier in Microbiology, we examine why some of these vaccines have failed, collecting reflections from leading researchers in the field.

Some of the key obstacles to vaccine development discussed in this issue include:

1. **The genetic diversity of the target pathogen.** In RNA viruses such as HIV and HCV, the error prone RNA dependent polymerase generates quasispecies (Chanzu and Ondondo, 2014; John and Gaudieri, 2014; Ondondo, 2014). In addition, influenza vaccines need to be reformulated annually, due to antigenic drift (Quinones-Parra et al., 2014). Over half a century of malaria vaccine development, despite awareness of the diversity of natural parasite populations, vaccines that have progressed to human clinical trials have only included a small fraction of the polymorphisms present in endemic regions. In addition to increasing the complexity of the immunogen, antigenic diversity of the organism in different geographic regions has major implications for vaccine efficacy. In this issue, Alyssa Barry and Alicia Arnott discuss the importance of population genetic studies in identifying functionally relevant polymorphisms, and argue that molecular epidemiological surveys are necessary to ensure that the vaccine strain corresponds to the local target parasite populations (Barry and Arnott, 2014). Targeting conserved pathogen antigens may help to overcome diversity, although these regions are often concealed and/or less accessible to immune effectors. Indeed, Chiu and colleagues herein showed that antibody titers to PfRh5 correlated with protection against *Plasmodium falciparum* in clinical trials in PNG (Chiu et al., 2014). Moreover, Quinones-Parra and colleagues also showed that broadly neutralizing antibodies targeting conserved regions, which developed naturally following the 2009 influenza pandemic, provide hints to the nature of the responses a successful vaccine should elicit (Quinones-Parra et al., 2014). As an example of this strategy, Drummer and colleagues proposed that the conserved regions within HCV E2, especially the residues that interact with the virus co-receptor CD81 (Drummer, 2014), may represent an attractive immunogen in a HCV vaccine.

OPEN ACCESS

Edited and reviewed by:
Ian Marriott,
University of North Carolina at Charlotte, USA

***Correspondence:**
Shuo Li
shuo.li@monash.edu

Specialty section:
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Microbiology

Received: *06 October 2015*
Accepted: *09 November 2015*
Published: *27 November 2015*

Citation:
Li S, Plebanski M, Smooker P and Gowans EJ (2015) Editorial: Why Vaccines to HIV, HCV, and Malaria Have So Far Failed—Challenges to Developing Vaccines Against Immunoregulating Pathogens. Front. Microbiol. 6:1318. doi: 10.3389/fmicb.2015.01318

2. **The discrepancy between immunogenicity and protection.** As highlighted in this issue, although many HIV vaccine candidates induce strong T and B cell responses in pre-clinical and Phase-I trials, these responses have thus far failed to correlate with protection in larger scale trials (Chanzu and Ondondo, 2014). The current immunological readouts, such as ELISPOT, intracellular cytokine staining or antibody levels do not appear to be adequate measures that predict vaccine success or failure. New strategies of clinical trial monitoring, such as those depicting immunogenicity from a new angle, or those that dissociate the effects of the vaccine vector from vaccine antigen, may need to be developed and applied. These could include analysis of the T and B cell immune repertoire (Li et al., 2013), which might identify the clonotype response to the vector independent of responses to the immunogen. In addition, whole genome transcription arrays and other recent high throughput assays are likely to provide new and unexpected insights. Overall, new concepts are required to define the best reference or readout of immunogenicity that may in turn predict protective efficacy.
3. **Vector or Immunogen, which one matters?** While an effective vaccine may need to be multivalent, comprising multiple alleles for a given polymorphic antigen, and/or the antigen derived from conserved regions, the delivery vectors are at least as important as the immunogen itself. The vectors modulate innate and adaptive immunity, hopefully enabling the vaccine antigen to elicit the right response (Ondondo, 2014). Prime-boost strategies using plasmid DNA or viral vector prime followed by protein or viral vector boost have been studied extensively. An important lesson was learned from the HIV STEP study, in which a highly immunogenic vaccine actually increased HIV acquisition, presumably due to preexisting immunity to the vector. Of the proposed explanations, a strong response to the vector may have activated $CD4^+$ T cells, which are targets for HIV. This seemingly unavoidable paradox highlights the challenges of HIV vaccine development.
4. **The discrepancy between local and systemic responses.** Rafferty and colleagues argue that of the vectors used in HIV vaccine design, viral vectors with mucosal tropism, e.g., adenoviruses and influenza viruses, are particularly interesting, given that genitorectal mucosa is the first site of contact in HIV transmission (Rafferty et al., 2014). Most systemic vaccines do not elicit mucosal responses, and it is uncertain if mucosal delivery of antigen can induce systemic immunity. Cytokines and chemokines have been used as adjuvants to encourage mucosal homing of immune effector cells, such as the "prime-pull" approach in animal models (Rafferty et al., 2014). Difficulties in studying mucosal immune responses, including low cell numbers, sample variation and invasiveness of mucosal sampling means that mucosal immune responses are often not examined in clinical trials, as discussed in this issue (Chanzu and Ondondo, 2014). This is an important area of future clinical trial monitoring and is being addressed. An effective HIV vaccine strategy may need to involve both systemic and mucosal approaches simultaneously. Indeed, women in third world countries share the major burden of HIV infection, and a vaccine that can effectively elicit mucosal immune responses in the female genital tract is more likely to protect women (Rafferty et al., 2014).
5. **Infant vaccination, how much do we know?** On a global scale, millions of infants receive around 20 vaccines during the first year of life, but relatively few studies have examined the development of immunity in this age group. The innate immune system does not reach full capacity until the teenage years, and as adaptive immunity in newborns is intrinsically skewed to a Th2-type, the neonatal and infant immune responses to many vaccines are suboptimal (Ndure and Flanagan, 2014). In addition, the naïve immune repertoire's initial response to a vaccine, which may engage both vaccine antigen-specific and non-specific T and B cells, promoted for example by inflammation via the TNFR2 receptor (Wilson et al., 2015), may play an important role in shaping the repertoire toward subsequent unrelated pathogens. Indeed, growing evidence suggests that vaccines can have heterologous effects, affecting an individual's subsequent responses to unrelated pathogens or vaccines (Flanagan et al., 2011). As vaccines which target major global diseases are eventually likely to be included in childhood vaccination, it is important to understand how vaccines modulate the naïve immune system and the long-term impact of this intervention.
6. **Immune subversion and immunosuppression.** Malaria-infected red blood cells have an amazing capacity to induce $FOXP3^+$ expression, a marker of highly suppressive regulatory T cells (Treg), on co-cultured autologous T cells, suggesting that widespread induction *in vivo* would not require direct contact with the parasite (Scholzen et al., 2014). In an exciting mechanistic insight, in this issue, Wykes and colleagues further show a role for PD-1 in malaria-induced loss of T cell function and/or apoptosis (Wykes et al., 2014). While it is not feasible to directly predict viral epitopes recognized by the T cell receptor, Moise and colleagues show that the JanusMatrix algorithm can be applied to achieve this by searching for virus-encoded human homologs, which theoretically can be recognized by Treg (Moise et al., 2014). We also propose that to design improved vaccines we need to better understand how genetic factors such as HLA can affect viral susceptibility. For example, in addition to HIV and HCV which interfere with HLA expression in host immune cells, could HLA intrinsically influence T cell repertoire development before the selection step?

Regardless of the chances of developing a prophylactic vaccine for every disease, the world needs vaccines to reduce current disease burdens and save lives. How to effectively mobilize innate immunity may be another focus for future vaccine design. Learning from our mistakes and understanding our limitations will help us in our ongoing battle against pathogens.

AUTHOR CONTRIBUTIONS

SL wrote the manuscript; MP, PS, and EG helped with opinions, suggestions, and editing.

REFERENCES

Barry, A. E., and Arnott, A. (2014). Strategies for designing and monitoring malaria vaccines targeting diverse antigens. *Front. Immunol.* 5:359. doi: 10.3389/fimmu.2014.00359

Chanzu, N., and Ondondo, B. (2014). Induction of potent and long-lived antibody and cellular immune responses in the genitorectal mucosa could be the critical determinant of HIV vaccine efficacy. *Front. Immunol.* 5:202. doi: 10.3389/fimmu.2014.00202

Chiu, C. Y., Healer, J., Thompson, J. K., Chen, L., Kaul, A., Savergave, L., et al. (2014). Association of antibodies to *Plasmodium falciparum* reticulocyte binding protein homolog 5 with protection from clinical malaria. *Front. Microbiol.* 5:314. doi: 10.3389/fmicb.2014.00314

Drummer, H. E. (2014). Challenges to the development of vaccines to hepatitis C virus that elicit neutralizing antibodies. *Front. Microbiol.* 5:329. doi: 10.3389/fmicb.2014.00329

Flanagan, K. L., Klein, S. L., Skakkebaek, N. E., Marriott, I., Marchant, A., Selin, L., et al. (2011). Sex differences in the vaccine-specific and non-targeted effects of vaccines. *Vaccine* 29, 2349-2354. doi: 10.1016/j.vaccine.2011.01.071

John, M., and Gaudieri, S. (2014). Influence of HIV and HCV on T cell antigen presentation and challenges in the development of vaccines. *Front. Microbiol.* 5:514. doi: 10.3389/fmicb.2014.00514

Li, S., Lefranc, M. P., Miles, J. J., Alamyar, E., Giudicelli, V., Duroux, P., et al. (2013). IMGT/HighV QUEST paradigm for T cell receptor IMGT clonotype diversity and next generation repertoire immunoprofiling. *Nat. Commun.* 4, 2333. doi: 10.1038/ncomms3333

Moise, L., Terry, F., Gutierrez, A. H., Tassone, R., Losikoff, P., Gregory, S. H., et al. (2014). Smarter vaccine design will circumvent regulatory T cell-mediated evasion in chronic HIV and HCV infection. *Front. Microbiol.* 5:502. doi: 10.3389/fmicb.2014.00502

Ndure, J., and Flanagan, K. L. (2014). Targeting regulatory T cells to improve vaccine immunogenicity in early life. *Front. Microbiol.* 5:477. doi: 10.3389/fmicb.2014.00477

Ondondo, B. O. (2014). The influence of delivery vectors on HIV vaccine efficacy. *Front. Microbiol.* 5:439. doi: 10.3389/fmicb.2014.00439

Quiñones-Parra, S., Loh, L., Brown, L. E., Kedzierska, K., and Valkenburg, S. A. (2014). Universal immunity to influenza must outwit immune evasion. *Front. Microbiol.* 5:285. doi: 10.3389/fmicb.2014.00285

Rafferty, H., Sibeko, S., and Rowland-Jones, S. (2014). How can we design better vaccines to prevent HIV infection in women? *Front. Microbiol.* 5:572. doi: 10.3389/fmicb.2014.00572

Scholzen, A., Cooke, B. M., and Plebanski, M. (2014). *Plasmodium falciparum* induces Foxp3hi CD4 T cells independent of surface PfEMP1 expression via small soluble parasite components. *Front. Microbiol.* 5:200. doi: 10.3389/fmicb.2014.00200

Wilson, K. L., Xiang, S. D., and Plebanski, M. (2015). Montanide, Poly I:C and nanoparticle based vaccines promote differential suppressor and effector cell expansion: a study of induction of CD8 T cells to a minimal *Plasmodium berghei* epitope. *Front. Microbiol.* 6:29. doi: 10.3389/fmicb.2015.00029

Wykes, M. N., Horne-Debets, J. M., Leow, C. Y., and Karunarathne, D. S. (2014). Malaria drives T cells to exhaustion. *Front. Microbiol.* 5:249. doi: 10.3389/fmicb.2014.00249

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.

Copyright © 2015 Li, Plebanski, Smooker and Gowans. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 28 July 2014
doi: 10.3389/fimmu.2014.00359

Strategies for designing and monitoring malaria vaccines targeting diverse antigens

Alyssa E. Barry [1,2] **** and Alicia Arnott*** [1,2]

[1] *Division of Infection and Immunity, Walter and Eliza Hall Institute of Medical Research, Parkville, VIC, Australia*
[2] *Department of Medical Biology, The University of Melbourne, Parkville, VIC, Australia*

Edited by:
Magdalena Plebanski, Monash University, Australia

Reviewed by:
Hajime Hisaeda, Gunma University, Japan
Shannon Takala Harrison, University of Maryland School of Medicine, USA

****Correspondence:***
Alyssa E. Barry, Division of Infection and Immunity, Walter and Eliza Hall Institute for Medical Research, 1G Royal Parade, Parkville, VIC 3052, Australia
e-mail: barry@wehi.edu.au

After more than 50 years of intensive research and development, only one malaria vaccine candidate, "RTS,S," has progressed to Phase 3 clinical trials. Despite only partial efficacy, this candidate is now forecast to become the first licensed malaria vaccine. Hence, more efficacious second-generation malaria vaccines that can significantly reduce transmission are urgently needed. This review will focus on a major obstacle hindering development of effective malaria vaccines: parasite antigenic diversity. Despite extensive genetic diversity in leading candidate antigens, vaccines have been and continue to be formulated using recombinant antigens representing only one or two strains. These vaccine strains represent only a small fraction of the diversity circulating in natural parasite populations, leading to escape of non-vaccine strains and challenging investigators' abilities to measure strain-specific efficacy in vaccine trials. Novel strategies are needed to overcome antigenic diversity in order for vaccine development to succeed. Many studies have now cataloged the global diversity of leading *Plasmodium falciparum* and *Plasmodium vivax* vaccine antigens. In this review, we describe how population genetic approaches can be applied to this rich data source to predict the alleles that best represent antigenic diversity, polymorphisms that contribute to it, and to identify key polymorphisms associated with antigenic escape. We also suggest an approach to summarize the known global diversity of a given antigen to predict antigenic diversity, how to select variants that best represent the strains circulating in natural parasite populations and how to investigate the strain-specific efficacy of vaccine trials. Use of these strategies in the design and monitoring of vaccine trials will not only shed light on the contribution of genetic diversity to the antigenic diversity of malaria, but will also maximize the potential of future malaria vaccine candidates.

Keywords: *Plasmodium falciparum, Plasmodium vivax*, malaria, vaccine, strain, diversity, polymorphism, clinical trials

INTRODUCTION

Malaria is the most devastating parasitic disease afflicting humankind. The disease results from infection with protozoan parasites of the genus, *Plasmodium* and is transmitted by female anophelene mosquitoes. Of the 3.4 billion people in 108 countries at risk of malaria, 1.2 billion are at high risk of disease. In 2012, it was estimated that this disease caused 2000 deaths per day, the majority (77%) being children <5 years of age in sub-Saharan Africa infected with *Plasmodium falciparum*, the most virulent of the five known human malaria parasites (1, 2). In addition to this enormous heath toll, malaria exerts a heavy economic burden contributing to the cycle of poverty in many resource-limited settings (3). Although less lethal than *P. falciparum*, the majority of malaria infections occurring outside of sub-Saharan Africa are caused by *Plasmodium vivax*, with as many as 2.3 billion people at risk of infection (4). Several unique features of *P. vivax* biology, including its dormant stage in the human liver, make it more resistant to malaria elimination. As a result, *P. vivax* is predicted to present the ultimate obstacle to malaria elimination in endemic countries (5). Nevertheless, research into this parasite lags far behind that of *P. falciparum* due to its relatively recent recognition as a serious threat to global public health and lack of a viable long term *in vitro* culture system (4, 6).

Intensified malaria control efforts, supported by the Roll Back Malaria campaign, have resulted in a 42% decrease in malaria deaths worldwide in the last decade and many previously endemic countries have now shifted from controlling malaria to an elimination agenda (1). In 2007, encouraged by the stunning impact of this campaign, major funding bodies united to issue the ultimate challenge, to eradicate malaria globally by progressive malaria elimination from different countries and regions (3, 7). From past malaria eradication attempts, it is clear that in order for this ambitious goal to be achieved, malaria transmission must be permanently interrupted. Interventions that reduce the parasite reservoir, limit the rate at which infections are spread and the duration of time that a human or mosquito host is infectious are therefore urgently needed (8). In concert with other malaria control interventions, this could be achieved with the development of a broadly effective malaria vaccine.

Malaria parasites are ancient organisms with abundant genetic polymorphisms, much of which have evolved to escape host immune responses and thus presents a major obstacle to the

Box 1 | Glossary of terms.

Strain: a parasite variant that is genetically unique and induces specific immune responses against one or more of its antigens.

Isolate: a parasite specimen derived from an infected individual that has been either adapted for *in vitro* culture or used directly for experiments. Different isolates from the same population may contain parasites that are genetically identical at one or more loci. Individual isolates may also contain one or more genetically distinct parasites if they have been collected from an individual with multiple infections.

Clone: a genetically homogeneous parasite isolate.

Polymorphism: variation in the population at a particular nucleotide or amino acid residue.

Allele: one variant of a particular genetic locus. It can refer to individual polymorphic sites within a nucleotide or amino acid sequence, or the combination of all polymorphic sites in a gene or gene region (known as the haplotype).

Haplotype: a combination of alleles in a gene or gene region carried by a particular parasite clone.

Serotype: a haplotype from a gene or gene region that is antigenically unique and induces strain-specific responses.

Monovalent vaccine: a vaccine containing only one distinct antigen or one allele of the same antigen.

Multivalent vaccine: a vaccine containing two or more distinct antigens, or two or more alleles of the same antigen.

development of a vaccine that provides broad protection against all, or at least the majority of strains (9). As with other pathogens, the challenge in developing an effective malaria vaccine will be to differentiate between diversity that is associated with immune escape and cross protection, and that which has no bearing on the immune response, having simply accumulated over time through genetic drift or through adaptation to diverse host environments (9). To date, the polymorphisms in malaria antigens targeted by functionally important antibodies remain poorly characterized (10). Very little is known of how sequence polymorphisms relate to antigenic diversity or the potential for polymorphisms to mediate vaccine escape for *Plasmodium* spp. (11). The key to success with other pathogens has been the identification of immunologically relevant diversity. This has been achieved by performing population genetic and structural studies to identify functionally relevant polymorphisms, followed by molecular epidemiological surveys or *in vitro* functional studies prior to development and testing of vaccines (9). Narrowing the focus to immunologically relevant polymorphisms would greatly reduce the diversity that must be considered when developing multivalent malaria vaccines covering a broad range of strains (2, 9, 12) (**Box 1**).

MALARIA VACCINES: PAST, PRESENT, AND FUTURE

A long lasting, broadly efficacious malaria vaccine would be the most sustainable approach to control and eventually eradicate malaria. That a malaria vaccine may be feasible is strongly supported by the fact that people living in malaria endemic areas develop protective immunity against malaria symptoms during childhood (13). By adulthood, decreases in the prevalence of infection and density of parasitemia are achieved indicating that this immunity eventually provides some protection against infection (14, 15). Passive transfer of immunoglobulin from hyper-immune African adults to non-immune children with severe malaria was shown to have curative properties, demonstrating that antibody responses are largely responsible for protection against clinical disease (16). Furthermore, vaccination of rodent and primate models with recombinant parasite antigens elicits high antibody titers that are associated with protection against subsequent malaria challenge (17–20). Although the development of malaria vaccines has been an active focus of the malaria research community over the last 50 years, a vaccine remains a missing component of malaria control and elimination strategies (21).

Despite numerous promising malaria vaccine candidates and several partially successful malaria vaccine trials (22–25), short-lived protection, limited funding, and a lack of key technologies has hampered further testing and the scale-up of clinical trials of novel malaria vaccine candidates. The first successful malaria vaccine trial, based on a "whole parasite" approach, was conducted in humans in the 1950s. Vaccination with irradiated sporozoites was shown to protect against both homologous and heterologous challenge in humans (26, 27). However, the need for large-scale production prevented further development of this approach. With the advent of molecular technologies in the 1980s, the focus shifted to so-called "subunit" approaches, including highly immunogenic parasite antigens as targets such as the circumsporozoite protein (CSP), which was identified as the parasite antigenic determinant targeted by immune responses induced by the sporozoite vaccine (28). Following these early studies, and on the back of highly promising pre-clinical studies, many small-scale subunit vaccine trials were conducted in humans. Efficacy was highly variable with many candidates demonstrating no protective effect, however, there were some promising candidates identified that continue to be further developed today (21), and these are discussed in more detail below.

In the last decade, there has been an attempt to assist and accelerate development of a malaria vaccine with the establishment of the PATH Malaria Vaccine Initiative (MVI), which has greatly progressed the evaluation and identification of promising malaria vaccine candidates. To further focus and unite the global vaccine effort, in 2006 the World Health Organization (WHO) launched the first malaria vaccine technology roadmap with the landmark goal:

> By 2015, develop and license a first generation malaria vaccine that has a protective efficacy of more than 50% against severe disease and death and lasts more than one year (29).

Currently, a single vaccine candidate is on track to meet this goal. Known as "RTS,S," this vaccine is based on the repeat region and T-cell epitopes of CSP. RTS,S is the subject of a large multi-centre Phase 3 trial involving more than 15,000 children over 11 sites in sub-Saharan Africa. The full results of the trial have not yet been published; however, after three doses, clinical malaria cases during the first 18 months of follow-up decreased by an estimated 46% (severe disease by 36%) in children 5–17 months of age at first vaccination, and 27% (severe disease by 15%) in infants aged 6–12 weeks of age at first vaccination (30), therefore, RTS,S efficacy is approaching the above-mentioned WHO criteria for a first generation malaria vaccine. As a result, this vaccine is likely to be licensed in 2015 for use in young African children and could lead to significant decreases in malaria morbidity and mortality in this high-risk population. Despite this positive outlook, it is cautioned that this vaccine is only partially protective against disease and wanes over time. New, second-generation vaccines will need to have major improvements in efficacy to meet the challenges ahead (10).

Recognizing the changing epidemiology of malaria in the context of a shrinking global malaria map and a shift in populations most at risk of infection (5), as well as the need for vaccines with higher efficacies than RTS,S if malaria elimination is to be achieved, the goals of the Malaria Vaccine Technology Roadmap were recently reset with two major objectives:

> By 2030, license vaccines targeting *P. falciparum* and *P. vivax* that encompass: (i) development of second-generation malaria vaccines that provide a protective efficacy of more than 75% against clinical (mild and severe) malaria . . . and (ii) development of malaria vaccines that reduce transmission of the parasite and thereby reduce the incidence of human infection . . . (31).

To achieve these new goals, a better understanding of the minimal requirements and mechanisms underlying development of immunity against *P. falciparum* and *P. vivax* (10, 12) as well as knowledge regarding the factors that influence transmission of both species, will be essential. As the development of vaccines against *P. vivax* is also a major goal, it will be important to consider the distinct features of this species, which underscores the need to intensify research efforts into this relatively neglected parasite.

APPROACHES TO MALARIA VACCINE DEVELOPMENT

Malaria parasites are complex eukaryotes comprised of many antigenic targets. It has been suggested that vaccines may need to be as complex as the parasite itself (32) and therefore there has been considerable interest in the whole parasite approach. As mentioned above, irradiated live sporozoite vaccinations have shown great success in clinical trials (27, 33, 34). Currently, different methods are being used to attenuate sporozoite stages including chemical and genetic modification, and these have been reviewed elsewhere (35). However, there are some challenges to overcome in addition to the technical difficulties and high costs associated with scaling up production including the dose required to induce long-lasting protective immunity, transport, and storage in the absence of a reliable cold chain for distribution to at risk populations (36). There is also a risk of reversion to virulence (12). Hence the alternative subunit vaccine approach continues to be vigorously evaluated (37). As indicated above, this involves individual recombinant parasite proteins administered as monovalent preparations or combinations of multiple proteins together with different vectors and adjuvants that enhance the immune response. As the majority of clinical trials conducted to date have been based on subunit vaccines, this rest of this review will focus on the development of this class of malaria vaccines and the challenges associated with this approach.

Several highly abundant parasite proteins were identified as targets of natural immunity many years ago but in recent years the list of possible candidates has expanded. These candidates have been extensively validated in pre-clinical studies using different approaches including the measurement of inhibitory antibody responses in *in vitro* growth and invasion assays (short term culture only for *P. vivax*) (18, 38–41) and by vaccinating animal models followed by challenge infections (17, 18, 42–45). Subunit vaccines have been or are being developed based on these advanced candidate antigens, which are expressed in almost every stage of the parasite lifecycle. They have been classified into one of three different groups based on the target lifecycle stage (**Figure 1**) (46):

(i) *pre-erythrocytic vaccines*: these vaccines aim to prevent infection by targeting the infective stage, known as the sporozoite (e.g., RTS,S). Alternatively, pre-erythrocytic vaccines can target antigens expressed by liver stage parasites to prevent the emergence of merozoites into the bloodstream, the stage of infection responsible for the clinical symptoms of malaria infection (**Figure 1**). The risk associated with targeting sporozoite antigens is that the antigen dose inoculated during a natural infection is very low, with only a small number of sporozoites injected by the vector (~20), and this may not be sufficient to elicit an effective host immune response. Additionally, only one sporozoite needs to escape the vaccine-mediated immune response and invade liver cells for ~10,000 infectious merozoites to be produced, resulting in blood stage infection and clinical disease (47).

(ii) *blood stage vaccines*: the vast majority of malaria vaccine candidates are designed to protect against the blood stage of infection (**Figure 1**). Since all of the symptoms of malaria occur during this stage, the majority of vaccines targeting antigens expressed during the blood stage are designed primarily to prevent disease. One approach is to target merozoite antigens to prevent red blood cell invasion and reduce the density and prevalence of parasites in the infected host (**Figure 1**). In principle, this reduction in parasite density may also reduce the density of transmission forms, known as gametocytes (i.e., the sexual stage of the parasite transmitted from human to mosquito host) (**Figure 1**). In addition to preventing clinical disease, an effective blood stage vaccine that reduces parasite density may therefore also contribute to reducing malaria transmission (10, 46, 48). Approaches are also being developed to target the major surface protein expressed on the *P. falciparum* infected red blood cell known as erythrocyte membrane protein 1 (PfEMP1). PfEMP1 mediates adhesion to host cells, a mechanism that is associated with severe malaria [reviewed by Hviid (49)].

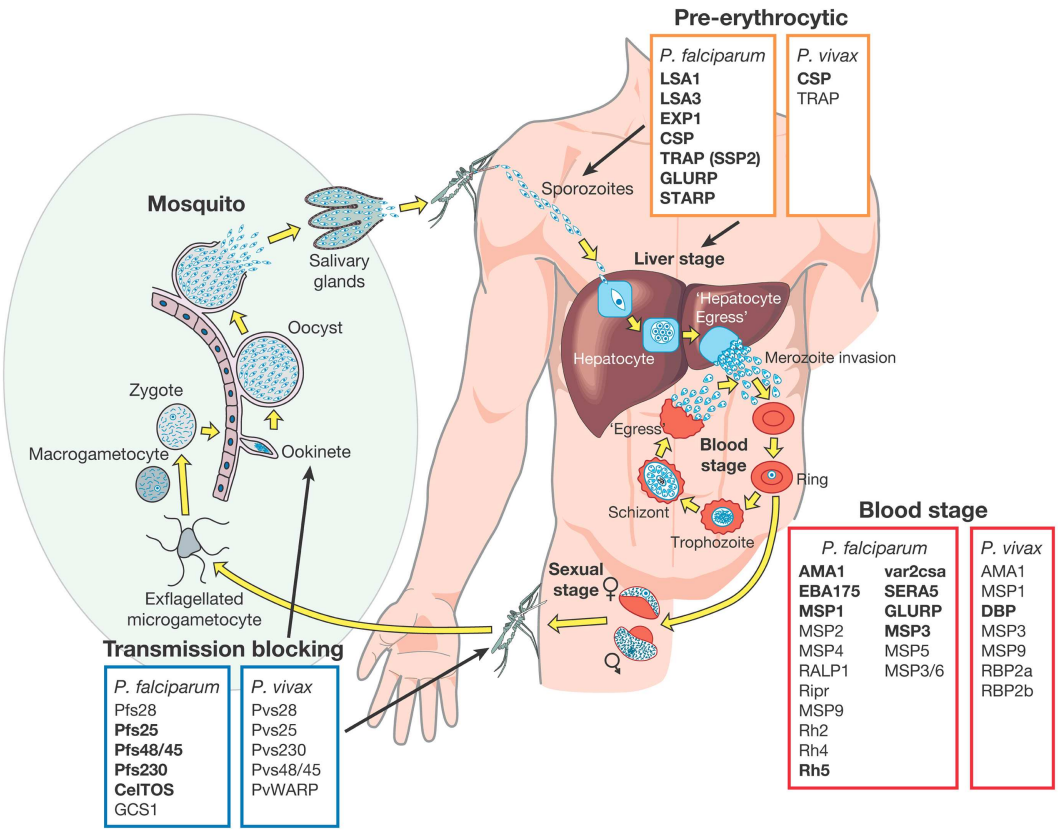

FIGURE 1 | Malaria vaccine candidate antigens are shown. All candidate antigens for *Plasmodium falciparum* and *Plasmodium vivax* are superimposed on the *Plasmodium* lifecycle, to indicate the category of malaria vaccine being developed and the lifecycle stage targeted. Antigens indicated in bold are those that are currently being evaluated in pre-clinical trials or have entered at least Phase 1 clinical trials according to the WHO malaria vaccine rainbow tables (50). The *P. vivax* latent stages known as "hyponozoites" are not shown but these occur in the liver stage.

(iii) *transmission-blocking vaccines*: the aim of transmission-blocking vaccines is to target antigens expressed during lifecycle stages in the mosquito host (e.g., gametocyte or oocyst antigens) (**Figure 1**). Although these vaccines would not directly prevent infection or clinical disease, they would greatly assist elimination efforts to prevent the onward transmission of infections that may be imported into an elimination zone (10, 46).

Development of a vaccine against *P. falciparum* is well advanced with 31 promising antigens identified (**Figure 1**). Currently, 27 subunit candidates comprising different domains and alleles for 22 different antigens are being tested in pre-clinical or clinical trials (50) (**Figure 1**; **Table 1**). However, the majority of candidates tested in clinical trials so far have been based on different formulations and regions of a handful of antigens identified many years ago, including CSP (51), the liver stage antigen 1 (LSA1) (52), thrombospondin-related antigen (TRAP, also known as sporozoite surface protein 2, SSP2) (53–55), merozoite surface protein 1 (MSP1) (56), MSP2 (57), MSP3 (58), and apical membrane antigen 1 (AMA1) (42, 59). As some of these antigens have shown promising pre-clinical profiles yet limited efficacy in human trials, they have been tested in many different formulations over the years (21, 60). For example, the most advanced blood stage antigens, AMA1 and MSP1, naturally induce protective immune responses (61–63), demonstrated using *in vitro* inhibition assays (64) and by vaccination of animal models (17, 18, 20, 65). However in humans, only limited clinical efficacy has been observed (66, 67). Similarly, CSP, the major component of the RTS,S vaccine, is the major surface antigen on the sporozoite surface, yet provides only partial and short-lived protection against the blood stage symptoms of malaria following vaccination of human volunteers. It does not protect against infection *per se* as would be expected by a pre-erythrocytic vaccine and therefore the precise mechanism of protection is not well understood (23, 68). These "historical" vaccine candidates are much further down the development pipeline (21) than more recently identified candidates such as the invasion ligands, the 175 kDa erythrocyte binding antigen (EBA175) (69), reticulocyte binding homolog 5 (RH5) (70, 71), and *P. vivax* Duffy binding protein (DBP) (72). In recent years, the *var2csa* variant of PfEMP1, which is the major parasite ligand involved in placental adhesion during pregnancy malaria (73); and the gametocyte antigens, Pfs25 and Pfs48/45 (74) representing promising transmission-blocking targets, have also come to the forefront. The elucidation of the complete parasite genome (75) has further advanced vaccine development by enabling identification

Table 1 | Diversity of malaria vaccine candidate antigens currently in clinical trials based on the WHO rainbow tables (50).

Antigen	Lifecycle stage	Domain analyzed	Number of continents surveyed	Number of countries surveyed	Number of isolates sampled (range)	Number of haplotypes identified (range)	Reference
PLASMODIUM FALCIPARUM							
CSP	Sporozoite	C-terminal	3	13	604 (9–143)	71 (3–20)	(2)
			1	1	157	n.r. (13–34)	(83)
			1	1	100	57	(84)
			3	17	1339 (9–336)	117 (1–40)	(85)
		Full length	2	7	485	n.r.	(86)
STARP	Sporozoite	Full length	1	1*	134 (10–24)	24	(87)
TRAP	Sporozoite	N-terminal	2	3	100 (8–48)	84 (8–37)	(2)
LSA1	Liver stage	N-terminal	3	4	74 (10–22)	13 (3–7)	(2)
GLURP	Sporozoite/gametocyte	Region 0	3	3	48 (9–11)	22 (2–9)	(2)
		Region 0 and 2	1	1	77 (R0); 79 (R2)	n.r.	(88)
AMA1	Merozoite	Domain I	3	11	572 (8–162)	181 (6–68)	(2)
			1	1	193 (9–100)	139 (6–58)	(89)
		Full length	2	7	459	n.r.	(86)
			1	1	21	11	(90)
			1	1	129	78	(91)
			1	1	315	168	(92)
EBA175	Merozoite	Region II	2	3	135 (30–48)	51 (15–23)	(2)
MSP1	Merozoite	$MSP1_{19}$	3	11	2237 (18–1368)	20 (1–15)	(2)
			1	1	136	12	(83)
			1	1	61 (9–15)	5	(93)
			1	1	300	19	(94)
		Block 2	1	1	35	23	(95)
			1	1	36	13	(96)
			1	1	128	14	(97)
		Full length	2	7	404	n.r.	(86)
MSP2	Merozoite	Blocks 2 and 3	2	3	392 (n.d)	275 (n.r.)	(2)
		Block 3	1	1	148	22	(97)
MSP3	Merozoite	Repeat region	2	2	124 (75–86)	21 (9–12)	(2)
MSP4	Merozoite	Full length	2	4	142 (12–42)	47 (9–23)	(2)
MSP3/6	Merozoite		1	2	117 (51–66)	n.r.	(81, 82)
Rh2	Merozoite	Binding region	1	1	33 (15)	n.r. (13)	(98)
Rh4	Merozoite	Binding region	1	1	23 (12)	9 (4)	(99)
RH5	Merozoite	Full length	3	6	227 (21–125)	n.r.	(100)
Pfs48/45	Gametocyte	Full length	3	4	55 (9–15)	19 (2–8)	(2)
Pfs28	Ookinete				No population data available		
Pfs25	Ookinete	Full length	2	2	41	n.r.	(101)
var2csa	Trophozoite	DBL3	2	3	124 (15–54)	n.r.	(102)
		DBL3X	1	1	108	79	(103)
		DBL5	1	2	70	n.r.	(104)
var	Trophozoite	DBLalpha	3	4	29–42 (32)	140–666 (449)**	(105)
SERA5	Trophozoite/schizont	Exon II–IV	4	9	445 (39–80)	133 (3–44)	(106)
PLASMODIUM VIVAX							
CSP	Pre-eryth	Central repeat (CR)	2	2	168 (31–137)	n.r. (13–25)	(107, 108)
			3	9	194	76 (5–23)	(109)
			1	1	84	23	(95)
DBP	Merozoite	Region II	2	8	675 (11–123)	n.r. (9–73)	(110)
			3	9	707 (11–200)	150 (8–59)	(111)
			1	1	63	16	(112)
			1	1	70	13	(113)
			1	1	22	8	(114)
			1	1	54	12	(115)
			3	7	402 (9–122)	138 (7–56)	(116)

**Also included a small number of strains from Brazil, Indonesia, Tanzania and Kenya.*

***Predicted total number of var alleles is much higher: 232–7565 (3564).*

of scores of novel antigens. Subsequent antigenic credentialing through functional studies (76), proteomic and immunological screening (63, 77–80), and population genetic analyses (81, 82), has resulted in prioritization of several of these promising candidates for further development.

Research on potential *P. vivax* vaccine candidates lags far behind that of *P. falciparum*. Currently, only two *P. vivax* vaccine candidates (based on PvCSP and PvDBP) have shown promise in pre-clinical studies and only one candidate has reached Phase 1a clinical trials (50) (**Figure 1**). If the new goals of the Malaria Vaccine Technology Roadmap are to be realized, significantly more resources need to be invested in identifying and validating promising *P. vivax* vaccine candidates for progression to clinical trials.

PARASITE DIVERSITY: A MAJOR OBSTACLE TO MALARIA VACCINE DEVELOPMENT

One often quoted explanation for the variable efficacy of subunit malaria vaccine candidates is parasite genetic diversity (2, 9, 12, 37) and the strain-specific nature of immunity to diverse parasite antigens (15, 117, 118). Theoretically, a malaria "strain" can be defined as a parasite variant that is genetically distinct and induces specific immune responses against one or more antigens (**Box 1**). However, exactly what defines a *Plasmodium* strain is not fully understood (119) because this definition becomes exceedingly complex when the whole parasite is considered. In the context of subunit vaccines, the term "strain" refers to the parasite isolate from which the vaccine antigen is derived, while the actual genetic variant of that antigen is known as the "allele" or "haplotype" (**Box 1**). The inclusion of only one allele in a subunit vaccine formulation elicits responses only against similar, cross-reactive alleles (the "serotype," **Box 1**) and runs the risk of selection for non-vaccine strains in the vaccinated host population, as discussed in detail below (22). Indeed, natural parasite populations have large numbers of alleles or haplotypes for single copy antigens, such as AMA1 and MSP1 (2, 11) (**Table 1**). However for PfEMP1, which is encoded by as many as 60 different genes per parasite genome, there are hundreds to thousands of distinct alleles even within local geographic areas $<10\,km^2$ (105). Extensive parasite antigenic diversity explains the slow development of naturally acquired immunity (120) with repeated exposure over several years necessary to build up a large repertoire of antibodies to the different serotypes circulating in an endemic area (14, 121). Given the high diversity of the available vaccine candidates (**Table 1**), a broadly effective malaria vaccine may need to be multivalent, comprising multiple alleles (or haplotypes) for a given polymorphic antigen (12), much like the vaccine approaches used to successfully combat other highly polymorphic pathogens such as influenza A and human papillomavirus.

On the other hand, some *Plasmodium* antigens are relatively conserved, such as RH5 (100), or have highly conserved functional regions that vaccine-developers may be able to exploit such as the AMA1 receptor-binding pocket (122, 123). Furthermore, antibodies against major surface antigens cross-react with different parasite strains including those from different geographic areas suggesting that conserved epitopes exist (65, 124). It has therefore been proposed that using a panel of peptides containing conserved epitopes would be one approach to induce immune responses that avoid dominant polymorphic epitopes (125).

An important priority in malaria vaccine development is therefore to not only confirm the diversity circulating in the target parasite population but also to understand the contribution of *genetic* (allelic/haplotypic) diversity to the *antigenic* (serotype) diversity that is relevant to malaria vaccine design for each candidate antigen (48). Whilst there are indeed multiple diverse alleles of many candidate antigens circulating within distinct populations, not all polymorphisms, will mediate antigenic escape, hence these must be identified and targeted for vaccine design. However, the relationship between allele and serotype has been dissected for only one candidate, AMA1 (126). More rigorous investigation of available candidates as well as the identification of novel relatively conserved antigenic targets is therefore absolutely required to develop a framework for selection and to prioritize antigens for further development as vaccine candidates.

DIVERSITY-COVERING VACCINE APPROACHES

Although well established, the extreme diversity of leading candidate antigens has rarely been considered when developing and testing candidate malaria vaccines [reviewed by Barry et al. (2)]. The majority of subunit vaccine candidates tested in clinical trials have been monovalent. Moreover, all vaccine candidates have been based on alleles from a handful of parasite isolates such as *3D7*, *FC27*, *FUP*, and *FVO* for *P. falciparum*, and *Sal1* for *P. vivax*, that have been propagated for decades *in vitro* (or in primate models for *P. vivax*), and poorly reflect the parasite strains circulating in natural populations (2, 110, 127–129). As a result, many malaria vaccine candidates do not adequately cover the diversity observed in natural parasite populations and this could explain the poor clinical efficacy observed in vaccine trials where efficacy endpoints include infection with any strain (21, 66, 127). A multivalent malaria vaccine comprised of multiple serotypes may perform better as it would be designed to protect against a wide range of parasite strains. However, for almost all malaria vaccine candidates, the polymorphisms that define the serotypes and the number of alleles that should be incorporated into a malaria vaccine to cover serotype diversity remain unknown.

Supporting the argument for a multivalent vaccine strategy, vaccine candidates based on a single allele for specific antigens have demonstrated more strongly protective responses when strain-specific endpoints (i.e., infection with a strain carrying the vaccine allele) have been measured as compared to standard endpoints (i.e., infection with any strain). One of the most successful vaccine trials conducted to date was that of the "Combination B" vaccine, conducted in 120 Papua New Guinean children. This vaccine contained only the *3D7* allele of MSP1, MSP2, and the ring-associated erythrocyte antigen (RESA), however, it resulted in a 62% reduction in parasite density in vaccinated children compared to those that received the placebo (22). MSP2 contains a central complex tandem repeat region and many different alleles that vary in size, however all alleles fall into two major families that form different serotypes (*3D7* and *FC27*) (130). Interestingly, at the time of vaccination, the prevalence of the *3D7*-type alleles was between 23 and 50% within each of the treatment groups, which could explain the high overall efficacy. Furthermore, vaccinees were less

frequently infected and had a lower rate of clinical episodes associated with *3D7*-type parasites compared to the control group (22). Similarly, volunteers from Mali vaccinated with FMP2.1, which is based on the *3D7* allele of AMA1, had a much higher risk of non-*3D7* infections (64%) (131) than any infection (20%) based on residues in the AMA1 cluster 1 loop (c1L) (66). The results of these trials highlight the danger of vaccine-induced selection pressure and its consequences for morbidity, and strongly argue for developing vaccines covering major serotypes circulating in natural parasite populations (9, 22, 127). The frequency of vaccine or vaccine-serotypes in the target parasite population is also likely to be important for significant vaccine efficacy. The bivalent candidate, AMA1-C1 (containing *3D7* and *FVO* haplotypes) has demonstrated a lack of protective efficacy against either of the two vaccine alleles in a Phase 2b trial (92). However, this lack of observed efficacy could be explained by a low frequency of these alleles in the target parasite population and the small sample size, with only 44 sequences analyzed for both the vaccine and control groups combined. Even when the analysis of polymorphisms was narrowed to the c1L cluster of polymorphisms, which have been implicated in antigenic escape as the basis of AMA1 serotypes (126), baseline vaccine-allele frequencies were <10% indicating that much larger sample sizes would be required to observe any shift in frequency after vaccination. No other multivalent vaccine trial results are currently available, but several trials are ongoing and the malaria vaccine community awaits the final results with interest.

Multivalent combination vaccines tested in animal models have shown promising and surprising results. Vaccination with combinations of four highly diverse AMA1 alleles was shown to overcome diversity by producing a broader inhibitory response compared to single allele vaccination, thought to have occurred partly by redirecting responses to conserved epitopes (65, 132). This phenomenon, which is analogous to "original antigenic sin," occurs because abundant, strain-specific AMA1 epitopes vary and are potentially replaced with each new infection, whereas the conserved regions remain constant. Hence, high levels of exposure to conserved epitopes with vaccination or repeated exposure during natural infections may enhance the antibody response against these regions.

Another approach to covering antigenic diversity has been to assemble all available global sequence data for an antigen target and to design a small number of synthetic protein constructs that together cover most of the diversity observed. Phase 1a and 1b trials have begun after promising pre-clinical results for a multivalent vaccine candidate (DiCo), consisting of fusion protein chimeras comprising three synthetic AMA1 molecules covering 97% of the amino acid variability, and these have been shown to elicit stronger antibody responses as a combination than alone (133). This approach has been further evaluated in pre-clinical studies together with a construct containing two allelic variants of the C-terminal 19-kDa region of merozoite surface protein 1 ($MSP1_{19}$) fused to the DiCo construct, and again enhanced antibody responses were induced (134). Clinical trial results are not yet available for the DiCo and $MSP1_{19}$-DiCo combination vaccines but it will be interesting to see whether the diversity-covering approach is more efficacious than the single allele approach.

PREDICTING SEROTYPES THROUGH POPULATION GENETIC ANALYSES

Population genetic studies are needed to guide vaccine design, by defining the diversity of candidate antigens, to predict polymorphisms that contribute to antigenic diversity (122, 127, 135) and to investigate the geospatial distribution of predicted serotypes (136, 137). Moreover, as a continuous *in vitro* culture system is yet to be developed for *P. vivax*, epidemiological studies currently represent an important tool with which to investigate the significance of polymorphism within vaccine candidate antigens (125). This approach has been used to identify correlations between specific polymorphic sites in two leading *P. falciparum* vaccine candidates, MSP1 and AMA1, with clinical infection (127, 138).

The extensive genetic diversity of malaria vaccine candidate antigens has been demonstrated by many studies investigating genetic polymorphism in samples ranging from small numbers of geographically disparate culture-adapted isolates to large numbers of natural parasite isolates from the same local geographic area or country. These results demonstrate the high numbers of haplotypes found in natural parasite populations for many antigens (**Table 1**). However, there are fewer haplotypes and vaccine alleles are far more common when individual amino acid polymorphisms or limited "haplotypes" (**Box 1**) comprising different combinations of amino acid alleles that might form critical epitopes are considered. For example, AMA1, which has 214 amino acid haplotypes in 1 African population, has only 25 serotypes based on the c1L cluster (127), demonstrating that if the haplotype can be refined to represent only antigenic escape polymorphisms, the number of alleles required in a potential multivalent vaccine could be reduced substantially. More recent studies have suggested that the majority of the antigenic escape diversity in AMA1 may even be explained by polymorphism in just one residue (130). Importantly, in vaccine trials, a lack of knowledge of the polymorphisms that mediate antigenic escape would result in an underestimate of strain-specific vaccine efficacy. Population genetic studies are therefore critical to gain more insight into antigenic diversity and to achieve the goal of a broadly efficacious malaria vaccine.

Another important point to remember is that the global *P. falciparum* and *P. vivax* populations are structured into geographically distinct subpopulations, therefore, local population-level analyses are required to fully understand diversity that would be relevant to the efficacy of a malaria vaccine in a defined endemic area (139, 140). Given the large number of parasite populations that are likely to exist worldwide it may not be feasible to design vaccines for every target population and therefore a universal approach to cover diversity is needed. Below, we provide a step-by-step guide of how to identify and characterize diversity within candidate antigens that is relevant to malaria vaccine design, and this is further summarized in **Figure 2**.

DATA COLLECTION

In order to understand the antigenic diversity impacting on vaccine efficacy and to identify potential serotypes, the target gene or gene region encoding the candidate antigen must be amplified, sequenced, and population genetic analyses completed including the determination of regions under balancing (immune) selection.

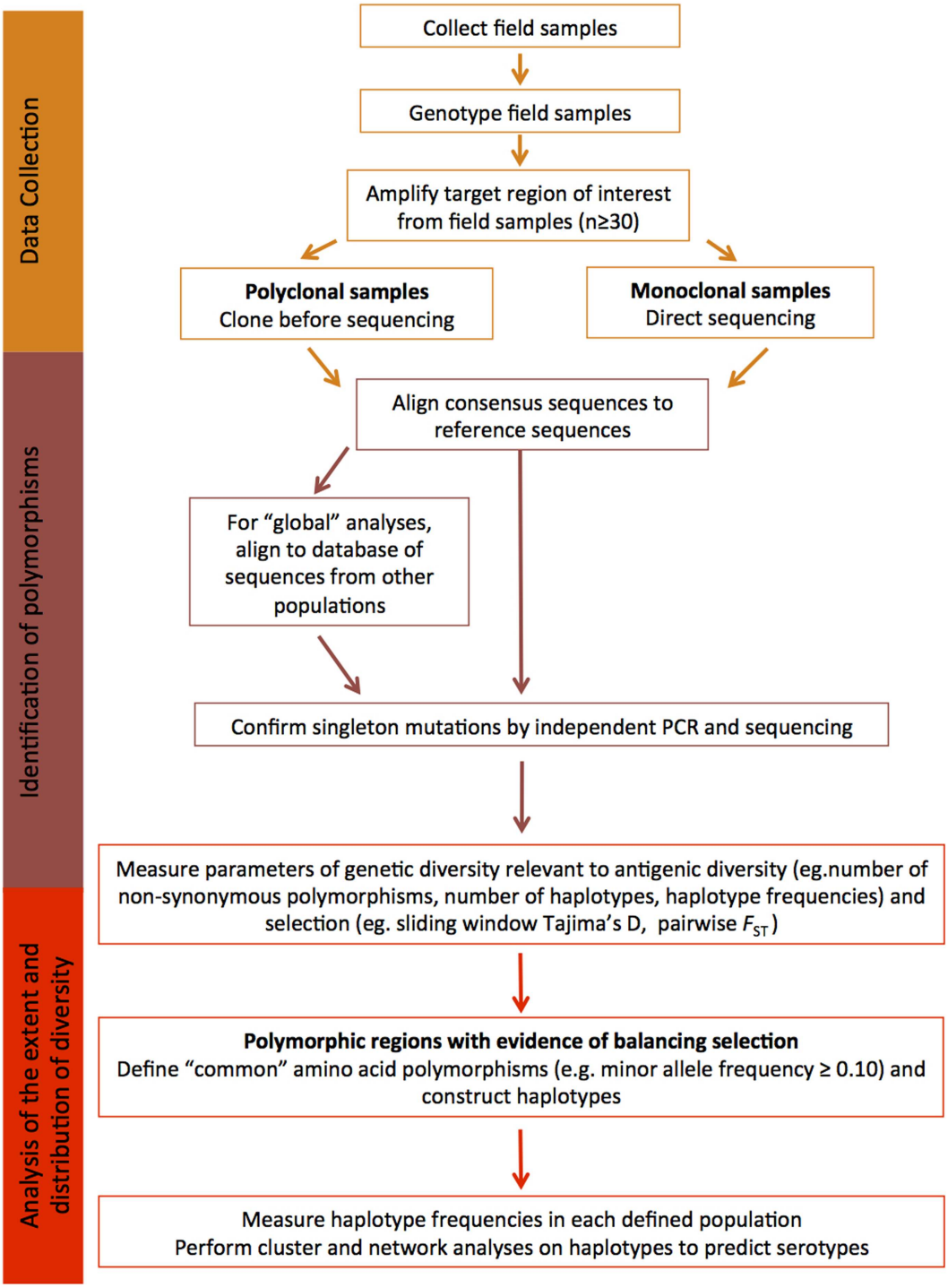

FIGURE 2 | Understanding diversity and predicting serotypes using population genetic analyses. A flow chart describing the step-by-step methodology to define the extent and distribution of parasite diversity and to predict antigenic escape polymorphisms and serotypes (see text for more details).

To accurately estimate natural allele frequencies, it is critical to collect sequence data from samples representing the natural parasite population of a defined geographic area. Use of samples collected in the same geographic area ensures that the diversity of the target sequence is accurately estimated for that region. As the most informative analyses of balancing selection are based on allele frequencies, it is also important that sequence data are obtained from a substantial dataset, at least 30–50 samples (81, 82).

Analysis of too few sequences can result in incorrect estimates of diversity, as alleles might be under-represented in very small population samples, and this can result in skewed allele frequencies and diversity estimates.

Prior to PCR amplification, it is essential that parasite isolates are genotyped using highly polymorphic microsatellite markers to determine the number of distinct parasite clones present in the sample (141, 142). Even samples collected from areas of low malaria transmission can contain multiple clones, especially for *P. vivax* with its frequent relapses (143). If only one clone is present, sequencing can be performed directly from amplicons. If the number of field samples containing a single clone is insufficient, or polyclonal infections are common, PCR products amplified from polyclonal samples can be sub-cloned prior to sequencing. However, sub-cloning often results in artifacts (144), therefore, any novel polymorphisms should be resolved by cloning and sequencing-independent PCR products. Another approach is to perform NextGen sequencing on samples containing multiple clones, once a prohibitively expensive approach that is becoming increasingly affordable. Briefly, amplicons are sheared and adaptors added, generating a library for each sample. Libraries can be sequenced separately or as pools with the addition of sample barcodes. Reads are subsequently mapped to the relevant reference genome. The advantage of this approach is that reads are quantitative and relate to clone abundance in the sample, therefore, haplotypes of the predominant clone(s) can be computationally reconstructed (145).

IDENTIFICATION OF POLYMORPHISM

From the raw sequence data, consensus sequences can be obtained and compared to the reference sequence for the defined candidate. Polymorphisms identified in consensus sequences will form the basis of all downstream population genetic analyses. Quality control of sequences is therefore essential, to ensure that polymorphic sites are accurately called and that any ambiguous sites or artifacts are identified. If a clear result cannot be obtained, the sample should be removed from the analysis.

At this point, all high quality consensus sequences from a given population can be compared and basic diversity parameters determined as outlined below. In order to place the dataset in context with the known diversity, published sequences of the target region collected from other natural populations in distinct geographical areas can also be added to the analysis (e.g., from GenBank). Multiple alignments of sequence data are straightforward if sequences are non-repetitive. However, if repeat length polymorphisms, which are common in parasite antigens, or insertions and deletion (indels) are found, then manual realignment needs to be done to ensure that gaps are correctly aligned, which can lead to an overestimate of the number of single nucleotide polymorphisms (SNPs). While repeat regions can be included in population genetic analyses, the expansion and contraction of repeat arrays does not impact on antigenic diversity as dramatically as amino acid changes (22, 131). However, by defining different alleles based on the number of repeats or in the case of indels, the presence or absence of a particular string of nucleotides, it is possible to predict whether such polymorphisms are modulated by immune selection.

Balancing selection, which is a result of immune selection pressure, favors the maintenance of diversity, with alleles at low to medium frequencies within populations, and balanced frequencies between populations (137). Polymorphic sites or regions that show such patterns are predicted to be under the influence of immune selection, and thus contribute to antigenic diversity. For some antigens under strong immune selection, similar alleles or clusters of alleles have been maintained across broad geographic areas (2, 110, 127, 129, 136, 146). The maintenance of moderate frequencies of allele clusters (and even individual alleles) across large geographic distances indicates that they represent distinct serotypes (2, 146).

In contrast, singleton polymorphisms and polymorphic sites with very low minor allele frequencies (MAFs) are indicative of deleterious mutations under purifying selection, or recent polymorphisms yet to increase in frequency. These polymorphisms will only be represented in a very small proportion of the parasite population. As the aim of diversity-covering vaccines is to encompass as many of the alleles (haplotypes) found as possible, several groups have chosen to exclude rare polymorphisms from population genetic analyses of vaccine antigens (110, 129, 145). Another cautionary note is that a large number of singleton polymorphisms in population datasets from the public databases may also indicate PCR artifacts, especially if the methodology includes a cloning step. If the validity of such data cannot be confirmed then it should be discarded from the comparative analyses.

ANALYSIS OF THE EXTENT AND DISTRIBUTION OF DIVERSITY

In order to determine the diversity of the candidate antigen, both overall and within parasite populations from different geographic areas (e.g., village, district or country), genetic diversity should be estimated for all sequences combined and for each population. Genetic differentiation (e.g., Wrights fixation index, F_{ST}) can be measured to determine whether allele frequencies vary and if not, populations from the same region or country can be considered as one population (2). Important diversity parameters to define for each population include the number and type of polymorphisms (SNP, indel, repeat length variation), the number and relative proportions of neutral (synonymous) and amino acid (non-synonymous) polymorphisms, and the number of haplotypes resulting from different combinations of amino acid polymorphisms. All of these statistics can be calculated using freely available population genetic analysis software and an excellent overview of these programs has been published (147). Conversion of polymorphisms to amino acid residues before haplotype definition ensures that complex codons and rare nucleotide polymorphisms within codons containing other more common polymorphisms are included. In any case, it is more appropriate to describe the amino acid haplotypes to predict serotypes, rather than the nucleotide haplotypes. Other more complex statistics such as the nucleotide and haplotype diversity can also be measured as an indication of the number and frequency of polymorphisms and haplotypes in the population, respectively; however, these are of more interest to the population geneticist than the vaccine developer.

To determine whether polymorphic sites are under the influence of balancing (immune) selection, statistical tests such as

Tajima's *D* can be performed using a sliding window approach, to identify specific domains or clusters of polymorphisms targeted by host immune responses (148). In addition, Wright's F_{ST}, has been applied to investigate whether allele frequencies at specific polymorphic sites, or gene domains, are balanced between populations (137). Polymorphisms under balancing selection and associated with immune escape will be non-synonymous, located in regions with a positive value of Tajima's *D* and low inter-population F_{ST} values. However, sampling is extremely important as both the Tajima's *D* and F_{ST} tests are based on allele frequencies. Populations must be of sufficient size so that the sampling error of the estimate is low, and panmictic (randomly mating) from the same geographic region, because any underlying geographic population structure may influence antigen allele frequencies (129, 136, 139, 140). Polymorphisms under balancing selection are also likely to have moderate MAFs, because the maintenance of diversity in the parasite population is advantageous for survival (137). The physical location of all polymorphisms identified as immune targets should then be mapped to the protein structure where possible, to help determine the potential functional relevance and implications of mutation at the site(s) identified, as has been successfully done for AMA1 [e.g., Ref. (126, 127)].

Determining the extent and distribution of immunologically relevant diversity for potential malaria vaccine candidates will help to determine the feasibility of whether a regionally or globally effective vaccine can be developed for the particular target. It is therefore important to focus the analysis to only those polymorphisms with the greatest likelihood of creating antigenic diversity to allow immune escape. Once polymorphisms predicted to be under balancing selection have been identified (see above and **Figure 2**), the distribution of unique haplotypes representing the predicted serotypes can be analyzed by determining haplotype frequencies in different geographic areas.

Different haplotypes will be related to differing degrees. Using network and clustering analyses, relationships between haplotypes from different populations, clusters of closely related haplotypes, and the extent and distribution of predicted serotypes can be investigated. By identifying the relationships among all of the different haplotypes in this way, the most distantly related alleles can be selected for further analysis or for inclusion in a vaccine to cover diversity. Previous studies have demonstrated the utility of these approaches to identify distinct clusters of alleles as a starting point to predict serotypes (11, 146, 149). These analyses identify the most distinct and common haplotypes and provide a basis upon which to select haplotypes that cover a large proportion of the population-wide diversity for a specific candidate antigen. The outcome of such analyses can help to determine: (i) whether it may be possible to cover all known diversity of the target sequence, (ii) the number of different haplotypes that would need to be included in a vaccine in order to cover diversity and (iii) the predicted efficacy of vaccine candidates. Inclusion of vaccine alleles in the analysis provides a reference point to estimate vaccine allele or serotype frequencies. The results of these analyses provide a simple diversity framework upon which to determine the parameters of allele specific and cross-reactive responses in pre-clinical studies (65, 127, 146) and to measure strain-specific efficacy in clinical trials (131). It is important to note that unlike other approaches that have clearly identified antigenic escape polymorphisms (126, 131), population genetic analyses provide a prediction, are simpler and less expensive, and can reduce the number of polymorphisms (and thus haplotypes) that need to be assessed to confirm their contribution to the serotype.

DEFINING ANTIGENIC DIVERSITY BY MONITORING THE DYNAMICS OF DIVERSITY IN NATURAL PARASITE POPULATIONS

Children living in malaria endemic areas are infected many times and have several episodes of clinical malaria before building up a large repertoire of antibodies that recognize a large number of strains (14, 15). Meanwhile, the rate of turnover of infections increases with age and is thought to be associated with the acquisition of antibodies to an increasing breadth of strains (150). Therefore, strains that children are yet to be exposed to pose a greater risk of clinical illness. Defining the dynamics of clinical infection in the context of parasite antigenic diversity may therefore provide insights into the specific genetic determinants required for antigenic escape, since the risk of a clinical episode due to infection with parasites representing a specific serotype would decrease after being exposed to that serotype (**Figure 3A**) (127, 138). Furthermore, analyses of strain-specific antibodies in conjunction with such analyses would confirm the status of immunity at a particular time point and allow associations to be made between gaps in the antibody repertoire and risk of a clinical episode (62, 98, 99, 151). Longitudinal studies in endemic regions that monitor children at regular time intervals for the presence of specific antibodies or the turnover of alleles at antigen loci could therefore be harnessed to identify the genetic determinants of antigenic diversity.

MEASURING STRAIN-SPECIFIC EFFICACY IN VACCINE TRIALS

Vaccines based on a single allele of the candidate antigen elicit strain-specific antibodies, so that vaccinated individuals continue to be at risk of infection with different strains but risk of infection with the vaccine strain is lower (**Figure 3B**) (22, 66, 131). Vaccine trials with strain-specific endpoints (i.e., infection with a particular parasite genotype) therefore represent a major opportunity to characterize the genetic determinants responsible for immune escape; however, this has been rarely attempted. Given the high diversity of most malaria vaccine candidates and the limited efficacy of single-strain vaccine candidates, for any candidate that elicits strain-specific responses it will be essential to measure strain-specific endpoints to ensure that antigenic escape is explored in vaccine testing. The advent of high throughput genotyping approaches (142, 152) has reduced the effort and funding required for molecular epidemiological studies, and therefore as long as relevant expertise is available, these investigations should be relatively simple to incorporate into a vaccine trial.

The primary efficacy endpoint in clinical trials of malaria vaccines has included a range of measurements such as time to first infection, occurrence of a clinical episode (mild and severe), and parasite density. However, only a few malaria vaccine trials have determined whether efficacy against vaccine or closely related strains has been achieved (22, 92, 131). Below, we cover some

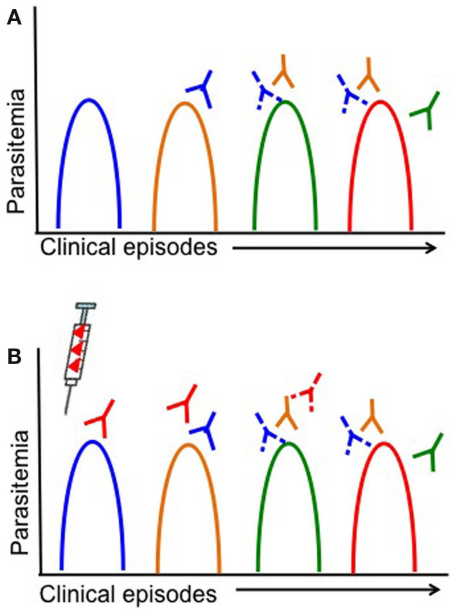

FIGURE 3 | Antigenic diversity, clinical malaria, and vaccine efficacy are shown. Simplified overview of the impact of parasite antigenic diversity on the dynamics of natural infection and the efficacy of vaccines. Peaks in parasitemia correspond with different clinical episodes and colors indicate different serotypes. Strain-specific acquired antibodies are shown in corresponding colors some time after each clinical episode. Solid lines represent a strong antibody response, while dashed lines represent limited antibody responses. **(A)** Dynamics of natural infection with recurrent episodes of clinical malaria in an individual that acquires only strain-specific antibodies after being infected. As individuals experience different strains through natural infection (or vaccination), they acquire strain-specific antibodies and have a lower risk of having a clinical episode due to the same strain. **(B)** Lack of vaccine efficacy in a vaccinated individual due to antigenic diversity. The syringe indicates vaccination with a single serotype (red). If a single-strain vaccine is given at baseline, individuals are more likely to experience clinical episodes due to other strains (blue, yellow, green) than the vaccine strain, until antibody responses decrease.

molecular epidemiological analyses that could be considered in the different stages of a vaccine trial.

VACCINE TRIAL RECONNAISSANCE

The low prevalence of vaccine alleles within natural populations has been proposed to limit vaccine efficacy (2, 11, 129, 138). Furthermore, a low prevalence of the vaccine allele will make it extremely difficult to measure allele specific efficacy, since changes may be very small and therefore require exceedingly large sample sizes (92). Therefore, even before a vaccine trial begins in a particular geographic area, molecular epidemiological surveys will be important to assess baseline population genetic characteristics, and in particular the allele- (or if known, the serotype-) frequencies to ensure that the vaccine to be tested is representative of the target parasite population.

STRAIN-SPECIFIC ENDPOINTS

In addition to measuring strain-specific endpoints by determining the time to first clinical episode with vaccine and non-vaccine alleles, the analysis of outcomes in control and vaccine groups provide an opportunity for more detailed analyses that can provide key insights into the complexities of parasite antigenic diversity (92, 127, 153). In principle, the same analyses can be done in longitudinal studies to achieve the same goal. These analyses will provide further functional evidence of antigenic escape polymorphisms, thus allowing refinement of the serotype. Different types of analyses that have been used to measure strain-specific efficacy in vaccine trials include:

(i) *measuring strain-specific protection* by assessing risk of clinical episode associated with parasites carrying the vaccine-allele compared to those carrying any non-vaccine allele;
(ii) *measuring cross-strain protection* by assessing risk of clinical infection with parasites carrying the vaccine-allele compared to those carrying different non-vaccine alleles;
(iii) *measuring vaccine-mediated selection* by:
  a. comparison of allele or haplotype frequencies before and after vaccination in the different vaccine groups;
  b. comparison of the incidence of infection with parasites carrying different haplotypes and individual polymorphisms before and after vaccination;
(iv) *assessment of vaccine-mediated strain-specific natural immunity* by measuring antibodies to vaccine alleles in vaccine and control groups.

VACCINE-MEDIATED SELECTION AND ANTIGENIC ESCAPE MUTANTS

The development and licensing of an effective malaria vaccine will require sustained and intensive surveillance to monitor for vaccine-mediated selection of non-vaccine alleles and to ensure that vaccine alleles are common enough for vaccines to remain effective. Regular population genetic surveys will be required in order to monitor allele frequencies, to ensure that alleles contained within the vaccine continue to represent the diversity of the parasite population and that new, potential antigenic escape mutants have not emerged. A genetic surveillance system would be difficult and costly to implement, but the development of low cost rapid assays to quickly genotype parasite isolates would facilitate such an approach (152). The need for simple and informative surveillance tools also highlights the importance of gaining knowledge into the precise determinants of antigenic diversity in any antigen to be included in a licensed malaria vaccine.

CONCLUSION

The trials and tribulations of malaria vaccine development have reached a critical juncture. The first licensed malaria vaccine is almost at hand and children in African countries stand to benefit greatly from its availability. However, this vaccine is only partially effective against the symptoms of malaria, not infection, and provides only short-lived protection (23). As a result of the success of the reinvigorated global malaria eradication program, many previously highly endemic countries no longer have high burdens of disease. By 2030, the goal is to have a second-generation vaccine(s) that can provide broad and long-lived protection against multiple species and diverse strains. Parasite antigenic diversity, one of the major reasons for the failures of past candidate malaria vaccines, remains a barrier to the efficacy of subunit and potentially

even whole parasite vaccine approaches (48), but there is light at the end of the tunnel: this review demonstrates how it may be possible to overcome parasite antigenic diversity. Successful identification of the critical genetic determinants of the serotype for one leading vaccine candidate, AMA1 (126, 127, 131) and proof that multivalent vaccines representing the majority of the diversity of this antigen can generate broad antibody responses further support the development of effective multi-allele vaccines and suggest that antigenic diversity in malaria may be overcome (65, 132). By consolidating the vast knowledge gained on the genetic diversity of candidate antigens, by harnessing high throughput genotyping tools and with careful design of longitudinal studies and vaccine trials, it is highly likely that it will also be possible to identify the critical genetic determinants underlying antigenic diversity (i.e., the serotype) for any candidate malaria vaccine antigen.

AUTHOR CONTRIBUTIONS

Alyssa E. Barry and Alicia Arnott wrote the paper, collated data, and prepared figures.

ACKNOWLEDGMENTS

The authors are grateful to all of our colleagues and mentors who have contributed to discussions about the concepts outlined in this review over the last several years, in particular, J. Reeder, J. Beeson, M. Good, and I. Mueller. The authors' research is supported by Project Grants awarded to Alyssa E. Barry by the National Health and Medical Research Council of Australia (No. 488211, 1005653 and 1003825). The work was made possible through Victorian State Government Operational Infrastructure Support and Australian Government NHMRC IRIISS.

REFERENCES

1. World Health Organisation. *World Malaria Report*. Geneva: World Health Organisation (2013).
2. Barry AE, Schultz L, Buckee CO, Reeder JC. Contrasting population structures of the genes encoding ten leading vaccine-candidate antigens of the human malaria parasite, *Plasmodium falciparum*. *PLoS One* (2009) **4**(12):e8497. doi:10.1371/journal.pone.0008497
3. Roll Back Malaria Partnership. *The Global Malaria Action Plan for a Malaria Free World*. Geneva: Roll Back Malaria Partnership (2008).
4. Gething PW, Elyazar IR, Moyes CL, Smith DL, Battle KE, Guerra CA, et al. A long neglected world malaria map: *Plasmodium vivax* endemicity in 2010. *PLoS Negl Trop Dis* (2012) **6**(9):e1814. doi:10.1371/journal.pntd.0001814
5. Cotter C, Sturrock HJ, Hsiang MS, Liu J, Phillips AA, Hwang J, et al. The changing epidemiology of malaria elimination: new strategies for new challenges. *Lancet* (2013) **382**(9895):900–11. doi:10.1016/S0140-6736(13)60310-4
6. Mueller I, Galinski MR, Baird JK, Carlton JM, Kochar DK, Alonso PL, et al. Key gaps in the knowledge of *Plasmodium vivax*, a neglected human malaria parasite. *Lancet Infect Dis* (2009) **9**(9):555–66. doi:10.1016/S1473-3099(09)70177-X
7. World Health Organization. *World Malaria Report*. Geneva: World Health Organization (2010).
8. Alonso PL, Brown G, Arevalo-Herrera M, Binka F, Chitnis C, Collins F, et al. A research agenda to underpin malaria eradication. *PLoS Med* (2011) **8**(1):e1000406. doi:10.1371/journal.pmed.1000406
9. Takala SL, Plowe CV. Genetic diversity and malaria vaccine design, testing and efficacy: preventing and overcoming "vaccine resistant malaria". *Parasite Immunol* (2009) **31**(9):560–73. doi:10.1111/j.1365-3024.2009.01138.x
10. Riley EM, Stewart VA. Immune mechanisms in malaria: new insights in vaccine development. *Nat Med* (2013) **19**(2):168–78. doi:10.1038/nm.3083
11. Barry AE, Beeson J, Reeder JC, Fowkes F, Arnott A. Using population genetics to guide malaria vaccine design. In: Okwa APO, editor. *Malaria Parasites*. Rijeka: InTech (2012). p. 231–64.
12. Richards JS, Beeson JG. The future for blood-stage vaccines against malaria. *Immunol Cell Biol* (2009) **87**(5):377–90. doi:10.1038/icb.2009.27
13. Koch R. Dritter Bericht uber die Thatigkeit der Malaria-Expedition. *Dtsch Med Wochenschr* (1900) **26**(17):281–4. doi:10.1055/s-0029-1203823
14. Marsh K, Kinyanjui S. Immune effector mechanisms in malaria. *Parasite Immunol* (2006) **28**(1–2):51–60. doi:10.1111/j.1365-3024.2006.00808.x
15. Doolan DL, Dobano C, Baird JK. Acquired immunity to malaria. *Clin Microbiol Rev* (2009) **22**(1):13–36. doi:10.1128/CMR.00025-08
16. Cohen S, Mc GI, Carrington S. Gamma-globulin and acquired immunity to human malaria. *Nature* (1961) **192**:733–7. doi:10.1038/192733a0
17. Darko CA, Angov E, Collins WE, Bergmann-Leitner ES, Girouard AS, Hitt SL, et al. The clinical-grade 42-kilodalton fragment of merozoite surface protein 1 of *Plasmodium falciparum* strain FVO expressed in *Escherichia coli* protects Aotus nancymai against challenge with homologous erythrocytic-stage parasites. *Infect Immun* (2005) **73**(1):287–97. doi:10.1128/IAI.73.1.287-297.2005
18. Dutta S, Sullivan JS, Grady KK, Haynes JD, Komisar J, Batchelor AH, et al. High antibody titer against apical membrane antigen-1 is required to protect against malaria in the Aotus model. *PLoS One* (2009) **4**(12):e8138. doi:10.1371/journal.pone.0008138
19. Gentil F, Bargieri DY, Leite JA, Francoso KS, Patricio MB, Espindola NM, et al. A recombinant vaccine based on domain II of *Plasmodium vivax* apical membrane antigen 1 induces high antibody titres in mice. *Vaccine* (2010) **28**(38):6183–90. doi:10.1016/j.vaccine.2010.07.017
20. Stowers AW, Kennedy MC, Keegan BP, Saul A, Long CA, Miller LH. Vaccination of monkeys with recombinant *Plasmodium falciparum* apical membrane antigen 1 confers protection against blood-stage malaria. *Infect Immun* (2002) **70**(12):6961–7. doi:10.1128/IAI.70.12.6961-6967.2002
21. Schwartz L, Brown GV, Genton B, Moorthy VS. A review of malaria vaccine clinical projects based on the WHO rainbow table. *Malar J* (2012) **11**:11. doi:10.1186/1475-2875-11-11
22. Genton B, Betuela I, Felger I, Al-Yaman F, Anders RF, Saul A, et al. A recombinant blood-stage malaria vaccine reduces *Plasmodium falciparum* density and exerts selective pressure on parasite populations in a phase 1-2b trial in Papua New Guinea. *J Infect Dis* (2002) **185**(6):820–7. doi:10.1086/339342
23. Agnandji ST, Lell B, Soulanoudjingar SS, Fernandes JF, Abossolo BP, Conzelmann C, et al. First results of phase 3 trial of RTS,S/AS01 malaria vaccine in African children. *N Engl J Med* (2011) **365**(20):1863–75. doi:10.1056/NEJMoa1102287
24. Sirima SB, Cousens S, Druilhe P. Protection against malaria by MSP3 candidate vaccine. *N Engl J Med* (2011) **365**(11):1062–4. doi:10.1056/NEJMc1100670
25. Laurens MB, Thera MA, Coulibaly D, Ouattara A, Kone AK, Guindo AB, et al. Extended safety, immunogenicity and efficacy of a blood-stage malaria vaccine in malian children: 24-month follow-up of a randomized, double-blinded phase 2 trial. *PLoS One* (2013) **8**(11):e79323. doi:10.1371/journal.pone.0079323
26. Clyde DF, Most H, McCarthy VC, Vanderberg JP. Immunization of man against sporozite-induced falciparum malaria. *Am J Med Sci* (1973) **266**(3):169–77. doi:10.1097/00000441-197312000-00001
27. Hoffman SL, Goh LM, Luke TC, Schneider I, Le TP, Doolan DL, et al. Protection of humans against malaria by immunization with radiation-attenuated *Plasmodium falciparum* sporozoites. *J Infect Dis* (2002) **185**(8):1155–64. doi:10.1086/339409
28. Nussenzweig V, Nussenzweig RS. Circumsporozoite proteins of malaria parasites. *Cell* (1985) **42**(2):401–3. doi:10.1016/0092-8674(85)90093-5
29. Malaria Vaccine Funders Group. *Malaria Vaccine Technology Roadmap*. Geneva: World Health Organization(2006).
30. Otieno L. Efficacy of RTS,S/AS01 vaccine candidate against malaria in African infants and children 8 months post-primary vaccination series: a phase III randomized, double-blind controlled trial. *Multilateral Initiative on Malaria Pan Africa Conference*. Durban (2013).
31. Malaria Vaccine Funders Group. *Malaria Vaccine Technology Roadmap*. Geneva: World Health Organization (2013).
32. Doolan DL, Aguiar JC, Weiss WR, Sette A, Felgner PL, Regis DP, et al. Utilization of genomic sequence information to develop malaria vaccines. *J Exp Biol* (2003) **206**(Pt 21):3789–802. doi:10.1242/jeb.00615
33. Roestenberg M, McCall M, Hopman J, Wiersma J, Luty AJ, van Gemert GJ, et al. Protection against a malaria challenge by sporozoite inoculation. *N Engl J Med* (2009) **361**(5):468–77. doi:10.1056/NEJMoa0805832

34. Teirlinck AC, McCall MB, Roestenberg M, Scholzen A, Woestenenk R, de Mast Q, et al. Longevity and composition of cellular immune responses following experimental *Plasmodium falciparum* malaria infection in humans. *PLoS Pathog* (2011) **7**(12):e1002389. doi:10.1371/journal.ppat.1002389
35. Butler NS, Vaughan AM, Harty JT, Kappe SH. Whole parasite vaccination approaches for prevention of malaria infection. *Trends Immunol* (2012) **33**(5):247–54. doi:10.1016/j.it.2012.02.001
36. Hill AV. Vaccines against malaria. *Philos Trans R Soc Lond B Biol Sci* (2011) **366**(1579):2806–14. doi:10.1098/rstb.2011.0091
37. Stanisic DI, Barry AE, Good MF. Escaping the immune system: how the malaria parasite makes vaccine development a challenge. *Trends Parasitol* (2013) **29**(12):612–22. doi:10.1016/j.pt.2013.10.001
38. Singh S, Miura K, Zhou H, Muratova O, Keegan B, Miles A, et al. Immunity to recombinant *Plasmodium falciparum* merozoite surface protein 1 (MSP1): protection in Aotus nancymai monkeys strongly correlates with anti-MSP1 antibody titer and in vitro parasite-inhibitory activity. *Infect Immun* (2006) **74**(8):4573–80. doi:10.1128/IAI.01679-05
39. Dent AE, Bergmann-Leitner ES, Wilson DW, Tisch DJ, Kimmel R, Vulule J, et al. Antibody-mediated growth inhibition of *Plasmodium falciparum*: relationship to age and protection from parasitemia in Kenyan children and adults. *PLoS One* (2008) **3**(10):e3557. doi:10.1371/journal.pone.0003557
40. King CL, Michon P, Shakri AR, Marcotty A, Stanisic D, Zimmerman PA, et al. Naturally acquired Duffy-binding protein-specific binding inhibitory antibodies confer protection from blood-stage *Plasmodium vivax* infection. *Proc Natl Acad Sci U S A* (2008) **105**(24):8363–8. doi:10.1073/pnas.0800371105
41. McCallum FJ, Persson KE, Mugyenyi CK, Fowkes FJ, Simpson JA, Richards JS, et al. Acquisition of growth-inhibitory antibodies against blood-stage *Plasmodium falciparum*. *PLoS One* (2008) **3**(10):e3571. doi:10.1371/journal.pone.0003571
42. Deans JA, Knight AM, Jean WC, Waters AP, Cohen S, Mitchell GH. Vaccination trials in rhesus monkeys with a minor, invariant, *Plasmodium knowlesi* 66 kD merozoite antigen. *Parasite Immunol* (1988) **10**(5):535–52. doi:10.1111/j.1365-3024.1988.tb00241.x
43. Renia L, Ling IT, Marussig M, Miltgen F, Holder AA, Mazier D. Immunization with a recombinant C-terminal fragment of *Plasmodium yoelii* merozoite surface protein 1 protects mice against homologous but not heterologous *P. yoelii* sporozoite challenge. *Infect Immun* (1997) **65**(11):4419–23.
44. Biswas S, Spencer AJ, Forbes EK, Gilbert SC, Holder AA, Hill AV, et al. Recombinant viral-vectored vaccines expressing *Plasmodium chabaudi* AS apical membrane antigen 1: mechanisms of vaccine-induced blood-stage protection. *J Immunol* (2012) **188**(10):5041–53. doi:10.4049/jimmunol.1101106
45. Lyon JA, Angov E, Fay MP, Sullivan JS, Girourd AS, Robinson SJ, et al. Protection induced by *Plasmodium falciparum* MSP1(42) is strain-specific, antigen and adjuvant dependent, and correlates with antibody responses. *PLoS One* (2008) **3**(7):e2830. doi:10.1371/journal.pone.0002830
46. Birkett AJ, Moorthy VS, Loucq C, Chitnis CE, Kaslow DC. Malaria vaccine R&D in the decade of vaccines: breakthroughs, challenges and opportunities. *Vaccine* (2013) **31**(Suppl 2):B233–43. doi:10.1016/j.vaccine.2013.02.040
47. Sultan AA, Thathy V, Nussenzweig V, Menard R. Green fluorescent protein as a marker in *Plasmodium berghei* transformation. *Infect Immun* (1999) **67**(5):2602–6.
48. The malERA Consultative Group on Vaccines. A research agenda for malaria eradication: vaccines. *PLoS Med* (2011) **8**(1):e1000398. doi:10.1371/journal.pmed.1000398
49. Hviid L. The role of *Plasmodium falciparum* variant surface antigens in protective immunity and vaccine development. *Hum Vaccin* (2010) **6**(1):84–9. doi:10.4161/hv.6.1.9602
50. World Health Organisation. *Malaria Vaccine Rainbow Tables* (2013). Available from: http://www.who.int/vaccine_research/links/Rainbow/en/index.html
51. Yoshida N, Nussenzweig RS, Potocnjak P, Nussenzweig V, Aikawa M. Hybridoma produces protective antibodies directed against the sporozoite stage of malaria parasite. *Science* (1980) **207**(4426):71–3. doi:10.1126/science.6985745
52. Guerin-Marchand C, Druilhe P, Galey B, Londono A, Patarapotikul J, Beaudoin RL, et al. A liver-stage-specific antigen of *Plasmodium falciparum* characterized by gene cloning. *Nature* (1987) **329**(6135):164–7. doi:10.1038/329164a0
53. Robson KJ, Hall JR, Jennings MW, Harris TJ, Marsh K, Newbold CI, et al. A highly conserved amino-acid sequence in thrombospondin, properdin and in proteins from sporozoites and blood stages of a human malaria parasite. *Nature* (1988) **335**(6185):79–82. doi:10.1038/335079a0
54. Rogers WO, Malik A, Mellouk S, Nakamura K, Rogers MD, Szarfman A, et al. Characterization of *Plasmodium falciparum* sporozoite surface protein 2. *Proc Natl Acad Sci U S A* (1992) **89**(19):9176–80. doi:10.1073/pnas.89.19.9176
55. Cowan G, Krishna S, Crisanti A, Robson K. Expression of thrombospondin-related anonymous protein in *Plasmodium falciparum* sporozoites. *Lancet* (1992) **339**(8806):1412–3. doi:10.1016/0140-6736(92)91229-2
56. Holder AA, Blackman MJ, Burghaus PA, Chappel JA, Ling IT, McCallum-Deighton N, et al. A malaria merozoite surface protein (MSP1)-structure, processing and function. *Mem Inst Oswaldo Cruz* (1992) **87**(Suppl 3):37–42. doi:10.1590/S0074-02761992000700004
57. Smythe JA, Coppel RL, Brown GV, Ramasamy R, Kemp DJ, Anders RF. Identification of two integral membrane proteins of *Plasmodium falciparum*. *Proc Natl Acad Sci U S A* (1988) **85**(14):5195–9. doi:10.1073/pnas.85.14.5195
58. Oeuvray C, Bouharoun-Tayoun H, Gras-Masse H, Bottius E, Kaidoh T, Aikawa M, et al. Merozoite surface protein-3: a malaria protein inducing antibodies that promote *Plasmodium falciparum* killing by cooperation with blood monocytes. *Blood* (1994) **84**(5):1594–602.
59. Hodder AN, Crewther PE, Matthew ML, Reid GE, Moritz RL, Simpson RJ, et al. The disulfide bond structure of *Plasmodium* apical membrane antigen-1. *J Biol Chem* (1996) **271**(46):29446–52. doi:10.1074/jbc.271.46.29446
60. Genton B, Reed ZH. Asexual blood-stage malaria vaccine development: facing the challenges. *Curr Opin Infect Dis* (2007) **20**(5):467–75. doi:10.1097/QCO.0b013e3282dd7a29
61. Fowkes FJ, Richards JS, Simpson JA, Beeson JG. The relationship between anti-merozoite antibodies and incidence of *Plasmodium falciparum* malaria: a systematic review and meta-analysis. *PLoS Med* (2010) **7**(1):e1000218. doi:10.1371/journal.pmed.1000218
62. Stanisic DI, Richards JS, McCallum FJ, Michon P, King CL, Schoepflin S, et al. Immunoglobulin G subclass-specific responses against *Plasmodium falciparum* merozoite antigens are associated with control of parasitemia and protection from symptomatic illness. *Infect Immun* (2009) **77**(3):1165–74. doi:10.1128/IAI.01129-08
63. Richards JS, Arumugam TU, Reiling L, Healer J, Hodder AN, Fowkes FJ, et al. Identification and prioritization of merozoite antigens as targets of protective human immunity to *Plasmodium falciparum* malaria for vaccine and biomarker development. *J Immunol* (2013) **191**(2):795–809. doi:10.4049/jimmunol.1300778
64. Baum J, Chen L, Healer J, Lopaticki S, Boyle M, Triglia T, et al. Reticulocyte-binding protein homologue 5 – an essential adhesin involved in invasion of human erythrocytes by *Plasmodium falciparum*. *Int J Parasitol* (2009) **39**(3):371–80. doi:10.1016/j.ijpara.2008.10.006
65. Dutta S, Dlugosz LS, Drew DR, Ge X, Ababacar D, Rovira YI, et al. Overcoming antigenic diversity by enhancing the immunogenicity of conserved epitopes on the malaria vaccine candidate apical membrane antigen-1. *PLoS Pathog* (2013) **9**(12):e1003840. doi:10.1371/journal.ppat.1003840
66. Thera MA, Doumbo OK, Coulibaly D, Laurens MB, Ouattara A, Kone AK, et al. A field trial to assess a blood-stage malaria vaccine. *N Engl J Med* (2011) **365**(11):1004–13. doi:10.1056/NEJMoa1008115
67. Ogutu BR, Apollo OJ, McKinney D, Okoth W, Siangla J, Dubovsky F, et al. Blood stage malaria vaccine eliciting high antigen-specific antibody concentrations confers no protection to young children in Western Kenya. *PLoS One* (2009) **4**(3):e4708. doi:10.1371/journal.pone.0004708
68. Fowkes FJ, Simpson JA, Beeson JG. Implications of the licensure of a partially efficacious malaria vaccine on evaluating second-generation vaccines. *BMC Med* (2013) **11**:232. doi:10.1186/1741-7015-11-232
69. El Sahly HM, Patel SM, Atmar RL, Lanford TA, Dube T, Thompson D, et al. Safety and immunogenicity of a recombinant nonglycosylated erythrocyte binding antigen 175 region II malaria vaccine in healthy adults living in an area where malaria is not endemic. *Clin Vaccine Immunol* (2010) **17**(10):1552–9. doi:10.1128/CVI.00082-10
70. Douglas AD, Williams AR, Illingworth JJ, Kamuyu G, Biswas S, Goodman AL, et al. The blood-stage malaria antigen PfRH5 is susceptible to vaccine-inducible cross-strain neutralizing antibody. *Nat Commun* (2011) **2**:601. doi:10.1038/ncomms1615

71. Crosnier C, Bustamante LY, Bartholdson SJ, Bei AK, Theron M, Uchikawa M, et al. Basigin is a receptor essential for erythrocyte invasion by *Plasmodium falciparum*. *Nature* (2011) **480**(7378):534–7. doi:10.1038/nature10606
72. Ntumngia FB, Schloegel J, McHenry AM, Barnes SJ, George MT, Kennedy S, et al. Immunogenicity of single versus mixed allele vaccines of *Plasmodium vivax* Duffy binding protein region II. *Vaccine* (2013) **31**(40):4382–8. doi:10.1016/j.vaccine.2013.07.002
73. Salanti A, Staalsoe T, Lavstsen T, Jensen AT, Sowa MP, Arnot DE, et al. Selective upregulation of a single distinctly structured var gene in chondroitin sulphate A-adhering *Plasmodium falciparum* involved in pregnancy-associated malaria. *Mol Microbiol* (2003) **49**(1):179–91. doi:10.1046/j.1365-2958.2003.03570.x
74. Rener J, Graves PM, Carter R, Williams JL, Burkot TR. Target antigens of transmission-blocking immunity on gametes of *Plasmodium falciparum*. *J Exp Med* (1983) **158**(3):976–81. doi:10.1084/jem.158.3.976
75. Gardner MJ, Hall N, Fung E, White O, Berriman M, Hyman RW, et al. Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature* (2002) **419**(6906):498–511. doi:10.1038/nature01097
76. Taechalertpaisarn T, Crosnier C, Bartholdson SJ, Hodder AN, Thompson J, Bustamante LY, et al. Biochemical and functional analysis of two *Plasmodium falciparum* blood-stage 6-cys proteins: P12 and P41. *PLoS One* (2012) **7**(7):e41937. doi:10.1371/journal.pone.0041937
77. Florens L, Washburn MP, Raine JD, Anthony RM, Grainger M, Haynes JD, et al. A proteomic view of the *Plasmodium falciparum* life cycle. *Nature* (2002) **419**(6906):520–6. doi:10.1038/nature01107
78. Sanders PR, Gilson PR, Cantin GT, Greenbaum DC, Nebl T, Carucci DJ, et al. Distinct protein classes including novel merozoite surface antigens in Raft-like membranes of *Plasmodium falciparum*. *J Biol Chem* (2005) **280**(48):40169–76. doi:10.1074/jbc.M509631200
79. Doolan DL, Mu Y, Unal B, Sundaresh S, Hirst S, Valdez C, et al. Profiling humoral immune responses to *P. falciparum* infection with protein microarrays. *Proteomics* (2008) **8**(22):4680–94. doi:10.1002/pmic.200800194
80. Persson KE, Fowkes FJ, McCallum FJ, Gicheru N, Reiling L, Richards JS, et al. Erythrocyte-binding antigens of *Plasmodium falciparum* are targets of human inhibitory antibodies and function to evade naturally acquired immunity. *J Immunol* (2013) **191**(2):785–94. doi:10.4049/jimmunol.1300444
81. Tetteh KK, Stewart LB, Ochola LI, Amambua-Ngwa A, Thomas AW, Marsh K, et al. Prospective identification of malaria parasite genes under balancing selection. *PLoS One* (2009) **4**(5):e5568. doi:10.1371/journal.pone.0005568
82. Ochola LI, Tetteh KK, Stewart LB, Riitho V, Marsh K, Conway DJ. Allele frequency-based and polymorphism-versus-divergence indices of balancing selection in a new filtered set of polymorphic genes in *Plasmodium falciparum*. *Mol Biol Evol* (2010) **27**(10):2344–51. doi:10.1093/molbev/msq119
83. Kariuki SK, Njunge J, Muia A, Muluvi G, Gatei W, Ter Kuile F, et al. Effect of malaria transmission reduction by insecticide-treated bed nets (ITNs) on the genetic diversity of *Plasmodium falciparum* merozoite surface protein (MSP-1) and circumsporozoite (CSP) in Western Kenya. *Malar J* (2013) **12**:295. doi:10.1186/1475-2875-12-295
84. Bailey JA, Mvalo T, Aragam N, Weiser M, Congdon S, Kamwendo D, et al. Use of massively parallel pyrosequencing to evaluate the diversity of and selection on *Plasmodium falciparum* csp T-cell epitopes in Lilongwe, Malawi. *J Infect Dis* (2012) **206**(4):580–7. doi:10.1093/infdis/jis329
85. Zeeshan M, Alam MT, Vinayak S, Bora H, Tyagi RK, Alam MS, et al. Genetic variation in the *Plasmodium falciparum* circumsporozoite protein in India and its relevance to RTS,S malaria vaccine. *PLoS One* (2012) **7**(8):e43430. doi:10.1371/journal.pone.0043430
86. Tanabe K, Mita T, Palacpac NM, Arisue N, Tougan T, Kawai S, et al. Within-population genetic diversity of *Plasmodium falciparum* vaccine candidate antigens reveals geographic distance from a Central sub-Saharan African origin. *Vaccine* (2013) **31**(9):1334–9. doi:10.1016/j.vaccine.2012.12.039
87. Jongwutiwes S, Putaporntip C, Karnchaisri K, Seethamchai S, Hongsrimuang T, Kanbara H. Positive selection on the *Plasmodium falciparum* sporozoite threonine-asparagine-rich protein: analysis of isolates mainly from low endemic areas. *Gene* (2008) **410**(1):139–46. doi:10.1016/j.gene.2007.12.006
88. Pratt-Riccio LR, Perce-da-Silva Dde S, Lima-Junior Jda C, Theisen M, Santos F, Daniel-Ribeiro CT, et al. Genetic polymorphisms in the glutamate-rich protein of *Plasmodium falciparum* field isolates from a malaria-endemic area of Brazil. *Mem Inst Oswaldo Cruz* (2013) **108**(4):523–8. doi:10.1590/S0074-02762013000400022
89. Basu M, Maji AK, Mitra M, Sengupta S. Natural selection and population genetic structure of domain-I of *Plasmodium falciparum* apical membrane antigen-1 in India. *Infect Genet Evol* (2013) **18**:247–56. doi:10.1016/j.meegid.2013.05.015
90. Mehrizi AA, Sepehri M, Karimi F, Djadid ND, Zakeri S. Population genetics, sequence diversity and selection in the gene encoding the *Plasmodium falciparum* apical membrane antigen 1 in clinical isolates from the south-east of Iran. *Infect Genet Evol* (2013) **17**:51–61. doi:10.1016/j.meegid.2013.03.042
91. Osier FH, Weedall GD, Verra F, Murungi L, Tetteh KK, Bull P, et al. Allelic diversity and naturally acquired allele-specific antibody responses to *Plasmodium falciparum* apical membrane antigen 1 in Kenya. *Infect Immun* (2010) **78**(11):4625–33. doi:10.1128/IAI.00576-10
92. Ouattara A, Mu J, Takala-Harrison S, Saye R, Sagara I, Dicko A, et al. Lack of allele-specific efficacy of a bivalent AMA1 malaria vaccine. *Malar J* (2010) **9**:175. doi:10.1186/1475-2875-9-175
93. Simpalipan P, Pattaradilokrat S, Siripoon N, Seugorn A, Kaewthamasorn M, Butcher RD, et al. Diversity and population structure of *Plasmodium falciparum* in Thailand based on the spatial and temporal haplotype patterns of the C-terminal 19-kDa domain of merozoite surface protein-1. *Malar J* (2014) **13**(1):54. doi:10.1186/1475-2875-13-54
94. Pan D, Hu J, Ma Q, Pan W, Li M. Diversity and prevalence of the C-terminal region of *Plasmodium falciparum* merozoite surface protein 1 in China. *Acta Trop* (2010) **116**(3):200–5. doi:10.1016/j.actatropica.2010.08.004
95. Lopez AC, Ortiz A, Coello J, Sosa-Ochoa W, Torres RE, Banegas EI, et al. Genetic diversity of *Plasmodium vivax* and *Plasmodium falciparum* in Honduras. *Malar J* (2012) **11**:391. doi:10.1186/1475-2875-11-391
96. Yuan L, Zhao H, Wu L, Li X, Parker D, Xu S, et al. *Plasmodium falciparum* populations from northeastern Myanmar display high levels of genetic diversity at multiple antigenic loci. *Acta Trop* (2013) **125**(1):53–9. doi:10.1016/j.actatropica.2012.09.008
97. Kang JM, Moon SU, Kim JY, Cho SH, Lin K, Sohn WM, et al. Genetic polymorphism of merozoite surface protein-1 and merozoite surface protein-2 in *Plasmodium falciparum* field isolates from Myanmar. *Malar J* (2010) **9**:131. doi:10.1186/1475-2875-9-131
98. Reiling L, Richards JS, Fowkes FJ, Barry AE, Triglia T, Chokejindachai W, et al. Evidence that the erythrocyte invasion ligand PfRh2 is a target of protective immunity against *Plasmodium falciparum* malaria. *J Immunol* (2010) **185**(10):6157–67. doi:10.4049/jimmunol.1001555
99. Reiling L, Richards JS, Fowkes FJ, Wilson DW, Chokejindachai W, Barry AE, et al. The *Plasmodium falciparum* erythrocyte invasion ligand Pfrh4 as a target of functional and protective human antibodies against malaria. *PLoS One* (2012) **7**(9):e45253. doi:10.1371/journal.pone.0045253
100. Bustamante LY, Bartholdson SJ, Crosnier C, Campos MG, Wanaguru M, Nguon C, et al. A full-length recombinant *Plasmodium falciparum* PfRH5 protein induces inhibitory antibodies that are effective across common PfRH5 genetic variants. *Vaccine* (2013) **31**(2):373–9. doi:10.1016/j.vaccine.2012.10.106
101. Da DF, Dixit S, Sattabonkot J, Mu J, Abate L, Ramineni B, et al. Anti-Pfs25 human plasma reduces transmission of *Plasmodium falciparum* isolates that have diverse genetic backgrounds. *Infect Immun* (2013) **81**(6):1984–9. doi:10.1128/IAI.00016-13
102. Hommel M, Elliott SR, Soma V, Kelly G, Fowkes FJ, Chesson JM, et al. Evaluation of the antigenic diversity of placenta-binding *Plasmodium falciparum* variants and the antibody repertoire among pregnant women. *Infect Immun* (2010) **78**(5):1963–78. doi:10.1128/IAI.01365-09
103. Talundzic E, Shah S, Fawole O, Owino S, Moore JM, Peterson DS. Sequence polymorphism, segmental recombination and toggling amino acid residues within the DBL3X domain of the VAR2CSA placental malaria antigen. *PLoS One* (2012) **7**(2):e31565. doi:10.1371/journal.pone.0031565
104. Gnidehou S, Jessen L, Gangnard S, Ermont C, Triqui C, Quiviger M, et al. Insight into antigenic diversity of VAR2CSA-DBL5epsilon domain from multiple *Plasmodium falciparum* placental isolates. *PLoS One* (2010) **5**(10):e13105. doi:10.1371/journal.pone.0013105
105. Chen DS, Barry AE, Leliwa-Sytek A, Smith TA, Peterson I, Brown SM, et al. A molecular epidemiological study of var gene diversity to characterize the reservoir of *Plasmodium falciparum* in humans in Africa. *PLoS One* (2011) **6**(2):e16629. doi:10.1371/journal.pone.0016629
106. Tanabe K, Arisue N, Palacpac NM, Yagi M, Tougan T, Honma H, et al. Geographic differentiation of polymorphism in the *Plasmodium falciparum*

malaria vaccine candidate gene SERA5. *Vaccine* (2012) **30**(9):1583–93. doi:10.1016/j.vaccine.2011.12.124
107. Patil A, Orjuela-Sanchez P, da Silva-Nunes M, Ferreira MU. Evolutionary dynamics of the immunodominant repeats of the *Plasmodium vivax* malaria-vaccine candidate circumsporozoite protein (CSP). *Infect Genet Evol* (2010) **10**(2):298–303. doi:10.1016/j.meegid.2010.01.006
108. Zakeri S, Abouie Mehrizi A, Djadid ND, Snounou G. Circumsporozoite protein gene diversity among temperate and tropical *Plasmodium vivax* isolates from Iran. *Trop Med Int Health* (2006) **11**(5):729–37. doi:10.1111/j.1365-3156.2006.01613.x
109. Dias S, Wickramarachchi T, Sahabandu I, Escalante AA, Udagama PV. Population genetic structure of the *Plasmodium vivax* circumsporozoite protein (Pvcsp) in Sri Lanka. *Gene* (2013) **518**(2):381–7. doi:10.1016/j.gene.2013.01.003
110. Nobrega de Sousa T, Carvalho LH, Alves de Brito CF. Worldwide genetic variability of the Duffy binding protein: insights into *Plasmodium vivax* vaccine development. *PLoS One* (2011) **6**(8):e22944. doi:10.1371/journal.pone.0022944
111. Chenet SM, Tapia LL, Escalante AA, Durand S, Lucas C, Bacon DJ. Genetic diversity and population structure of genes encoding vaccine candidate antigens of *Plasmodium vivax*. *Malar J* (2012) **11**(1):68. doi:10.1186/1475-2875-11-68
112. Valizadeh V, Zakeri S, Mehrizi AA, Djadid ND. Population genetics and natural selection in the gene encoding the Duffy binding protein II in Iranian *Plasmodium vivax* wild isolates. *Infect Genet Evol* (2014) **21**:424–35. doi:10.1016/j.meegid.2013.12.012
113. Ju HL, Kang JM, Moon SU, Bahk YY, Cho PY, Sohn WM, et al. Genetic diversity and natural selection of Duffy binding protein of *Plasmodium vivax* Korean isolates. *Acta Trop* (2013) **125**(1):67–74. doi:10.1016/j.actatropica.2012.09.016
114. Kano FS, Sanchez BA, Sousa TN, Tang ML, Saliba J, Oliveira FM, et al. *Plasmodium vivax* Duffy binding protein: baseline antibody responses and parasite polymorphisms in a well-consolidated settlement of the Amazon region. *Trop Med Int Health* (2012) **17**(8):989–1000. doi:10.1111/j.1365-3156.2012.03016.x
115. Ju HL, Kang JM, Moon SU, Kim JY, Lee HW, Lin K, et al. Genetic polymorphism and natural selection of Duffy binding protein of *Plasmodium vivax* Myanmar isolates. *Malar J* (2012) **11**:60. doi:10.1186/1475-2875-11-60
116. Premaratne PH, Aravinda BR, Escalante AA, Udagama PV. Genetic diversity of *Plasmodium vivax* Duffy binding protein II (PvDBPII) under unstable transmission and low intensity malaria in Sri Lanka. *Infect Genet Evol* (2011) **11**(6):1327–39. doi:10.1016/j.meegid.2011.04.023
117. Collins WE, Jeffery GM, Roberts JM. A retrospective examination of reinfection of humans with *Plasmodium vivax*. *Am J Trop Med Hyg* (2004) **70**(6):642–4.
118. Jeffery GM. Epidemiological significance of repeated infections with homologous and heterologous strains and species of *Plasmodium*. *Bull World Health Organ* (1966) **35**(6):873–82.
119. McKenzie FE, Smith DL, O'Meara WP, Riley EM. Strain theory of malaria: the first 50 years. *Adv Parasitol* (2008) **66**:1–46. doi:10.1016/S0065-308X(08)00201-7
120. Bull PC, Lowe BS, Kortok M, Molyneux CS, Newbold CI, Marsh K. Parasite antigens on the infected red cell surface are targets for naturally acquired immunity to malaria. *Nat Med* (1998) **4**(3):358–60. doi:10.1038/nm0398-358
121. Day KP, Marsh K. Naturally acquired immunity to *Plasmodium falciparum*. *Immunol Today* (1991) **12**(3):A68–71. doi:10.1016/S0167-5699(05)80020-9
122. Bai T, Becker M, Gupta A, Strike P, Murphy VJ, Anders RF, et al. Structure of AMA1 from *Plasmodium falciparum* reveals a clustering of polymorphisms that surround a conserved hydrophobic pocket. *Proc Natl Acad Sci U S A* (2005) **102**(36):12736–41. doi:10.1073/pnas.0501808102
123. Collins CR, Withers-Martinez C, Hackett F, Blackman MJ. An inhibitory antibody blocks interactions between components of the malarial invasion machinery. *PLoS Pathog* (2009) **5**(1):e1000273. doi:10.1371/journal.ppat.1000273
124. Nielsen MA, Pinto VV, Resende M, Dahlback M, Ditlev SB, Theander TG, et al. Induction of adhesion-inhibitory antibodies against placental *Plasmodium falciparum* parasites by using single domains of VAR2CSA. *Infect Immun* (2009) **77**(6):2482–7. doi:10.1128/IAI.00159-09
125. Ntumngia FB, King CL, Adams JH. Finding the sweet spots of inhibition: understanding the targets of a functional antibody against *Plasmodium vivax* Duffy binding protein. *Int J Parasitol* (2012) **42**(12):1055–62. doi:10.1016/j.ijpara.2012.09.006
126. Dutta S, Lee SY, Batchelor AH, Lanar DE. Structural basis of antigenic escape of a malaria vaccine candidate. *Proc Natl Acad Sci U S A* (2007) **104**(30):12488–93. doi:10.1073/pnas.0701464104
127. Takala SL, Coulibaly D, Thera MA, Batchelor AH, Cummings MP, Escalante AA, et al. Extreme polymorphism in a vaccine antigen and risk of clinical malaria: implications for vaccine development. *Sci Transl Med* (2009) **1**(2):2ra5. doi:10.1126/scitranslmed.3000257
128. Reeder JC, Wapling J, Mueller I, Siba PM, Barry AE. Population genetic analysis of the *Plasmodium falciparum* 6-cys protein Pf38 in Papua New Guinea reveals domain-specific balancing selection. *Malar J* (2011) **10**:126. doi:10.1186/1475-2875-10-126
129. Arnott A, Mueller I, Ramsland PA, Siba PM, Reeder JC, Barry AE. Global population structure of the genes encoding the malaria vaccine candidate, *Plasmodium vivax* apical membrane antigen 1 (PvAMA1). *PLoS Negl Trop Dis* (2013) **7**(10):e2506. doi:10.1371/journal.pntd.0002506
130. Taylor RR, Smith DB, Robinson VJ, McBride JS, Riley EM. Human antibody response to *Plasmodium falciparum* merozoite surface protein 2 is serogroup specific and predominantly of the immunoglobulin G3 subclass. *Infect Immun* (1995) **63**(11):4382–8.
131. Ouattara A, Takala-Harrison S, Thera MA, Coulibaly D, Niangaly A, Saye R, et al. Molecular basis of allele-specific efficacy of a blood-stage malaria vaccine: vaccine development implications. *J Infect Dis* (2013) **207**(3):511–9. doi:10.1093/infdis/jis709
132. Kusi KA, Faber BW, Thomas AW, Remarque EJ. Humoral immune response to mixed PfAMA1 alleles; multivalent PfAMA1 vaccines induce broad specificity. *PLoS One* (2009) **4**(12):e8110. doi:10.1371/journal.pone.0008110
133. Remarque EJ, Faber BW, Kocken CH, Thomas AW. A diversity-covering approach to immunization with *Plasmodium falciparum* apical membrane antigen 1 induces broader allelic recognition and growth inhibition responses in rabbits. *Infect Immun* (2008) **76**(6):2660–70. doi:10.1128/IAI.00170-08
134. Faber BW, Younis S, Remarque EJ, Rodriguez Garcia R, Riasat V, Walraven V, et al. Diversity covering AMA1-MSP119 fusion proteins as malaria vaccines. *Infect Immun* (2013) **81**(5):1479–90. doi:10.1128/IAI.01267-12
135. Coley AM, Parisi K, Masciantonio R, Hoeck J, Casey JL, Murphy VJ, et al. The most polymorphic residue on *Plasmodium falciparum* apical membrane antigen 1 determines binding of an invasion-inhibitory antibody. *Infect Immun* (2006) **74**(5):2628–36. doi:10.1128/IAI.74.5.2628-2636.2006
136. Conway DJ, Cavanagh DR, Tanabe K, Roper C, Mikes ZS, Sakihama N, et al. A principal target of human immunity to malaria identified by molecular population genetic and immunological analyses. *Nat Med* (2000) **6**(6):689–92. doi:10.1038/76272
137. Weedall GD, Conway DJ. Detecting signatures of balancing selection to identify targets of anti-parasite immunity. *Trends Parasitol* (2010) **26**(7):363–9. doi:10.1016/j.pt.2010.04.002
138. Takala SL, Coulibaly D, Thera MA, Dicko A, Smith DL, Guindo AB, et al. Dynamics of polymorphism in a malaria vaccine antigen at a vaccine-testing site in Mali. *PLoS Med* (2007) **4**(3):e93. doi:10.1371/journal.pmed.0040093
139. Anderson TJ, Haubold B, Williams JT, Estrada-Franco JG, Richardson L, Mollinedo R, et al. Microsatellite markers reveal a spectrum of population structures in the malaria parasite *Plasmodium falciparum*. *Mol Biol Evol* (2000) **17**(10):1467–82. doi:10.1093/oxfordjournals.molbev.a026247
140. Imwong M, Nair S, Pukrittayakamee S, Sudimack D, Williams JT, Mayxay M, et al. Contrasting genetic structure in *Plasmodium vivax* populations from Asia and South America. *Int J Parasitol* (2007) **37**(8–9):1013–22. doi:10.1016/j.ijpara.2007.02.010
141. Koepfli C, Mueller I, Marfurt J, Goroti M, Sie A, Oa O, et al. Evaluation of *Plasmodium vivax* genotyping markers for molecular monitoring in clinical trials. *J Infect Dis* (2009) **199**(7):1074–80. doi:10.1086/597303
142. Falk N, Maire N, Sama W, Owusu-Agyei S, Smith T, Beck HP, et al. Comparison of PCR-RFLP and Genescan-based genotyping for analyzing infection dynamics of *Plasmodium falciparum*. *Am J Trop Med Hyg* (2006) **74**(6):944–50.
143. Betuela I, Rosanas-Urgell A, Kiniboro B, Stanisic DI, Samol L, de Lazzari E, et al. Relapses contribute significantly to the risk of *Plasmodium vivax* infection and disease in Papua New Guinean children 1-5 years of age. *J Infect Dis* (2012) **206**(11):1771–80. doi:10.1093/infdis/jis580
144. Barry AE, Leliwa A, Choi M, Nielsen KM, Hartl DL, Day KP. DNA sequence artifacts and the estimation of time to the most recent common ancestor (TMRCA)

of *Plasmodium falciparum*. *Mol Biochem Parasitol* (2003) **130**(2):143–7. doi:10.1016/S0166-6851(03)00164-6

145. Chan ER, Menard D, David PH, Ratsimbasoa A, Kim S, Chim P, et al. Whole genome sequencing of field isolates provides robust characterization of genetic diversity in *Plasmodium vivax*. *PLoS Negl Trop Dis* (2012) **6**(9):e1811. doi:10.1371/journal.pntd.0001811
146. Duan J, Mu J, Thera MA, Joy D, Kosakovsky Pond SL, Diemert D, et al. Population structure of the genes encoding the polymorphic *Plasmodium falciparum* apical membrane antigen 1: implications for vaccine design. *Proc Natl Acad Sci U S A* (2008) **105**(22):7857–62. doi:10.1073/pnas.0802328105
147. Excoffier L, Heckel G. Computer programs for population genetics data analysis: a survival guide. *Nat Rev Genet* (2006) **7**(10):745–58. doi:10.1038/nrg1904
148. Tajima F. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* (1989) **123**(3):585–95.
149. Parobek CM, Bailey JA, Hathaway NJ, Socheat D, Rogers WO, Juliano JJ. Differing patterns of selection and geospatial genetic diversity within two leading *Plasmodium vivax* candidate vaccine antigens. *PLoS Negl Trop Dis* (2014) **8**(4):e2796. doi:10.1371/journal.pntd.0002796
150. Bruce MC, Galinski MR, Barnwell JW, Donnelly CA, Walmsley M, Alpers MP, et al. Genetic diversity and dynamics of *Plasmodium falciparum* and *P. vivax* populations in multiply infected children with asymptomatic malaria infections in Papua New Guinea. *Parasitology* (2000) **121**(Pt 3):257–72. doi:10.1017/S0031182099006356
151. Stanisic DI, Javati S, Kiniboro B, Lin E, Jiang J, Singh B, et al. Naturally acquired immune responses to *P. vivax* merozoite surface protein 3alpha and merozoite surface protein 9 are associated with reduced risk of *P. vivax* malaria in young Papua New Guinean children. *PLoS Negl Trop Dis* (2013) **7**(11):e2498. doi:10.1371/journal.pntd.0002498
152. Barnadas C, Kent D, Timinao L, Iga J, Gray LR, Siba P, et al. A new high-throughput method for simultaneous detection of drug resistance associated mutations in *Plasmodium vivax* dhfr, dhps and mdr1 genes. *Malar J* (2011) **10**:282. doi:10.1186/1475-2875-10-282
153. Fluck C, Schopflin S, Smith T, Genton B, Alpers MP, Beck HP, et al. Effect of the malaria vaccine combination B on merozoite surface antigen 2 diversity. *Infect Genet Evol* (2007) **7**(1):44–51. doi:10.1016/j.meegid.2006.03.006

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.

Received: 12 May 2014; accepted: 13 July 2014; published online: 28 July 2014.
*Citation: Barry AE and Arnott A (2014) Strategies for designing and monitoring malaria vaccines targeting diverse antigens. Front. Immunol. **5**:359. doi: 10.3389/fimmu.2014.00359*
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology.
Copyright © 2014 Barry and Arnott. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 22 August 2014
doi: 10.3389/fmicb.2014.00439

The influence of delivery vectors on HIV vaccine efficacy

Beatrice O. Ondondo*

Nuffield Department of Medicine, The Jenner Institute, University of Oxford, Oxford, UK

Edited by:
Shuo Li, The University of Melbourne, Australia

Reviewed by:
Andrew Lucas, Murdoch University, Australia
Cody Charles Allison, Walter and Eliza Hall Institute, Australia

****Correspondence:***
Beatrice O. Ondondo, Nuffield Department of Medicine, The Jenner Institute, University of Oxford, ORCRB, Roosevelt Drive, Oxford, OX3 7DQ, UK
e-mail: beatrice.ondondo@ndm.ox.ac.uk

Development of an effective HIV/AIDS vaccine remains a big challenge, largely due to the enormous HIV diversity which propels immune escape. Thus novel vaccine strategies are targeting multiple variants of conserved antibody and T cell epitopic regions which would incur a huge fitness cost to the virus in the event of mutational escape. Besides immunogen design, the delivery modality is critical for vaccine potency and efficacy, and should be carefully selected in order to not only maximize transgene expression, but to also enhance the immuno-stimulatory potential to activate innate and adaptive immune systems. To date, five HIV vaccine candidates have been evaluated for efficacy and protection from acquisition was only achieved in a small proportion of vaccinees in the RV144 study which used a canarypox vector for delivery. Conversely, in the STEP study (HVTN 502) where human adenovirus serotype 5 (Ad5) was used, strong immune responses were induced but vaccination was more associated with increased risk of HIV acquisition than protection in vaccinees with pre-existing Ad5 immunity. The possibility that pre-existing immunity to a highly promising delivery vector may alter the natural course of HIV to increase acquisition risk is quite worrisome and a huge setback for HIV vaccine development. Thus, HIV vaccine development efforts are now geared toward delivery platforms which attain superior immunogenicity while concurrently limiting potential catastrophic effects likely to arise from pre-existing immunity or vector-related immuno-modulation. However, it still remains unclear whether it is poor immunogenicity of HIV antigens or substandard immunological potency of the safer delivery vectors that has limited the success of HIV vaccines. This article discusses some of the promising delivery vectors to be harnessed for improved HIV vaccine efficacy.

Keywords: HIV-1, vaccines, delivery vectors, MVA, NYVAC, adenovirus, ALVAC, DNA

INTRODUCTION

Thirty years after the discovery of HIV/AIDS, the search for a safe and effective vaccine has intensified, as a number of promising candidate vaccines progressing to phase IIb/III clinical trials have failed to show efficacy. One of the greatest barriers to HIV vaccine development is the enormous virion diversity (depicted by the existence of numerous clades and subtypes in distinct geographic demarcations) and the continuous evolution which generates numerous quasi-species within an infected individual (Hemelaar et al., 2011). This not only makes it challenging to create immunogens which are effectively matched to the circulating target viruses, but also provides room for immune escape of HIV from potent vaccine-induced immune responses. Therefore, it has emerged that immunogens derived from the most conserved regions of HIV and covering multiple variants (conserved mosaics) stand out as the most suitable candidates for T-cell based vaccines, while immunogens covering the most potent and broadly neutralizing and non-neutralizing antibody epitopes are better for antibody-based vaccines (Emini and Koff, 2004; Robinson and Amara, 2005; McMichael, 2006; Letourneau et al., 2007; Thorner and Barouch, 2007; Sekaly, 2008; Korber et al., 2009; Barouch et al., 2010; Santra et al., 2010; Borthwick et al., 2014). However, the development of a vaccine based on conserved antibody epitopes to provide protective global coverage and to minimize immune escape is hampered by inaccessibility of the highly shielded conserved envelope domains. Furthermore, the observation that development of broadly neutralizing antibodies requires prolonged stimulation with higher antigenic loads from divergent virus species (van Gils and Sanders, 2013) implies that HIV vaccine strategies must provide a continuous high level expression of a cocktail of immunogens. Although the use of polyvalent T-cell and B-cell mosaic constructs or the conserved consensus sequences may effectively overcome the challenges of HIV diversity and significantly improve vaccine efficacy (Santra et al., 2010, 2012), the lack of clearly defined correlates of efficacy means that it remains unclear what immune responses an HIV vaccine should aim to induce. Recently, a non-human primate (NHP) study based on the RhCMV vector induced exceptionally broad and persistent atypical CD8+ T cells which effectively cleared SIV and maintained durable suppression of virus replication (Hansen et al., 2009, 2011, 2013), suggesting that HIV vaccine development research may have to adapt immunogen design and delivery strategies that stimulate similar responses.

Delivery vectors are vital and integral components of a successful vaccine as they play an important role in modulating both innate and adaptive immunity. Therefore, vaccine vectors can significantly influence the magnitude and breadth, as well

as the phenotypic and functional qualities of vaccine-induced immune responses. Moreover, as the type of delivery vector, in conjunction with the route of vaccine administration often determine whether or not vaccine-specific immune responses persist within the systemic and/or mucosal compartments (Masopust et al., 2001; Kiyono and Fukuyama, 2004; Ranasinghe et al., 2007; Czerkinsky and Holmgren, 2012), vector choice remains a critical determinant of the overall efficacy of any given vaccine. A part from the immunostimulatory potential to induce strong and persistent immunity, several other factors such as stability and ease of large scale manufacturing, safety, capacity for transgene insertion and pre-existing immunity also influence vector choice. It is now well-documented that pre-existing anti-vector immunity (especially neutralizing antibodies) can prevent transduction and/or expression of vaccine transgenes thus reducing vaccine-specific immune induction (Xiang et al., 2002; Fitzgerald et al., 2003; Lasaro and Ertl, 2009). This is a common phenomenon, clearly demonstrated with certain vectors which show superior immunogenicity in animal models yet induce only modest immune responses due to neutralization by pre-existing antibodies in humans (McCoy et al., 2007). Additionally, pre-existing immunity can alter the natural course of infection leading to catastrophic consequences such as enhanced HIV acquisition and possibly accelerated disease progression (Buchbinder et al., 2008; McElrath et al., 2008). Thus strategies that concurrently maximize vaccine immunogenicity while minimizing safety concerns remain an urgent priority in the development of a safe and efficacious vaccine for HIV/AIDS.

A good number of HIV vaccine candidates (both prophylactic and therapeutic) employing a broad range of vaccine delivery vectors have been tested and some have progressed to evaluation of potential efficacy in phase IIb/III trials. Of significant relevance as far as safety is the STEP trial that used human adenovirus serotype 5 (Ad5) to deliver a well-designed HIV immunogen expressing Gag/Pol/Nef, which was associated with increased risk of HIV acquisition in uncircumcised male vaccinees with pre-existing immunity to Ad5 (Buchbinder et al., 2008; McElrath et al., 2008). This unexpected and rather worrisome finding prompted the premature halting of two related efficacy trials due to futility (Gray et al., 2011; Hammer et al., 2013). As disappointing as this might have been at the time, invaluable lessons have been learned and there is still great optimism as these lessons are now taken on board. Focussing on some of the promising HIV vaccine candidates in preclinical and clinical development, this review discusses pertinent issues relating to safety and immunogenicity of replicating and non-replicating viral vectors, pre-existing anti-vector immunity and how these can potentially influence the natural history of HIV infection and progression. In particular, this article highlights the safety profiles, immuno-stimulatory potential and possible limitations of plasmid DNA, MVA (modified vaccinia virus Ankara), ALVAC (canarypox virus), NYVAC (New York attenuated vaccinia virus), influenza virus and adenovirus vectored vaccines in preclinical and clinical studies for HIV vaccines. Some of the delivery vectors evaluated in clinical studies are summarized in **Table 1**, while those in preclinical development are summarized in **Table 2**.

RECOMBINANT DNA VACCINE VECTORS

DNA plasmid vaccines can induce both T and B cell immune responses, and are popular for their safety, stability, versatility and ease of large scale production. Most importantly is the fact that they can be used repetitively to boost immunity (Valentin et al., 2010) without the risk of immune interference as is the case with viral vectors with high prevalence of pre-existing immunity. However, on their own DNA plasmid vaccines have exhibited very limited immunostimulatory capacity and often induced sub-optimal immune responses. Recent advances in DNA delivery such as intramuscular, skin or intradermal electroporation (Selby et al., 2000; Widera et al., 2000; Brave et al., 2010; Vasan et al., 2011; Kopycinski et al., 2012) or use of other physical delivery methods such as gene gun and biojector devices (Drape et al., 2006; Wang et al., 2008a; Graham et al., 2013), together with concurrent use of cytokine adjuvants including IL-2, IL-12, and IL-15 (Winstone et al., 2011; Kalams et al., 2012, 2013) have greatly improved the immunogenic potential of DNA vaccines. In particular, IL-12 was shown to significantly augment the frequency, magnitude and breadth of Gag-specific immune responses in healthy volunteers immunized with a recombinant DNA vaccine expressing HIV-1 Gag (Kalams et al., 2012, 2013). Similarly, when macaques were co-immunized with a plasmid encoding IL-12 and a DNA plasmid expressing SIV-Gag, strong antibody and cellular responses which correlated with a better clinical outcome were induced (Boyer et al., 2005; Chong et al., 2007). More impressively, co-delivery of a plasmid encoding GM-CSF with a DNA vaccine expressing SIV genes induced strong neutralizing antibody responses and ADCC, which protected against infection with SIVsmE660 (Lai et al., 2011). The use of strong adjuvants such as glucopyranosyl lipid A (a TLR4 agonist) in a DNA/MVA/protein immunization regimen was shown to enhance both antibody and T cell responses (McKay et al., 2014), while plasmids encoding the TLR5 agonist, flagellin, enhanced both antibody and T cell immunity to influenza virus (Applequist et al., 2005)

Other significant improvements in DNA vaccine technology include codon optimization, use of stronger promoters/enhancers and signal peptides such as the tissue plasminogen activator (tPA) and lysosome associated membrane protein (LAMP1), all of which significantly enhance transgene expression and trafficking, thus leading to increased vaccine immunogenicity (Wang et al., 2006a; Yan et al., 2007; Wallace et al., 2013). Furthermore, ease of DNA manipulation provides a platform to deliver polyvalent or multi-gene vaccine components which can increase the breadth and depth of vaccine-induced immunity to reduce immune escape. This strategy showed remarkable success in rabbit experiments where a polyvalent gp120 vaccine induced broadly neutralizing antibody responses as opposed to the monovalent vaccine (Wang et al., 2006b). Similarly, polyvalent mosaic plasmid DNA vaccines have demonstrated enhanced immunogenicity in mice (Kong et al., 2009) and rhesus monkeys (Santra et al., 2010).

Several studies indicate that delivery of DNA vaccines by electroporation induces both cellular and humoral immune responses which are long-lived and can persist for several years with or without subsequent heterologous boosting (Cristillo et al.,

Table 1 | Representative clinical studies.

Study name and phase	Immunogen	Vectors, regimen and route of immunization	Immune responses generated	References
(I) HETEROLOGOUS PRIME-BOOST STUDIES				
HIVCORE002 (Phase I study)	HIVconsv (T cell immunogen based on conserved regions)	ChAdV63/MVA (i.m.) DNA/ChAdV63/MVA (i.m.) DNA/MVA/ChAdV63 (i.m.)	-CD4+ and CD8+ T cells -*In vitro* virus inhibition	Borthwick et al., 2014
HVTN 505 (Phase IIb study)	VRC-HIVDNA016-00-VP/VRC-HIVADV014-00-VP	DNA-prime (i.m. biojector device)/rAd5 boost (i.m. needle and syringe)	-T cells and gp140 binding IgG antibodies	Hammer et al., 2013
HVTN 503/Phambili (Phase IIb study)	MRKAd5 HIV-1 Gag/Pol/Nef	DNA-prime (i.m.)/Ad5 boost (i.m.)	-CD8+ and CD4+ T cells	Gray et al., 2011
Phase 1 study	Gag and Env DNA and recombinant trimeric Env glycoprotein	DNA-prime (i.m.)/Protein boost with MF59 adjuvant	-Robust B and T cells -Strong NAbs to SF162 -ADCC and neutralization of tier 2 strains	Spearman et al., 2011
Phase I/II study	Multi-clade, multigene: DNA/HIV-1 gp160, p17/p24 Gag and MVA/HIV-1 Gag/Pol	Low dose (i.d.) DNA-prime (x3)/MVA-boost (i.m. x2) (DDDMM)	-High magnitude and broad CD4+ and CD8+ T cell responses -Env antibodies	Bakari et al., 2011
Phase I study DP6-001	Multigene polyvalent gp120 and Gag DNA and polyvalent gp120 protein	i.m. or i.d. Polyvalent DNA-prime/i.m. protein-boost (with QS21 adjuvant)	-High titer binding and BNAbs, ADCC and multifunctional T cells	Bansal et al., 2008; Vaine et al., 2010
RV144 (Phase III study)	ALVAC-HIV vCP1521/AIDSVAX gp120 B/E	ALVAC-prime (i.m.)/gp120 protein-boost	-T cells and non-neutralizing antibodies to V1/V2 loop	Rerks-Ngarm et al., 2009
Phase I study	Multigenic HIV DNA (gp160- A/B/C; Rev B, Gag A/B and RT- B and HIV-MVA Env/Gag/Pol)	DNA- prime (i.d. with Biojector)/MVA-boost (i.d./i.m.); with or without GM-CSF adjuvant	-Broad and potent cellular immune responses	Sandstrom et al., 2008; Gudmundsdotter et al., 2009
HVTN 502/STEP Study (Phase IIb)	MRKAd5 HIV-1 Gag/Pol/Nef	DNA-prime (i.m.)/Ad5 boost (i.m.)	-Strong CD8+ T cell responses	Buchbinder et al., 2008; McElrath et al., 2008
Phase 1 study	HIVA (HIV-1 clade A and a CTL epitope string)	DNA-prime (i.m.)/MVA-boost (i.m.)	-Multifunctional CD4+ and CD8+ T cells	Mwau et al., 2004; Goonetilleke et al., 2006
Phase I study (EuroVacc: EV02)	HIV-1 clade C-Env/Gag/Pol/Nef (DNA-C and NYVAC-C)	DNA-prime (i.m.)/NYVAC- boost (i.m.)	-Durable, broad and poly-functional CD4+ and CD8+ T cells	Harari et al., 2008; McCormack et al., 2008
Phase I study	ALVAC-HIV(vCP300) gp120/gp41, Gag, Pro, Nef, Pol and SF-2 rgp120	ALVAC-prime (i.m.)/i.m. Protein-boost (with MF59 adjuvant)	-Durable CTLs -Antibody responses	Evans et al., 1999
Phase I study	ALVAC-HIV(vCP205) gp120/gp41, Gag, Pol and SF-2 rgp120	ALVAC-prime (i.m.)/i.m. Protein-boost (with MF59 adjuvant)	-Strong CD8+ T cell responses and NAbs	Belshe et al., 1998

(Continued)

Table 1 | Continued

Study name and phase	Immunogen	Vectors, regimen and route of immunization	Immune responses generated	References
(II) HOMOLOGOUS PRIME-BOOST OR SINGLE DOSE STUDIES				
HVTN-070 and -080 Phase I studies	PV (PENNVAX(R)-B DNA expressing Gag, Pol, Env and DNA/IL-12	DNA+IL-12 (i.m. or by electroporation)	-CD4+ and CD8+ T cell responses	Kalams et al., 2013
IPCAVD-001	Ad26.ENVA.01	Intramuscular delivery of rAd26	-Binding antibodies -Multiple CD8+ and CD4+ T cell responses -ADCC and virus inhibition	Baden et al., 2013; Barouch et al., 2013
HVTN 090 Phase Ia study	$VSV_{IN}N4CT1_{Gag}1$ (recombinant VSV expressing HIV-1 Gag)	Dose-escalating i.m. delivery	Low level T cell responses detected following initial dosing	Fuchs et al., 2012, 2013
Phase I study	Ad35-GRIN (Gag, RT, Integrase, Nef) and Ad35-GRIN/ENV	Intramuscular delivery of Ad35-GRIN/Env or Ad35-GRIN	-Robust, broad and polyfunctional CD4 and CD8+ T cells	Keefer et al., 2012
Phase I/II study (RISVAC02)	MVA-B (monomeric gp120 and clade B Gag/Pol/Nef poly-protein)	Three doses of MVA (i.m.)	-Durable antibody and cellular immune responses	Garcia et al., 2011; Gomez et al., 2011
Phase I study	ADVAX (multigenic HIV-1 DNA vaccine)	DNA by i.m. electroporation	-CD4 and CD8+ T cells with multiple cytokines	Vasan et al., 2011
VAX 003 (Phase III study)	Bivalent recombinant gp120 vaccine: AIDSVAX B/E	Seven i.m. injections; with Alum adjuvant	-Binding and neutralizing antibodies to gp120	Pitisuttithum et al., 2006
VAX 004 (Multicentre Phase III study)	Bivalent recombinant gp120 vaccine: AIDSVAX B/B	Seven i.m. injections; with Alum adjuvant	-Binding and neutralizing antibodies to gp120	Flynn et al., 2005; Gilbert et al., 2005

i.m., intramuscular; i.n., intranasal; i.d., intradermal; s.c., subcutaneous; i.p., intraperitoneal; ADCC, antibody dependent cytotoxicity; NAbs, neutralizing antibodies; BNAbs, broadly neutralizing antibodies.

2008; Patel et al., 2010; Jalah et al., 2014). In particular, the level of HIV-specific immune responses to the multigenic ADVAX vaccine was increased by up to 70-fold when electroporation was used for delivery (Vasan et al., 2011). Nonetheless, DNA vaccines consistently show much better immunogenicity when used as priming components in conjunction with viral vectors such as adenoviruses (Shiver et al., 2002; Hammer et al., 2013; Borthwick et al., 2014), MVA (Sandstrom et al., 2008; Gudmundsdotter et al., 2009; Bakari et al., 2011; Borthwick et al., 2014), fowlpox (Kent et al., 1998), and NYVAC (Hel et al., 2001) in heterologous prime boost regimens delivering the same vaccine inserts, or in co-immunization strategies that combine DNA-prime with protein boosting (Kennedy et al., 2008; Wang et al., 2008b). As a matter of fact, prime-boost regimens still remain the most successful strategies that emphasize the potential of DNA vaccines. It was recently shown that a DNA-prime/protein-boost regimen was significantly better than either DNA/DNA or protein/protein alone regimens for generating long-term protection of mice against *Leishmania donovani* (Mazumder et al., 2011). The DNA and protein co-immunization modalities are particularly desirable as they maximize induction of long-lived humoral and cellular immune responses which can disseminate to mucosal sites, including the genito-rectal mucosae (Patel et al., 2013; Jalah et al., 2014). A recent study has demonstrated in small animal models that concurrent, multiple-route DNA vaccinations comprising DNA prime by electroporation, followed with intranasal, intramuscular, subcutaneous or transcutaneous homologous protein boost induced strong HIV-specific B and T cell responses (Mann et al., 2014). Independently, another study showed enhancement of HIV gp120-specific IgA responses in serum and mucosal secretions following a DNA env-prime and gp120 protein-boost delivered with novel carbohydrate-based adjuvants (Advax-M and Advax-P) which were specifically designed for mucosal and systemic immune enhancement (Cristillo et al., 2011). The tremendous effect of a DNA prime in enhancing antibody responses to protein vaccines was also documented in a Phase 1 clinical study, where intramuscular delivery of a DNA priming vaccine followed with recombinant protein boost stimulated higher frequencies of B and T cells, as well as higher neutralizing antibody titres and ADCC in contrast to immunization with protein alone (Spearman et al., 2011). Perhaps the most exciting of the DNA-prime/protein-boost studies is the 6-plasmid polyvalent

Table 2 | Representative preclinical studies.

Animals	Immunogen	Vectors, regimen and route of immunization	Immune responses generated	Outcomes	References
(I) HETEROLOGOUS PRIME-BOOST STUDIES					
Mice and rabbits	HIV Env/Gag-Pol-Nef DNA, MVA-C (HIV Env/Gag-Pol-Nef and CN54gp140 protein)	Intramuscular delivery of DNA/MVA/Protein with TLR4 (GLA-AF adjuvant) for protein boost	Antibody and T cell responses	–	McKay et al., 2014
Rhesus macaques	SIVmac239 Env/Gag DNA, rmIL-12 DNA and SIVmac239 protein vaccines	DNA-prime (by electroporation)/i.m. or i.d. Protein-boost, or DNA and protein co-immunization	Persistent mucosal Envelope-specific antibody responses	Enhanced immunity by the co-immunization modality	Jalah et al., 2014
Rhesus macaques	SIV-Gag mosaic SIV-Env mosaic SIV_{mac239} Env	DNA-prime (x3, i.m.) Ad5-boost (i.m.)	-NAbs -ADCC -Cellular responses	Protection against SIVsmE660 challenge	Roederer et al., 2014
Rhesus monkeys	DNA expressing SIVmac239 antigens + rmIL-12 and inactivated SIVmac239 virus particles as protein	DNA prime (i.m. followed by *in vivo* electroporation) /protein-boost	-SIV-specific CTLs -CD4+ and CD8+ memory T cells -Binding antibodies	-Protection from $SIV_{SM}E660$ acquisition -Reduced peak and chronic phase viremia	Patel et al., 2013
Mice	pCCMp24 rddVTT-CCMp24	DNA prime/Tiantan boost (i.m.)	Antibody and T cells	–	Excler et al., 2010; Liu et al., 2013
Rhesus macaques	SIV_{SME543}-Gag/Pol/Env	Prime-boost (i.m.) with: Ad26/MVA, Ad35/Ad26, DNA/MVA, MVA/Ad26	-NAbs -Binding antibodies -Cellular responses	Protection from SIVmac251 acquisition or disease progression	Barouch et al., 2012
Mice	Ad35-GRIN/ENV and MVA-C (Gag/Env/Pol)	Ad35-GRIN/ENV-prime (i.m.)/MVA-boost (i.m.)	Polyfunctional CD8+ T cells	–	Ratto-Kim et al., 2012
Macaques	SIV DNA/GM-CSF (SIV239 Gag/PR/RT/Env/Tat/Rev) and MVA-SIVgpe	DNA/GM-CSF- prime (i.m.)/MVA-boost (i.m.)	-Neutralizing antibody responses -ADCC	Sterile protection after SIVsmE660 challenge	Lai et al., 2011
Murine	DNA-Env and gp120 protein vaccines	DNA Env-prime/gp120 protein-boost (i.m. and i.n.) (Advax-M and Advax-P adjuvants)	-Persistent mucosal and systemic Abs -T cell responses	–	Cristillo et al., 2011
New-born and adult mice	BCG-HIVA, MVA-HIVA and HAdV5.HIVA	BCG-prime (i.p./i.d./s.c.) followed with i.m. MVA- or HAdV5- boost	-Strong, cytotoxic CD8+ T cell responses	–	Hopkins et al., 2011a; Saubi et al., 2011
Rhesus macaques	VSV and SFV replicon expressing SIV-Gag/Env	VSV-prime (i.m. and i.n.)/SFVG-boost (i.m.)	-High titer NAbs to Env proteins and weak cellular responses	-Sterilizing immunity Control of SIVsmE660 breakthrough infections	Schell et al., 2011
New-born macaques	VSV-SIVgpe (rVSV-Gag/Pol/Env) and MVA-SIVgpe	VSV-prime (oral)/MVA-boost (i.m.)	-Systemic Abs, both systemic and local cellular responses	–	Van Rompay et al., 2010

(Continued)

Table 2 | Continued

Animals	Immunogen	Vectors, regimen and route of immunization	Immune responses generated	Outcomes	References
Mice, rabbits and macaques	Consensus or Polyvalent mosaic DNA and protein (gp120) vaccines	DNA-prime (i.m.)/i.m. and i.d. rVaccinia-boost. DNA-prime (gene gun)/ Protein-boost (i.d.) + IFA	-Broadly neutralizing antibodies and CD8+ T cell responses	Enhanced immunogenicity	Wang et al., 2006b; Santra et al., 2010
Rhesus macaques	VSV-SHIVGag/Pol/Env MVA-SHIVGag/Pol/Env	VSV-prime (i.m.)/MVA-boost (i.m.)	-Persistent multi-functional CD8+ T cells and NAbs	Durable (over 5 years) control of SHIV89.6P replication	Rose et al., 2001 Schell et al., 2009
Rabbits macaques	HIV-1 Env gp120	DNA (electroporation)/gp120 protein boost	-Persistent Th1, CTL and Env responses	Neutralization of sensitive SHIV isolates	Cristillo et al., 2008
Rhesus macaques	CMV-SHIVdEN and SeV-Gag	DNA prime (i.m.)/Sendai Virus boost (i.n.)	-CD8+ T cells	Durable control of SIVmac239 and SHIV89.6PD	Matano et al., 2001; Takeda et al., 2003; Kawada et al., 2007
Rhesus Macaques	replication-defective SHIV particles and MVA-SHIV (SIV Gag, SIV Pol and HIV Env)	Intrarectal DNA prime/MVA boost	-Antibodies in plasma -Cellular responses	-Preserved CD4 T cells -Reduced disease progression after SHIV 89.6P challenge	Wang et al., 2004
Rhesus macaques	SHIV-DNA plus IL-2 and rMVA	DNA + IL-12-prime (i.n.)/MVA-boost (i.n.)	-Mucosal and systemic antibody and cellular responses	Protection from SHIV 89.6P challenge	Bertley et al., 2004
Mice and monkeys	E1/E3-deleted AdHu5 and E1-deleted AdC7 or AdC6, expressing Gag37	i.m. prime-boost with: AdC7/AdC6/AdHu5 or AdHu5/AdC6/AdC7	-Robust CD8+ CD4+ T cells -Antibody responses	–	Reyes-Sandoval et al., 2004
Cynomolgus macaques	DNA- HIV-1 IIIB Env/Gag/RT/Rev/Tat/Nef, MVA- HIV-1 IIIB Nef-Tat-Rev, SIVmacJ5 Gag/Pol and Vaccinia HIV-1 Env	DNA prime/MVA boost (i.m. or mucosally)	-Antibody and cellular responses	Protection from infection	Makitalo et al., 2004
Mice	HIV-1 Env IIIB Ag (DNA-Env and MVA-Env)	DNA-Env-prime/MVA-Env-boost (i.n. with Cholera toxin adjuvant)	-Mucosal CD8+ T cells, mucosal and systemic antibodies -Beta-chemokines	–	Gherardi et al., 2004
Rhesus monkeys	DNA, MVA and Ad5 vectors expressing SIVmac239 Gag	DNA Prime (i.m.)/MVA- or Ad5- boost (i.m.)	-Robust CD8+ T cells with cytotoxic activity	Pronounced attenuation of SHIV infection and mitigated disease progression	Shiver et al., 2002
Macaques	DNA and NYVAC SIV-gpe (Gag/Pol/Env)	DNA-prime (i.m.)/NYVAC-boost (i.m.)	-Durable CD8+ T cell responses	–	Hel et al., 2001
(II) HOMOLOGOUS PRIME-BOOST OR SINGLE DOSE STUDIES					
Mice and rabbits	Ad4Env160 Ad4Env140 Ad4Env120	i.m., i.n., or s.c. delivery of rAd4	-T cell and antibody responses	Neutralization of tier-1 and tier-2 pseudoviruses	Alexander et al., 2013

(Continued)

Table 2 | Continued

Animals	Immunogen	Vectors, regimen and route of immunization	Immune responses generated	Outcomes	References
Mice	Ad35-GRIN/ENV and MVA- Gag/Env/Pol	Ad35-prime (i.m.)/Ad35-boost i.m.): MVA-prime (i.m.)/MVA-boost (i.m.)	-Polyfunctional CD8+ T cells	–	Ratto-Kim et al., 2012
Rhesus macaques	SIV_{SME543}-Gag/Pol/Env	MVA-prime (i.m.)/MVA-boost (i.m.)	-Neutralizing Abs, binding antibodies and cellular responses	Protection from SIVmac251 acquisition or disease progression	Barouch et al., 2012
Rhesus macaques	RhCMV-SIV/Gag, Rev/Nef/Tat, Pol, Env	RhCMV vectors delivered by s.c. injection	-Strong and persisting, polyfunctional effector memory CD8+ and CD4+ cells	Viral clearance and durable protection from SIVmac239 disease progression	Hansen et al., 2009, 2011
Rhesus monkeys	SIV-Gag, SIV-Env and SIV Rev-Tat-Nef fusion protein	Intravenous delivery of recombinant Rhadinovirus	-Persistent effector memory CD8+ T cells	Control of SIVmac239 replication	Bilello et al., 2011
Rhesus macaques	Rabies virus (RV) expressing SIVmac239 Gag/Pol or Env	Intramuscular delivery of rRV constructs	-Polyfunctional CD8+ T cells in the mucosa -NAbs	Control of SIVmac251-CX challenge	Faul et al., 2009
Rhesus and Cynomolgus macaques	SIV-Gag DNA + rIL-12 DNA vaccines	Intramuscular DNA delivery	T cell and Antibody responses	Improved clinical outcome after SHIV[89.6P] challenge	Boyer et al., 2005; Chong et al., 2007
Juvenile and Infant Rhesus macaques	ALVAC-SIV and MVA-SIV both expressing SIV-Gag/Pol/Env	Multiple immunizations with ALVAC-SIV (i.m.) or MVA-SIV (i.m.)	-High titres of binding antibodies, low-level T cell responses	Protection from oral SIVmac251 challenge, and reduced viremia in breakthrough infections	Van Rompay et al., 2005
Mice	HIV-1 Env IIIB Ag (DNA-Env and MVA-Env)	MVA-Env/MVA-Env DNA-Env/DNA-Env (i.n. with Cholera toxin adjuvant)	-Mucosal CD8+ T cells, mucosal and systemic antibodies -Beta-chemokines	–	Gherardi et al., 2004
Mice	Influenza virus expressing HIV-1 ELDKWA epitope	i.n. prime/boost with chimeric influenza virus, followed with i.p. boost with live virus	-Neutralizing antibodies	Neutralization of distantly related HIV-1 isolates	Muster et al., 1994

i.m., intramuscular; i.n., intranasal; i.d., intradermal; s.c., subcutaneous; i.p., intraperitoneal; ADCC, antibody dependent cytotoxicity; NAbs, neutralizing antibodies; BNAbs, broadly neutralizing antibodies.

DNA vaccine expressing gp120 and Gag, followed by QS21-adjuvanted polyvalent gp120 protein boost (DP6-001 study) in which multifunctional T cells and high-titre gp120-specific binding and broadly-neutralizing antibodies as well as ADCC were induced (Graham et al., 2006; Bansal et al., 2008; Wang et al., 2008b; Vaine et al., 2010).

Apart from effective delivery strategies and routes of immunization, there is evidence showing that expression of DNA vaccines and subsequent immunogenicity in humans and other primates can be limited by serum amyloid P component (SAP), a protein found in blood and known to bind strongly to DNA (Wang et al., 2011, 2012). In small animals this protein either binds weakly or does not exist at all. Thus, depletion of SAP protein prior to administration of DNA vaccines is another new strategy being tested to improve DNA vaccine immunogenicity. This concept has been proven in mice, where depletion of SAP using the bis-d-proline compound CPHPC (Bodin et al., 2010; Gillmore et al., 2010) was shown to augment antibody and cellular immune responses to a DNA vaccine expressing Hepatitis B surface antigens (Wang et al., 2012). The concept is currently

being tested in a Phase 1 clinical trial (HIVCORE003) of healthy adults using the T-cell based HIV candidate vaccine, HIVconsv.

Although the efficacy of an HIV DNA vaccine is yet to be demonstrated in humans, various studies (prophylactic and therapeutic) in the macaque model have reported protective immune responses which controlled SIV/SHIV replication or protected from infection (Rosati et al., 2005, 2009; von Gegerfelt et al., 2007; Valentin et al., 2010; Patel et al., 2013). In particular, a study combining a DNA/MVA mucosal delivery of a DNA construct expressing replication-defective SHIV particles and MVA expressing SIV-Gag/Pol and HIV Env (MVA-SHIV) demonstrated significant protection from disease progression after a SHIV89.6P challenge (Wang et al., 2004). Furthermore, mucosal co-delivery of a DNA priming vaccine together with an IL-2 encoding vector, followed by MVA boost also induced protective immunity against SHIV89.6P challenge (Bertley et al., 2004). The results in these macaque models, together with the documented efficacy of DNA vaccines against animal diseases [e.g., equine West Nile Virus (WNV) (Davis et al., 2001), melanoma in dogs (Bergman et al., 2003) and infectious hematopoietic necrosis virus (IHNV) in salmon (Garver et al., 2005; Kurath et al., 2006)] raise hopes that with the right immunogen and effective delivery strategies (including adjuvants), plasmid DNA vaccines for HIV/AIDS could achieve efficacy in clinical trials, when used alone, but more realistically in prime-boost combinations with live viral-vectored or protein vaccines.

NON-REPLICATING RECOMBINANT VIRAL VECTORS

ADENOVIRUS VACCINE VECTORS

Adenoviruses are the most powerful vectors for inducing both antibody and cell-mediated immunity to inserted transgenes and are known to elicit between 5- and 10-fold stronger T cell responses compared to conventional naked DNA or MVA/pox-like virus vectors (Xiang et al., 1996; He et al., 2000; Fitzgerald et al., 2003; Casimiro et al., 2003a, 2004; Tatsis and Ertl, 2004; Catanzaro et al., 2006). The Adenovirus vectors use either the Coxsackie and Adenovirus Receptor (CAR) or CD46 receptors (Bergelson et al., 1997; Gaggar et al., 2003) and can infect a wide variety of cells, including dendritic cells. In particular, group B adenoviruses such as Ad35 recognize CD46 surface protein and infect DCs more efficiently than group C isolates. These vectors achieve higher levels of transgene expression which in turn results in stronger and persistent immune effector functions (Zhang et al., 2001; Hutnick et al., 2010; Suleman et al., 2011). Several studies indicate that adenoviruses predominantly stimulate persistent effector memory CD8+ T cell responses (Yang et al., 2003a, 2007a; Tatsis et al., 2007a) which are more suitable for immediate control of invading pathogens at peripheral entry sites such as the genital mucosa (Cerwenka et al., 1999; Sallusto et al., 2004; Huster et al., 2006), and have shown tremendous success in animal studies (Liu et al., 2009). In addition to the effector memory T cells, stable central memory CD8+ T cell populations are also generated, thus providing surveillance in both peripheral and lymphoid sites. Although persisting adenovirus-driven immune responses could also be due to the long-term presentation of antigens by non-haematopoietic cells serving as unlimited antigen depot (Finn et al., 2009; Kim et al., 2010; Bassett et al., 2011), long-lived immunity is largely attributed to persisting low-level expression of inserted immunogens. Adenovirus genomes are known to persist for prolonged periods in various cell types (including those at inoculation sites) where they remain transcriptionally active and continuously produce low-levels of antigen to prime naïve T cells while also maintaining the effector memory T cells (Yang et al., 2006, 2007b; Tatsis et al., 2007a). Furthermore, the arising effector memory T cells express the IL-7 receptor (CD127) which allows their prolonged survival in the absence of antigen. Besides induction of potent adaptive immune responses, adenoviruses also stimulate innate immunity via highly inflammatory responses which involve TLR2, TLR9, NOD-like receptors and the type 1 interferon pathways that result in abundant cytokine and chemokine secretion (Hensley et al., 2005; Nazir and Metcalf, 2005; Appledorn et al., 2008; Muruve et al., 2008). Another attractive feature of adenovirus vectors is their ability to induce both systemic and mucosal immune responses following parenteral delivery, as well as their suitability for mucosal immunization (Sharpe et al., 2002; Xiang et al., 2003; Bangari and Mittal, 2006; Haut et al., 2010).

The most well-characterized of the adenovirus vectors is human Ad5, successfully used as a delivery vector for a rabies vaccine and found to be very good at inducing protective virus neutralizing antibodies concurrently with CD8+ and CD4+ T cells (Xiang et al., 1995, 1996). In the HIV field, Ad5 was used as a booster immunization following DNA priming and induced strong CD8+ T cell responses in a large proportion of the STEP study vaccinees (Buchbinder et al., 2008; McElrath et al., 2008). However, clinical efficacy may have been significantly compromised by pre-existing neutralizing antibodies (ranging from 40 to 70% in developed countries and greater than 90% in developing countries) and cellular immunity (Fitzgerald et al., 2003; Holterman et al., 2004; Bangari and Mittal, 2006; Xiang et al., 2006; Lasaro and Ertl, 2009; Ersching et al., 2010; Mast et al., 2010; Barouch et al., 2011). These results were recapitulated in a non-human primate study using low-dose penile exposure to SIVmac251 in Ad5 seropositive animals immunized with SIV-Gag/Pol/Nef (Qureshi et al., 2012). Possibly, adenovirus vaccination boosted the numbers of activated CD4+ T cells which are targets for HIV-1 (Benlahrech et al., 2009). While this might seem a plausible explanation, especially when considering the potential of such activated targets to traffic to the genito-rectal mucosae (Tatsis et al., 2007a; Benlahrech et al., 2009), this argument is strongly contested by observations that other vaccine carriers such as DNA and MVA do stimulate CD4+ T cell activation but have not been associated with increased HIV acquisition. However, it is worth noting that DNA/MVA vaccines are yet to be tested for efficacy in large clinical trials and as such their potential to enhance HIV acquisition has never assessed. Furthermore, DNA/MVA vaccines combinations have not been associated with long-term persistence of activated T cells or mucosal homing. Another postulated theory is the formation of adenovirus-specific antibody immune complexes that activate both dendritic and CD4+ T cells hence fuelling infection (Perreau et al., 2008). In this study, Ad5 immune complexes were strongly correlated with higher HIV infection in the *in vitro* cultures, thus supporting a stronger likelihood of enhanced HIV acquisition. Should either

or both of these theories be true, this would have dire consequences for other clinical trials using Ad5 to deliver non-HIV immunogens such as malaria (Sedegah et al., 2011; Tamminga et al., 2011; Chuang et al., 2013) and TB (Smaill et al., 2013) vaccines which will induce similar phenotypes and pre-dispose the vaccinees to increased HIV acquisition risk, although this may not be apparently detectable as these studies may not monitor HIV acquisition.

Apart from the issue of pre-existing immunity, immunization with Ad5 can induce neutralizing antibodies in naïve individuals which can be a hindrance for successive immunizations with the same or cross-reactive adenoviral vectors (Casimiro et al., 2003b; Bangari and Mittal, 2006). Thus new rare adenovirus vectors with lower pre-existing immunity such as Ad26 and Ad35 are becoming more attractive (Holterman et al., 2004; Abbink et al., 2007; Barouch et al., 2012; Zhang et al., 2013), although these are relatively less immunogenic compared to Ad5 (Colloca et al., 2012). Besides the lower sero-prevalence, Ad26 neutralizing antibody titres are usually very low compared to Ad5 (Abbink et al., 2007; Chen et al., 2010; Mast et al., 2010). As an HIV vaccine delivery vector, Ad26 was shown to induce broadly functional cellular and antibody responses with viral inhibitory capacity in a first-in-human (IPCAVD-001) clinical trial of an HIV envelope immunogen (Ad26.ENVA.01) (Baden et al., 2013; Barouch et al., 2013). In this study, a dose-dependent expansion of the magnitude, breadth, and epitopic diversity of Env-specific binding antibody responses were observed. The responses comprised multiple CD8+ and CD4+ T cell memory subpopulations and cytokine secretion phenotypes. Antibody-dependent cell-mediated phagocytosis and degranulation functional activity were also observed. Ad35 has also shown high immunogenicity in healthy volunteers, eliciting robust and polyfunctional CD8+ and CD4+ T cells in a majority of volunteers immunized with Ad35-GRIN (an immunogen based on Gag, RT, integrase and nef) or Ad35-GRIN/ENV (premixed Ad35-GRIN and Ad35-ENV vaccines) (Keefer et al., 2012). Similarly, in BALB/c mice, an Ad35-GRIN/ENV-prime followed by a boost with rMVA containing Gag/Env/Pol genes from various HIV-1 clades induced polyfunctional CD8+ Gag-specific central and effector memory T cells which were superior to those elicited in homologous Ad35/Ad35 or MVA/MVA prime boosts (Ratto-Kim et al., 2012).

Other rare adenovirus vectors include human Ad6, chimpanzee Ad3, Ad63, and Ad68 (Barnes et al., 2012; Colloca et al., 2012; Dicks et al., 2012; O'Hara et al., 2012; Roshorm et al., 2012). The chimpanzee adenoviruses remain attractive in particular due to their high immunological potency and low sero-prevalence, as well as extremely low or virtually absent cross-reactivity with human adenoviruses (Xiang et al., 2006; Chen et al., 2010; Colloca et al., 2012). Furthermore, chimpanzee adenoviruses induce stronger T and B cell responses in heterologous prime-boost regimens even in the presence of pre-existing immunity to Ad5 (Tatsis et al., 2009). Apart from using these naturally occurring human and chimpanzee adenoviruses, new derivatives of adenovirus vectors that have equivalent immunogenicity but with significantly lower pre-existing antibodies are currently being developed (Dicks et al., 2012; Lopez-Gordo et al., 2014). However, it is worth noting that pre-existing cellular immunity (CD8+ and CD4+ T cells) may be a major deterrent as unlike antibodies, these cells are highly cross-reactive across adenovirus serotypes because they are directed to conserved sequences of adenovirus (Olive et al., 2002; Fitzgerald et al., 2003; Frahm et al., 2012). Nevertheless, some studies indicate that Ad5 and Ad26 vectors can still elicit significant systemic and mucosal responses even in people with pre-existing immunity (Barouch et al., 2013; Smaill et al., 2013). Immunogenic adenoviruses faced with significant pre-existing immunity problems can be improved by modification of the antibody-binding sites, especially within the variable hexon loops in order to reduce NAb binding whilst maintaining immunogenicity (Bruder et al., 2012). This can be achieved via point mutations or complete replacement (Roberts et al., 2006; Abe et al., 2009; Pichla-Gollon et al., 2009; Bruder et al., 2013).

Besides their immunogenicity when used alone, adenovirus vaccines are also very immunogenic when used to prime responses which are then boosted by other vaccine vectors (Tatsis et al., 2007b; Ratto-Kim et al., 2012). In particular, adenovirus-prime followed with MVA-boost can induce high frequencies of much more long-lived, potent T cells (Reyes-Sandoval et al., 2008, 2010; Capone et al., 2010; Hill et al., 2010). A Phase I clinical trial of a T-cell HIV vaccine based on the conserved regions was recently shown to elicit exceptionally high magnitude and polyfunctional T cell responses (circa 5000 IFN-γ ELISPOT SFU/million cells) in HIV-negative healthy volunteers when primed with chimpanzee Ad63 (ChAdV63-HIVconsv) followed with MVA-HIVconsv boost (Borthwick et al., 2014). The vaccine-induced CD8+ T cells exhibited potent *in vitro* antiviral activity. This study also demonstrated that the magnitude and functional capacity of T cells induced in a regimen comprising three priming doses of DNA followed with ChAdV63 and MVA (DDDCM) did not differ significantly from those in a simplified ChAdV63-prime and MVA-boost (CM) regimen. The superior immunogenicity of this regimen is not unique to HIV immunogens, as it has also been demonstrated in preclinical and clinical studies of experimental malaria vaccines (Dunachie et al., 2006; Draper et al., 2010). Such repeated heterologous immunizations with the same transgene are known to increase both the magnitude and functional quality of vaccine-specific T cells and to allow more efficient migration to mucosal-associated tissues (Tatsis et al., 2007b). This is important in HIV infection, as effector immune cells in mucosal sites could block HIV transmission. It has also been shown that DNA priming followed with adenovirus boosting can reduce the level of anti-vector antibodies and increase transgene-specific immune responses (Xiang et al., 1999; Yang et al., 2003b), although this is questionable when considering the STEP study which employed a DNA-prime/Ad5-boost regimen. However, it is possible that this regimen effectively reduced the anti-vector antibody effect, thus curtailing a potentially worse outcome in the absence of DNA priming. Furthermore, prime-boost regimens with various combinations of adenovirus vectors were shown to induce robust frequencies of HIV-1 Gag-specific CD8+ T cells in nonhuman primates (Reyes-Sandoval et al., 2004), although it has to be appreciated that the level of pre-existing Ad5 immunity in NHPs would be lower or absent.

Adenoviruses are only associated with benign human pathologies, but their greatest limitation is pre-existing immunity which dampens vaccine-specific immunity by limiting transgene expression, while potentially exacerbating HIV acquisition. However, all else considered, Adenoviruses remain by far the most promising vaccine carriers for HIV-1, because unlike other vectors, they induce exceptionally high and persistent frequencies of vaccine specific T cells, which is a requirement for sustained HIV control. Although their efficacy has probably been hampered by high sero-prevalence, this no longer seems an insurmountable hurdle in light of the enormous amount of research efforts directed at finding strategies to circumvent the problems of pre-existing immunity (Gabitzsch et al., 2009). Additionally, replicating adenoviruses such as AdH4 and AdHu7 which can be delivered orally in the form of edible capsules might help to overcome pre-existing immunity (Xiang et al., 2003). Moreover, intranasal or oral delivery of adenoviruses has been shown to provide superior protection in animal models, and might trigger mucosal immune responses well-situated for preventing HIV acquisition. Perhaps adenovirus vectors engineered not to induce CD4+ T cells could be an alternative to overcome increased HIV-1 acquisition risk, although lacking CD4+ T cell help for the CD8+ T cells might compromise the differentiation and stability and thus efficacy of both CD8+ T cells and antibody responses (Yang et al., 2007b).

RECOMBINANT MVA (rMVA) VECTORS

Apart from their excellent safety profile, inherent adjuvant properties and ease of large scale production, recombinant vaccinia virus vectors are also popular for their large genomes which facilitate insertion of larger immunogens (Smith and Moss, 1983). MVA does not replicate in humans (Carroll and Moss, 1997) due to serial passaging in chick embryo fibroblasts which resulted in loss of more than 10% of its genome (Meyer et al., 1991), and its safety was well-documented during the smallpox eradication campaign (Mahnel and Mayr, 1994). MVA's potent immunostimulatory properties are achieved in a cascade of events involving induction of type 1 interferons, various chemokines for cell migration and activation of several cellular signaling pathways (Price et al., 2013). The immunostimulatory potency of MVA is largely attributed to the absence of genes involved in immune evasion (such as those that interfere with IFN-α, IFN-β, and TNF-α), thus allowing for stronger innate immunity to be generated (Antoine et al., 1998). MVA vectors are particularly important for generating strong T cell immunity against intracellular pathogens and cancers, but have also been shown to induce potent, high titre antibodies in a variety of disease models including SIV and malaria (Gherardi et al., 2003; Draper et al., 2008, 2013; Barouch et al., 2012). However, it is now well established that MVA vectors are more suited for boosting rather than priming, and depending on the priming vector (e.g., DNA or live vectors such as fowlpox and adenoviruses), MVA can induce various phenotypes of T cells, either predominated by CD4+ or CD8+ subsets or a combination of both.

In pre-clinical and clinical studies of malaria, recombinant MVA was shown to be highly immunogenic as it induced strong (and protective) cellular and antibody responses to malaria antigens, either on its own or when used to boost responses primed by vectors such as DNA, fowlpox or AdHu5 (Schneider et al., 1998, 1999; Gilbert et al., 1999, 2002; McConkey et al., 2003; Anderson et al., 2004; Webster et al., 2005; Bejon et al., 2007; Sheehy et al., 2011). Recombinant MVA85A (expressing the mycobacterial antigen Ag85A) was also shown to induce strong and durable T cell responses in various clinical studies (Scriba et al., 2012; Tameris et al., 2013, 2014). Furthermore, it was demonstrated that MVA expressing influenza A virus antigens (MVA-NP+M1) efficiently boosted CD8+ T cell responses to achieve clinical efficacy in humans (Berthoud et al., 2011; Lillie et al., 2012). As a therapeutic vaccine for cancer, recombinant MVA expressing the human papilloma virus antigens E2, E6, or E7, with or without IL-12 was shown to induce T and B cell immunity resulting in controlled HPV load and subsequent regression or complete elimination of precancerous lesions in a majority of vaccinees (Corona Gutierrez et al., 2004; Garcia-Hernandez et al., 2006; Albarran et al., 2007). Additionally, MVA expressing 5T4 antigen (TroVax) induced 5T4-specific antibody and cellular responses which correlated with tumor regression in a clinical trial of patients with advanced colorectal cancer (Harrop et al., 2006).

Although there is clear demonstration of the clinical efficacy of prophylactic and therapeutic MVA-vectored vaccines for malaria, TB, influenza virus and cancer, MVA vaccines for HIV are yet to be evaluated for clinical efficacy. However, Phase I and II studies of MVA expressing HIV antigens, either alone or in various prime-boost combinations indicate modest to strong immunogenicity (Guimaraes-Walker et al., 2008; Howles et al., 2010; Bakari et al., 2011; Garcia et al., 2011; Goepfert et al., 2011; Gomez et al., 2011). In particular, the MVA-B candidate HIV vaccine expressing monomeric gp120 and Gag-Pol-Nef poly-protein of clade B where MVA was administered without prior priming, induced long-lasting robust and polyfunctional effector memory T cell and antibody responses in Phase I/II studies (Garcia et al., 2011; Gomez et al., 2011). Furthermore, MVA has shown much higher immunogenicity when combined in prime-boost regimens with other priming vectors such as DNA, fowlpox or adenovirus (Goepfert et al., 2011; Keefer et al., 2011; Borthwick et al., 2014). In Phase 1 studies of the HIVA immunogen (based on HIV clade A and a string of CTL epitopes), priming with DNA (pTHr.HIVA) followed with MVA boosting (MVA.HIVA) was found to be immunogenic, inducing multifunctional and proliferative CD8+ and CD4+ T cell responses in greater than 70% of the vaccinees (Mwau et al., 2004; Goonetilleke et al., 2006).

As discussed earlier, a Phase I study combining DNA- and/or ChAdV63-prime followed with MVA boost to deliver an HIV-1 T cell immunogen induced high magnitude T cell responses with potent antiviral capacity (Borthwick et al., 2014). This study and similar studies of malaria vaccines (Sheehy et al., 2011, 2012; O'Hara et al., 2012) showed that the magnitude of T cell responses induced by ChAdV63 alone were modest, but significant boosting was achieved following MVA administration, thus highlighting the superior immunogenic potential of MVA when combined with appropriate priming vectors such as BCG (Whelan et al., 2009; Scriba et al., 2012), natural influenza A virus (Berthoud et al., 2011) or ChAdV63 (Colloca et al., 2012).

Remarkably, a DNA/MVA prime boost of a vaccine expressing multiple HIV antigens induced responses in about 90% of volunteers and demonstrated strong immunogenicity despite pre-existing immunity to vaccinia virus (Sandstrom et al., 2008). As a therapeutic HIV vaccine vector, rMVA was found to be safe and to significantly augment HIV-specific CD4+ and CD8+ T cell responses in HAART-treated HIV-infected volunteers immunized with the MVA.HIVA candidate vaccine (Dorrell et al., 2006; Ondondo et al., 2006; Yang et al., 2007c). Furthermore MVA was found to be safe in neonates in a Phase 1 trial where MVA.HIVA was administered to infants born to HIV-infected or uninfected mothers (Afolabi et al., 2013). Therapeutic administration of MVA prime followed with fowlpox boost expressing Env, Gag, Tat, Rev, and Nef-RT fusion antigens increased the frequencies and breadth of T cell responses in young adults (Greenough et al., 2008).

One very attractive feature of rMVA (and other poxvirus vectors) is their ability to induce mucosal immune responses when administered via mucosal routes (Gherardi and Esteban, 1999, 2005). In particular, murine and macaques studies using rMVA vectors demonstrated induction of protective HIV-specific immune responses within the genito-rectal mucosae, which in some cases correlated with reduced disease progression (Belyakov et al., 1998a; Makitalo et al., 2004; Wang et al., 2004). Enhanced immunogenicity of rMVA in combination with DNA priming was also achieved by using the non-toxic B subunit of cholera toxin (CTB) as mucosal adjuvant (Gherardi et al., 2004). Thus, even though MVA may be inadequate as a stand-alone delivery platform, it definitely shows greater potential as a boosting vector (especially for the chimpanzee adenoviruses) and should be evaluated for efficacy in advanced HIV vaccine trials.

RECOMBINANT NYVAC VACCINE VECTORS

NYVAC vector is also a vaccinia-based vector which was highly attenuated by deletion of 18 genes involved in host range virulence. It has been shown to induce mainly CD4+ T cell responses, in contrast to MVA which has a stronger immunostimulatory potential and is known to induce both CD8+ and CD4+ responses (Mooij et al., 2008). However, in a trial of chronically infected patients on HAART, a NYVAC-based vaccine expressing Gag/Pol/Nef/Env from an HIV-1 clade B isolate (NYVAC-B) was found to be highly immunogenic and induced high magnitude, broad and polyfunctional CD4+ and CD8+ T cells (Harari et al., 2012). Similar to MVA, NYVAC elicits greater immune responses when used in prime-boost combinations rather than on its own (Harari et al., 2008; McCormack et al., 2008). In these EuroVacc studies, priming with DNA-C followed with NYVAC-C boost elicited broad, polyfunctional and durable CD4+ T cell responses in greater than 90% of volunteers, compared to only 40% when NYVAC was used alone (Harari et al., 2008). Moreover, in a preclinical study with a DNA prime followed with NYVAC boost, responses to a vaccine expressing SIV-Gag/Pol/Env were boosted 10-fold with improved quality and quantity of T cell responses (Hel et al., 2001). A NYVAC/SIV-gpe vaccine (expressing SIV Gag/Pol/Env) also elicited mucosal immune responses in macaques following both mucosal and systemic delivery (Stevceva et al., 2002). Despite the skewing toward CD4+ T cell responses, NYVAC has potential to stimulate and boost more balanced immune responses when combined with other vectors, and its potential should be fully explored, especially for therapeutic HIV vaccines which require re-invigoration of CD4+ T cell functions (and frequencies).

CANARYPOX (ALVAC) VACCINE VECTORS

ALVAC is an attenuated derivative of the canarypox virus that was repeatedly passaged in chick embryo fibroblasts and thus has restricted tropism with very minimal pathogenicity in humans (Yu et al., 2006). Despite the comparatively lower immunogenicity with respect to other poxvirus vectors such as MVA (Zhang et al., 2007) and NYVAC, the fact that ALVAC has no potential pre-existing immunity in humans makes it a more attractive HIV vaccine delivery vector. The ALVAC vector (vCP205) was shown to be safe and to induce strong CD8+ CTL and antibody responses to an HIV vaccine expressing gp120/41 and Gag/Pol sequences [ALVAC-HIV(vCP205)] in a Phase 1 clinical trial in the USA in the 1990 s (Belshe et al., 1998). A related ALVAC-based vaccine expressing multiple HIV antigens comprising Gag, Env, Nef, Pol and Pro [ALVAC-HIV(vCP300)] also induced durable CTL responses in healthy volunteers (Evans et al., 1999). In preclinical studies, ALVAC expressing SIV Gag/Pol/Env protected against low-dose oral SIVmac251 challenge of neonate rhesus macaques in a study design aiming to mimic HIV transmission through breast milk (Van Rompay et al., 2005). More recently ALVAC-based HIV vaccines have been tested in both adults and infants, where they have shown modest immunogenicity (Kintu et al., 2013; Kaleebu et al., 2014) and in the RV144 trial of ALVAC prime [ALVAC-HIV(vCP1521)] and protein boost (AIDSVAX B/E rgp120), the only HIV vaccine candidate to show efficacy (Rerks-Ngarm et al., 2009, 2013).

While it is unclear whether the modest success of RV144 was due to the immunostimulatory potential of canarypox virus vector or immunogenicity of the vaccine inserts, the fact that the immunogens in the RV144 trial vaccines are not significantly distinct from those used in other HIV vaccines in the field eliminates the "immunogen effect," thus leaving the vectors and delivery methods as possible explanations. But, as the AIDSVAX vaccine (recombinant gp120) showed no efficacy in earlier trials (VAX003 and VAX004), the success of RV144 points to the delivery vector (ALVAC) and possibly the benefits of a combined viral vector and protein immunization regimen as opposed to homologous boosts. This might suggest that combined live vector-priming and protein-boost immunization modalities could be further refined to achieve greater potential for increased efficacy. Alternatively, protection by the combined vaccines could be attributed to T cell help for the antibody responses. It must however be noted that unlike the RV144 study, VAX003, and VAX004 were conducted in high-risk populations, which might be a strong confounding factor, although this might as well be reflective of the very limited efficacy of stand-alone protein subunit vaccines for HIV. Despite the modest efficacy of RV144, the immune responses waned within a short time indicating that ALVAC may not be a particularly suitable vector to induce long-lived anti-HIV immunity, unless it is combined with other powerful vectors. In

direct comparison of immunogenicity, ALVAC was found to be less immunogenic than MVA, possibly due to MVA's enhanced antigen expression within dendritic cells (Zhang et al., 2007). Nonetheless, ALVAC is still quite promising for HIV vaccine delivery, as it is also already licensed for delivery of several veterinary vaccines including the feline leukemia virus (FeLV) and feline rabies vaccine (PUREVAX) and RECOMBITEK vaccine which protects against canine distemper, equine influenza and West Nile Virus.

MYCOBACTERIUM BOVIS BACILLUS CALMETTE-GUERIN (BCG) VACCINE VECTORS

Prevention of breast milk transmission of HIV-1 remains an important goal for HIV vaccine researchers. BCG is an attenuated vaccine proven to be safe and has for many years been administered to new-born babies to immunize against Mycobacterium tuberculosis (Mtb). As such, BCG provides a platform to co-deliver HIV immunogens in neonates to potentially protect against mother-to-child transmission of HIV-1. The potential use of BCG as an HIV vaccine vector was explored in preclinical studies of adult and new-born BALB/c mice using the HIV-1 clade A Gag immunogen (HIVA) (Mwau et al., 2004). Priming with recombinant BCG expressing HIVA (BCG.HIVA) induced HIV-specific T cell responses which were efficiently boosted with rMVA (MVA.HIVA) (Hopkins et al., 2011a,b; Saubi et al., 2011, 2012). In further related studies, priming with BCG.HIVA and boosting with a combination vaccine expressing HIVA and the Mtb antigen 85A (mMVA.HIVA.85A) induced robust IFN-γ-producing T cells to both HIV-1 and Mtb antigens. Moreover, in adult mice, BCG.HIVA primed weak HIV-1-specific CD8+ T cell responses, which were strongly boosted with either Ad5 (HAdV5.HIVA) or rMVA (MVA.HIVA). Thus, immunization of neonates with recombinant BCG expressing HIV-1 immunogens, followed with an MVA boost expressing the same HIV immunogen might concurrently protect against Mtb and HIV-1. It remains to be seen how these rBCG-vectored HIV-1 vaccines will perform in clinical studies.

REPLICATION-COMPETENT VIRAL VECTORS

The unprecedented success of the SIVmac239Δnef experimental vaccine in rhesus macaques (Reynolds et al., 2008, 2010) gives a hint that possibly, a successful HIV vaccine will require a live delivery vector, as these are known to induce high magnitude, durable and broadly effective immunity. But as exciting as this may sound, there are significant challenges in terms of balancing the safety and immunogenicity vs. replicative capacity. Of the adenoviruses, Ad4 and Ad7 have been tested in clinical studies (by oral delivery) and were successfully used for the prevention of respiratory and enteric illnesses (Hoke and Snyder, 2013). These replication competent adenoviruses naturally infect and replicate in mucosal tissues (Patterson and Robert-Guroff, 2008) and could thus be quite relevant for HIV vaccines. Preclinical studies of recombinant Ad4 expressing HIV-1 clade C envelope gp160 (Ad4Env160), gp140 (Ad4Env140), and gp120 (Ad4Env120) demonstrated induction of envelope-specific T cells in mice and antibody responses in rabbits (Alexander et al., 2013). Serum from the rabbits was able to neutralize a tier 1 clade C pseudovirus and to a lesser extent, homologous and heterologous tier 2 pseudoviruses.

A replicating CMV vectored SIV vaccine (RhCMV-SIV/Gag, Rev/Nef/Tat, Pol, Env) was shown to persist in vaccinated rhesus macaques and conferred durable protection from disease progression owing to induction of high magnitude effector memory CD8+ T cells, despite pre-existing CMV immunity (Hansen et al., 2009, 2011, 2013). Other replication-competent viruses in clinical development include the TianTan vaccinia virus (TT), Vesicular stomatitis virus (VSV), a derivative of NYVAC (NYVAC-C-KC) and Sendai virus (SeV). The TianTan vaccinia virus was used in a DNA-prime (pCCMp24)/Tiantan boost (rddVTT-CCMp24) regimen where it was shown to induce antibody and HIV-specific T cell responses (including memory phenotypes) following intramuscular delivery and has now been advanced to Phase II clinical study in China (Excler et al., 2010; Liu et al., 2013). The NYVAC-C-KC vectors have shown superior cellular and humoral immunity compared to the non-replicating NYVAC, at least in mice (Kibler et al., 2011; Gomez et al., 2012).

A Sendai virus vector expressing SIV Gag (SeV-Gag) administered intranasally as a boost following intramuscular priming with an envelope-independent DNA vaccine (CMV-SHIVdEN) demonstrated very strong suppression of intravenous SIVmac239 challenge in rhesus macaques, which was extended over a 3-year period (Matano et al., 2001; Takeda et al., 2003; Kawada et al., 2007). Clinical investigations of a SeV-based candidate HIV vaccine expressing Gag [SeV-G (NP)] are ongoing in Rwanda, Kenya and the UK, and it is expected that results of these trials will provide a feel of the potential of Sendai virus as an HIV vaccine vector. Attenuated VSV is a non-pathogenic, low sero-prevalence vector that was also found to be quite promising as it achieved virus control during SHIV89.6P challenge experiments in rhesus macaques immunized with rVSV expressing Gag and Env (Rose et al., 2001). Recombinant VSV vector was shown to induce strong memory CTL responses to HIV-1 Gag and Env in mice, which were significantly amplified by boosting with heterologous recombinant vaccinia virus vectors (Haglund et al., 2002). It is postulated that intranasal delivery of rVSV vaccines in combination with IL-12 administered during DNA priming may elicit mucosal immunity for HIV (Egan et al., 2004, 2005). Priming with rVSV-Gag/Pol/Env (VSV-SIVgpe) followed with MVA-Gag/Pol/Env (MVA-SIVgpe) boost was shown to induce strong and long-lived antibody and cellular responses that achieved long-term control of SHIV replication (Schell et al., 2009; Van Rompay et al., 2010). An ongoing phase 1 trial of rVSV-HIV-1 Gag vaccine (HVTN090) has demonstrated clinical safety and T cell immunogenicity following intramuscular delivery (Fuchs et al., 2012, 2013), although the magnitude of responses was limited and will most likely require priming (or boosting) with suitable vectors.

Other vectors being explored include rhadinovirus (Bilello et al., 2011), yellow fever virus (Bonaldo et al., 2010), rabies virus (Faul et al., 2009), Venezuelan equine encephalitis virus (VEEV) (Caley et al., 1997) and Semliki Forrest virus (Schell et al., 2011), all of which have shown strong immunogenicity, with some achieving efficacy in NHP challenge protection models. Influenza virus vaccine vectors have also been

studied extensively and have been successfully used as delivery vehicles for several experimental HIV vaccines (Li et al., 1993a, 2013; Muster et al., 1994, 1995; Garcia-Sastre and Palese, 1995; Palese et al., 1997; Sexton et al., 2009). As natural mucosal pathogens, influenza virus vectors are well-adapted for stimulating robust mucosal and systemic immunity comprising both antibody and cellular immune responses (Garcia-Sastre and Palese, 1995; Palese et al., 1997; Li et al., 2013). Mucosal immunization of mice with chimeric influenza virus vectors expressing the HIV-1 gp120 V3 loop peptide (IHIGPGRAFTYTT) (Li et al., 1993a) or the gp41 epitope (ELDKWA) (Muster et al., 1993, 1994, 1995) was shown to induce persistent antibody and CTL responses. Influenza virus vectors might be successfully combined in prime-boost regimens as demonstrated in influenza virus-prime and MVA-boost studies in mice (Gherardi et al., 2003), although they have a limited capacity for immunogen insertion.

HETEROLOGOUS PRIME-BOOST STRATEGIES FOR ENHANCED HIV VACCINE EFFICACY

Repeated vaccination in heterologous prime boost approaches employing different vector combinations in a specific order is widely accepted as the most efficient means to induce superior quality and quantity of vaccine-specific immune responses (Li et al., 1993b; Ramshaw and Ramsay, 2000; Estcourt et al., 2002; McShane, 2002; Newman, 2002). Heterologous prime boost regimes allow immune boosting without creating problems of anti-vector immunity. Furthermore, heterologous prime-boosts result in increased frequencies of memory T cells, and it has been shown that the number of immunizations can significantly influence the phenotype of vaccine-specific memory T cells, with secondary and tertiary immunizations generating effector-like memory T cells which preferentially accumulate in non-lymphoid organs (Masopust et al., 2006; Nolz and Harty, 2011). These findings have huge implications on the quality and potential of mucosal surveillance of cells induced in prime-boost vaccination protocols.

Distinct live viral vectors can be combined in prime-boost regimes to maximize immune responses. In most studies DNA has been used for priming, but recently a number of virus vectors including Adenoviruses, influenza viruses as well as fowlpox and canarypox have been tested in prime-boost regimens. Prime-boost regimens comprising Adenovirus and MVA or heterologous Adenovirus strains have recently been shown to induce both cellular and humoral immune responses to SIV and malaria antigens (Draper et al., 2008; Liu et al., 2009; Tatsis et al., 2009; Barouch et al., 2012). In particular, impressive protection against SIV acquisition in rhesus monkeys was achieved following immunization with a SIV_{SME543}-Gag/Pol/Env vaccine delivered by Ad26/MVA and Ad35/Ad26 prime-boost regimens which induced a mixture of neutralizing and binding antibody as well as cellular immune responses (Barouch et al., 2012). This study further demonstrated induction of both systemic and mucosal immune responses and achieved protection from both acquisition and disease progression, thus providing proof of concept that HIV-1 acquisition and post-infection control might be achieved by improved immunogen design and delivery strategies. Heterologous or homologous regimens comprising DNA/MVA, MVA/Ad26, and MVA/MVA were comparatively less efficacious than Ad26/MVA or Ad35/Ad26, which reduced viral load set-points by greater than 100-fold. A Phase 1 clinical trial (B003/IPCAVD-004) assessing the immunogenicity of various prime-boost combinations of Ad26 and Ad35 is ongoing, and will inform the field on the clinical utility of these two promising human adenovirus vector combinations. Another NHP study employing three doses of plasmid DNA followed with Ad5 to deliver various immunogens comprising SIV-Gag, SIV-Env mosaic immunogens or SIV_{mac239} Env also induced cellular and antibody responses (neutralizing antibodies and ADCC) and achieved significant protection against intrarectal challenge of rhesus macaques with SIV_{smE660} that was a mismatch of the vaccine strain (Roederer et al., 2014). Moreover, superior immunogenicity of prime-boost combinations using DNA/ChAdV63/MVA or ChAdV63/MVA has been demonstrated in a Phase I study (Borthwick et al., 2014).

The success of a viral vector for priming has already been demonstrated in the RV144 study which used ALVAC to prime antibody and T cell responses, followed with a protein boost (Rerks-Ngarm et al., 2009). Although priming with DNA has always seemed a better strategy as it focuses the immune response to the immunogen transgene, as opposed to viral vectors which carry multitudes of immunogenic antigens within their backbones, the efficacy of viral-vector priming followed by protein boosting in the RV144 study and the superior immunogenicity of virus-prime/virus-boost in the studies discussed above support the use of viral vectors for both priming and boosting. Therefore, heterologous prime-boost regimens combining DNA, Adenovirus and MVA or ALVAC are likely to achieve efficacy against HIV in clinical trials, although this will require that HIV Env or genes encoding NAb epitopes are included in the immunogen formulations (Barouch et al., 2012, 2013). Preclinical studies investigating the potential of combined chimpanzee adenovirus, MVA and protein prime-boost regimens to deliver immunogens which can stimulate broadly neutralizing antibodies such as BG505 are underway. The success of recombinant adenovirus vector priming followed with MVA boost in inducing high-titre antibodies either on their own or in conjunction with molecular adjuvants has already been proven in preclinical studies of malaria (Draper et al., 2008). Possibly the persistence of adenovirus ensures continuous antigen supply which is suitable for B cell priming. It is envisaged that optimal delivery modalities which combine HIV immunogens eliciting BNAbs with those that stimulate strong T cell immunity will achieve enhanced vaccine efficacy. Of course a major caveat of combining strong T cell vectors with antibody-producing immunogens is the possible immune interference of antibody production by these vectors. Nevertheless, this can be optimized perhaps by employing several protein boosts with powerful adjuvants in order to deliver the most balanced immune responses.

POTENTIAL VACCINE-ASSOCIATED RISK OF HIV ACQUISITION

The increased risk of HIV-1 acquisition in the STEP and HVTN505 trial vaccinees despite strong immune responses has

raised many unanswered questions as to whether the vaccine delivery modalities, suboptimal potency of the HIV immunogens or other unknown external factors are responsible for vaccine failure. As far as immunogen design, the vaccine construct used in the STEP, Phambili and HVTN505 studies represents one of the most comprehensive immunogens with broad coverage, as it comprised a 6-plasmid DNA and rAd5 vectors expressing Gag/Pol/Nef/Env proteins from multiple clades. Other immunogens based on similar or far less comprehensive HIV protein coverage have also been tested and showed varied degrees of immunogenicity. Thus, an understanding on whether the outcomes of the STEP/Phambili/HVTN505 studies (efficacy, immunogenicity or increased risk of acquisition) would have been different if other delivery vectors (such as DNA/MVA, DNA/ALVAC or DNA/Ad35/Ad26 or even a replicating CMV vector) had been used to deliver the same immunogens in these trials is key for further progression in the field. An Alternative way to look at this is to ask whether the results of RV144 trial would have been worse if Ad5 was used instead of ALVAC, assuming that the prevalence of Ad5 neutralizing antibodies in the RV144 population does not differ significantly from the STEP and Phambili study populations.

The finding that the vaccine was not at all efficacious amongst men who were circumcised or in uncircumcised men who did not have pre-existing Ad5 immunity raises doubts as to whether efficacy was genuinely hindered by Ad5 serostatus. This is further supported by the results of HVTN505 study which tested only circumcised individuals without Ad5 antibodies, yet no protection was observed. Moreover, the absence of Ad5 antibodies in the HVTN505 study participants (which should in theory allow for higher immunogenicity) was not associated with any significant enhancement of the magnitude and quality of immune responses over those seen in the STEP and Phambili studies. Therefore, Ad5 serostatus can be safely removed from the equation, leaving the only plausible explanation for vaccine failure to be the quality and quantity of immune responses. If this can be fully documented beyond doubt then it implies that either the Ad5 delivery vector or the HIV-1 antigens used were not immunogenic enough to afford protection from infection or post-infection virus control. However, considering that Ad5 is one of the most immunogenic vectors currently available, (and that the immunogen used in these studies was comprehensive and well-designed), this would have serious implications for vaccine design, as it sets the bar really high for new candidate vaccines which would be expected to stimulate responses of extremely higher magnitudes and superior qualitative properties in order to achieve even the minimal efficacy. On a brighter side, this would perhaps instigate intense scrutiny of the current methods used for assessing vaccine immunogenicity in order to standardize and synchronize with those for efficacy measurements.

One other interesting question is whether (and how) Ad5 sero-positivity is intrinsically associated with HIV acquisition. Although studies of uncircumcised men document increased risk of natural HIV acquisition due to a high frequency of CD4+CCR5+ target cells in the foreskin (Prodger et al., 2012), how this relates their Ad5 sero-positivity and titre levels with infection risk is not very clear. However, the fact that the risk of HIV-1 acquisition in the STEP study diminished with time after immunization, and eventually leveled up with placebo recipients (Buchbinder et al., 2008) might in actual fact support a role for vaccine-induced immune activation in HIV acquisition (Tenbusch et al., 2012). Perhaps this could be as a result of generalized immune activation or induction of activated vaccine-specific HIV-1 targets with mucosal-homing properties. Should this be the case, then this would not be unique to Ad5 vectors alone and it would therefore be expected to equally affect other delivery vectors capable of inducing activated mucosal-homing target cells. However, as there were no notable differences in activated circulating T cells between vaccinees and placebos, it is unlikely that generalized vaccine-induced immune activation played a role, although it remains possible that there could have been significant differences in activated targets at mucosal sites which were not measured.

This then raises another interesting question as to whether the outcome of the STEP/Phambili/HVTN505 studies would have been significantly worse (or better) had the vaccines been administered mucosally. This question might have two sides to it, in the sense that mucosal delivery would probably have generated higher frequencies of activated HIV targets at the genital mucosae, hence increasing the potential of fuelling infection. On the other hand, induction of robust and polyfunctional effector immune responses at mucosal portals of HIV entry would probably have cleared the incoming HIV before infection became established. Although these questions have no clear cut answers and cannot be addressed retrospectively in the context of the clinical trials they relate to, they however highlight the extreme challenges in HIV vaccine delivery, and new studies designed to directly tackle these issues will be quite informative for future vaccine development research. Studies looking at whether the most promising delivery vectors (and the respective immunogens) can concurrently induce activated HIV-1 target cells that preferentially home to and persist in the genito-rectal and GALT mucosae, and whether or not such vaccine-induced cells become highly permissive to HIV infection will be of particular interest in efforts aimed at limiting the risk of vaccine-induced HIV-1 acquisition and accelerated disease progression.

PERSPECTIVES AND CONCLUSION

Ideally, vectors for HIV-1 vaccines should directly target antigen presenting cells (APCs) or other immune cells to induce long-lived, strong antibody and cellular responses that can broadly disseminate to systemic and mucosal compartments. The vaccine-specific T cells in particular should be broad and contain activated effector, effector memory and central memory phenotypes in various proportions in order to achieve a proper balance between immediate virus clearance and sustained immune-surveillance for long-term protection, as demonstrated by the RhCMV-SIV vaccine which controlled and cleared pathogenic SIV infection (Hansen et al., 2009, 2011, 2013). Furthermore, vectors which can stimulate polyfunctional CD4+ and CD8+ T cells that act in concert with B cells to inhibit HIV replication through a variety of mechanisms would be more successful than those inducing only mono-functional T cells of either subset alone.

Of particular relevance to protection from infection would be vaccine vectors associated with homing and long-term persistence of vaccine-induced immune responsive cells at the genito-rectal mucosae (Chanzu and Ondondo, 2014) as well as other mucosal sites serving as HIV reservoirs. This remains a very important priority in consideration of the significant rapid CD4+ T cell depletion in the intestinal mucosa despite successful HAART (Brenchley et al., 2004; Mehandru et al., 2004). Thus, vaccine vectors which naturally infect cells within mucosal inductive sites, especially the replication-competent viruses such as adenovirus and influenza virus vectors (Gherardi et al., 2003; Sexton et al., 2009) which can be administered mucosally to trigger mucosal immunity, would be more suited for HIV vaccine delivery. Alternatively, delivery of vaccines via routes which enhance mucosal immunity (Holmgren et al., 2003; Holmgren and Czerkinsky, 2005; Czerkinsky and Holmgren, 2012) or vectors possessing an inherent ability to induce mucosal immunity in addition to systemic immune responses following parenteral or mucosal vaccine delivery (Moser et al., 2007) may be employed. Virosome vectors for instance, possess intrinsic adjuvant properties and a unique ability to target antigen presenting cells, hence have been very successful at inducing protective mucosal immunity in SHIV challenge models (Moser et al., 2007; Bomsel et al., 2011; Leroux-Roels et al., 2013). Other vectors suitable for mucosal vaccine delivery include VEEV (Caley et al., 1997). In the absence of mucosal delivery vectors, new delivery technologies such as the "prime and pull" approach may be utilized in conjunction with systemic delivery methods to enhance mucosal homing and subsequent immunity (Azizi et al., 2010; Shin and Iwasaki, 2012; Tregoning et al., 2013). In this approach, specialized chemokines are administered in mucosal compartments following parenteral immunization in order to chemo-attract the activated vaccine-specific immune cells from the systemic compartments. Furthermore, use of mucosal adjuvants such as CTB and LT-B (Albu et al., 2003; Yuki and Kiyono, 2003), pro-inflammatory cytokines (IL-1α, IL-12, and IL-18) (Belyakov et al., 1998b; Bradney et al., 2002; Albu et al., 2003) or immunostimulatory CpG motifs (Horner et al., 2001; Dumais et al., 2002; Daftarian et al., 2003; Jiang et al., 2005) which target recruitment of immune cells to the mucosal sites would be useful. Co-delivery of vaccines with genes encoding CCL19 and CCL28 was also shown to enhance HIV-1-specific T and B cell responses in the systemic as well as mucosal compartments (Hu et al., 2013).

In consideration of both safety and immunogenicity goals as already discussed, and with particular emphasis on the pivotal role of CTL responses in controlling HIV replication, it seems that non-replicating viral vectors with lower sero-prevalence would be highly desirable, mainly due to excellent safety profiles and potent adjuvant effect allowing for induction of very strong, high quality and long-lived cellular and humoral immunity. However, although safety and reduced immune interference would be guaranteed, a major caveat would be that these lower sero-prevalence vectors may not be adequately immunogenic. Perhaps these vectors can be re-engineered to improve their immunogenic potential. For instance, the immunogenicity of vectors such as MVA and NYVAC can be improved by removal of genes associated with immune evasion which counteract immune responses to the vaccine (Kibler et al., 2011; Gomez et al., 2012; Garcia-Arriaza et al., 2013). In other cases, addition of cytokine-encoding genes such as type 1 interferons, IL-12 or GM-CSF can enhance vaccine efficacy (Gherardi et al., 1999, 2000; Rodriguez et al., 1999; Ramshaw and Ramsay, 2000; Bayer et al., 2011). Furthermore, chemokines such as CCL3 which recruits professional APCs can be co-delivered with HIV antigens to enhance vaccine immunogenicity (Lietz et al., 2012).

Alternatively, vectors capable of inducing substantial immunogenicity in the presence of pre-existing natural or vaccine-induced anti-vector immunity may be worth considering, although it is expected that finding highly attenuated vectors which are safe and remain immunologically potent will be equally challenging. As discussed earlier, combining some of the most promising vectors in heterologous prime-boost regimens will significantly enhance the quantity, quality and protective efficacy of immune responses. However, in consideration of the possible catastrophic effects of elevated immune activation likely to arise from various vector combinations, it would be expected that suitable HIV vaccine vectors maintain lower levels of immune activation to limit the numbers of activated HIV-1 targets (Perreau et al., 2008; Benlahrech et al., 2009) likely to fuel infection in the event of exposure. Furthermore, it is documented that in the absence of a very strong protective immune responses to counteract the incoming virus, the presence of vaccine-specific T cells which are activated and hence more susceptible to infection may increase the risk of acquisition (Tenbusch et al., 2012). Whether it is possible to achieve potent immunostimulatory capacity but with minimal immune activation still remains a subject of intense investigation.

When safety and versatility are considered, and in full view of the enormous technology advancements in DNA plasmid formulations and delivery, in conjunction with other immunomodulatory interventions such as SAP depletion and use of molecular adjuvants, recombinant DNA vaccines remain very attractive, although efforts to improve stimulation of long-lived effector/memory CD8+ T cell phenotypes are still needed to achieve long-term efficacy. Undoubtedly, repeated immunizations or combining DNA vaccines with persistent (replicating) vectors or vectors with slow immunogen release features would induce durable immunity. Nonetheless, replicating vectors with lower sero-prevalence and minimal pathogenicity (Rose et al., 2001; Kawada et al., 2007; Fuchs et al., 2013; Liu et al., 2013) are being considered as they would provide a persistent pool of HIV vaccine-specific effector memory phenotype cytotoxic T cells which are critical for long-term protection from disease progression (Hansen et al., 2009, 2011, 2013). Such effector memory responses would otherwise be expected to wane with time, in the absence of antigen. Replicating vectors may also be better-suited for induction of broadly neutralizing antibodies since persisting expression of the Env antigens is likely to drive high levels of somatic mutations required for affinity maturation of these antibodies (van Gils and Sanders, 2013). A new strategy that has been proven to induce durable and protective antibody responses in humanized mice challenged with high doses of diverse HIV strains is vectored immunoprophylaxis, which involves insertion of immunoglobulin genes into viral vectors such as the

adeno-associated virus (AAV) to provide long-term expression of neutralizing antibodies (Balazs et al., 2012, 2014). Moreover, inclusion of Th2 cytokines such as IL-4, IL-5, and IL-6 which enhance B cell maturation into long-lived antibody secreting cells is yet another strategy already shown to induce high titres of neutralizing antibodies which protected mice from Friend Virus (Ohs et al., 2013). Other possible strategies include use of lentiviral vectors expressing B cell receptor genes encoding neutralizing antibodies to HIV-1 to transduce haematopoietic stem cells (Luo et al., 2009).

Since optimum induction of immune responses to vaccines strongly depends on innate immune triggering as well as the levels of transgene expression, vectors with natural adjuvant properties and therefore capable of strongly inducing innate immunity are particularly immunogenic and thus highly desirable. However, care must be taken to balance between strong innate function stimulation and the potential risk of inducing potent stimulation of immuno-pathological effects, including immune hyper-activation.

In conclusion, a successful vaccine for HIV will have to stimulate potent antibody and CTL responses broad enough to cover multiple HIV variants and with potential to neutralize, bind or suppress HIV-1 replication for sustained (possibly infinite) lengths of time. Of utmost importance, however is generation of vaccine-specific immune responses in the genito-rectal mucosae, the major portals of HIV entry. Emerging evidence strongly suggests that non-pathogenic, low-level replicating viral vectors which can mimic live attenuated vaccines, but with low seroprevalence might be the best way to achieve HIV vaccine efficacy. As these vectors persist long after immunization, they are capable of inducing and maintaining effector/memory CTLs for continued immune surveillance that is necessary to protect from infection, disease progression and to clear or prevent establishment of latent reservoirs. Thus, to achieve protective efficacy HIV vaccine development will need ingenious state of the art technologies to create the very best of T cell and antibody immunogens, delivered by the most potent but safe vectors possessing remarkably high capacity to induce both systemic and mucosal immunity, but without significant immune activation likely to fuel HIV acquisition. Recent significant advances in vaccine delivery technologies and HIV immunogen design provide hope that this is not far from reality.

ACKNOWLEDGMENTS

Beatrice O. Ondondo is a senior HIV vaccine development immunologist employed on a research grant supported by MRC UK.

REFERENCES

Abbink, P., Lemckert, A. A., Ewald, B. A., Lynch, D. M., Denholtz, M., Smits, S., et al. (2007). Comparative seroprevalence and immunogenicity of six rare serotype recombinant adenovirus vaccine vectors from subgroups B and D. *J. Virol.* 81, 4654–4663. doi: 10.1128/JVI.02696-06

Abe, S., Okuda, K., Ura, T., Kondo, A., Yoshida, A., Yoshizaki, S., et al. (2009). Adenovirus type 5 with modified hexons induces robust transgene-specific immune responses in mice with pre-existing immunity against adenovirus type 5. *J. Gene Med.* 11, 570–579. doi: 10.1002/jgm.1332

Afolabi, M. O., Ndure, J., Drammeh, A., Darboe, F., Mehedi, S. R., Rowland-Jones, S. L., et al. (2013). A phase I randomized clinical trial of candidate human immunodeficiency virus type 1 vaccine MVA.HIVA administered to Gambian infants. *PLoS ONE* 8:e78289. doi: 10.1371/journal.pone.0078289

Albarran, Y. C. A., de la Garza, A., Cruz Quiroz, B. J., Vazquez Zea, E., Diaz Estrada, I., Mendez Fuentez, E., et al. (2007). MVA E2 recombinant vaccine in the treatment of human papillomavirus infection in men presenting intraurethral flat condyloma: a phase I/II study. *BioDrugs* 21, 47–59. doi: 10.2165/00063030-200721010-00006

Albu, D. I., Jones-Trower, A., Woron, A. M., Stellrecht, K., Broder, C. C., and Metzger, D. W. (2003). Intranasal vaccination using interleukin-12 and cholera toxin subunit B as adjuvants to enhance mucosal and systemic immunity to human immunodeficiency virus type 1 glycoproteins. *J. Virol.* 77, 5589–5597. doi: 10.1128/JVI.77.10.5589-5597.2003

Alexander, J., Mendy, J., Vang, L., Avanzini, J. B., Garduno, F., Manayani, D. J., et al. (2013). Pre-clinical development of a recombinant, replication-competent adenovirus serotype 4 vector vaccine expressing HIV-1 envelope 1086 clade C. *PLoS ONE* 8:e82380. doi: 10.1371/journal.pone.0082380

Anderson, R. J., Hannan, C. M., Gilbert, S. C., Laidlaw, S. M., Sheu, E. G., Korten, S., et al. (2004). Enhanced CD8+ T cell immune responses and protection elicited against Plasmodium berghei malaria by prime boost immunization regimens using a novel attenuated fowlpox virus. *J. Immunol.* 172, 3094–3100. doi: 10.4049/jimmunol.172.5.3094

Antoine, G., Scheiflinger, F., Dorner, F., and Falkner, F. G. (1998). The complete genomic sequence of the modified vaccinia Ankara strain: comparison with other orthopoxviruses. *Virology* 244, 365–396. doi: 10.1006/viro.1998.9123

Appledorn, D. M., Patial, S., McBride, A., Godbehere, S., Van Rooijen, N., Parameswaran, N., et al. (2008). Adenovirus vector-induced innate inflammatory mediators, MAPK signaling, as well as adaptive immune responses are dependent upon both TLR2 and TLR9 *in vivo*. *J. Immunol.* 181, 2134–2144. doi: 10.4049/jimmunol.181.3.2134

Applequist, S. E., Rollman, E., Wareing, M. D., Liden, M., Rozell, B., Hinkula, J., et al. (2005). Activation of innate immunity, inflammation, and potentiation of DNA vaccination through mammalian expression of the TLR5 agonist flagellin. *J. Immunol.* 175, 3882–3891. doi: 10.4049/jimmunol.175.6.3882

Azizi, A., Ghunaim, H., Diaz-Mitoma, F., and Mestecky, J. (2010). Mucosal HIV vaccines: a holy grail or a dud? *Vaccine* 28, 4015–4026. doi: 10.1016/j.vaccine.2010.04.018

Baden, L. R., Walsh, S. R., Seaman, M. S., Tucker, R. P., Krause, K. H., Patel, A., et al. (2013). First-in-human evaluation of the safety and immunogenicity of a recombinant adenovirus serotype 26 HIV-1 Env vaccine (IPCAVD 001). *J. Infect. Dis.* 207, 240–247. doi: 10.1093/infdis/jis670

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

Balazs, A. B., Chen, J., Hong, C. M., Rao, D. S., Yang, L., and Baltimore, D. (2012). Antibody-based protection against HIV infection by vectored immunoprophylaxis. *Nature* 481, 81–84. doi: 10.1038/nature10660

Balazs, A. B., Ouyang, Y., Hong, C. M., Chen, J., Nguyen, S. M., Rao, D. S., et al. (2014). Vectored immunoprophylaxis protects humanized mice from mucosal HIV transmission. *Nat. Med.* 20, 296–300. doi: 10.1038/nm.3471

Bangari, D. S., and Mittal, S. K. (2006). Development of nonhuman adenoviruses as vaccine vectors. *Vaccine* 24, 849–862. doi: 10.1016/j.vaccine.2005.08.101

Bansal, A., Jackson, B., West, K., Wang, S., Lu, S., Kennedy, J. S., et al. (2008). Multifunctional T-cell characteristics induced by a polyvalent DNA prime/protein boost human immunodeficiency virus type 1 vaccine regimen given to healthy adults are dependent on the route and dose of administration. *J. Virol.* 82, 6458–6469. doi: 10.1128/JVI.00068-08

Barnes, E., Folgori, A., Capone, S., Swadling, L., Aston, S., Kurioka, A., et al. (2012). Novel adenovirus-based vaccines induce broad and sustained T cell responses to HCV in man. *Sci. Transl. Med.* 4, 115ra1. doi: 10.1126/scitranslmed.3003155

Barouch, D. H., Kik, S. V., Weverling, G. J., Dilan, R., King, S. L., Maxfield, L. F., et al. (2011). International seroepidemiology of adenovirus serotypes 5, 26, 35, and 48 in pediatric and adult populations. *Vaccine* 29, 5203–5209. doi: 10.1016/j.vaccine.2011.05.025

Barouch, D. H., Liu, J., Li, H., Maxfield, L. F., Abbink, P., Lynch, D. M., et al. (2012). Vaccine protection against acquisition of neutralization-resistant SIV challenges in rhesus monkeys. *Nature* 482, 89–93. doi: 10.1038/nature10766

Barouch, D. H., Liu, J., Peter, L., Abbink, P., Iampietro, M. J., Cheung, A., et al. (2013). Characterization of humoral and cellular immune responses elicited

by a recombinant adenovirus serotype 26 HIV-1 Env vaccine in healthy adults (IPCAVD 001). *J. Infect. Dis.* 207, 248–256. doi: 10.1093/infdis/jis671

Barouch, D. H., O'Brien, K. L., Simmons, N. L., King, S. L., Abbink, P., Maxfield, L. F., et al. (2010). Mosaic HIV-1 vaccines expand the breadth and depth of cellular immune responses in rhesus monkeys. *Nat. Med.* 16, 319–323. doi: 10.1038/nm.2089

Bassett, J. D., Swift, S. L., and Bramson, J. L. (2011). Optimizing vaccine-induced CD8(+) T-cell immunity: focus on recombinant adenovirus vectors. *Expert Rev. Vaccines* 10, 1307–1319. doi: 10.1586/erv.11.88

Bayer, W., Lietz, R., Ontikatze, T., Johrden, L., Tenbusch, M., Nabi, G., et al. (2011). Improved vaccine protection against retrovirus infection after co-administration of adenoviral vectors encoding viral antigens and type I interferon subtypes. *Retrovirology* 8:75. doi: 10.1186/1742-4690-8-75

Bejon, P., Ogada, E., Mwangi, T., Milligan, P., Lang, T., Fegan, G., et al. (2007). Extended follow-up following a phase 2b randomized trial of the candidate malaria vaccines FP9 ME-TRAP and MVA ME-TRAP among children in Kenya. *PLoS ONE* 2:e707. doi: 10.1371/journal.pone.0000707

Belshe, R. B., Gorse, G. J., Mulligan, M. J., Evans, T. G., Keefer, M. C., Excler, J. L., et al. (1998). Induction of immune responses to HIV-1 by canarypox virus (ALVAC) HIV-1 and gp120 SF-2 recombinant vaccines in uninfected volunteers. NIAID AIDS Vaccine Evaluation Group. *AIDS* 12, 2407–2415.

Belyakov, I. M., Ahlers, J. D., Brandwein, B. Y., Earl, P., Kelsall, B. L., Moss, B., et al. (1998b). The importance of local mucosal HIV-specific CD8(+) cytotoxic T lymphocytes for resistance to mucosal viral transmission in mice and enhancement of resistance by local administration of IL-12. *J. Clin. Invest.* 102, 2072–2081. doi: 10.1172/JCI5102

Belyakov, I. M., Derby, M. A., Ahlers, J. D., Kelsall, B. L., Earl, P., Moss, B., et al. (1998a). Mucosal immunization with HIV-1 peptide vaccine induces mucosal and systemic cytotoxic T lymphocytes and protective immunity in mice against intrarectal recombinant HIV-vaccinia challenge. *Proc. Natl. Acad. Sci. U.S.A.* 95, 1709–1714.

Benlahrech, A., Harris, J., Meiser, A., Papagatsias, T., Hornig, J., Hayes, P., et al. (2009). Adenovirus vector vaccination induces expansion of memory CD4 T cells with a mucosal homing phenotype that are readily susceptible to HIV-1. *Proc. Natl. Acad. Sci. U.S.A.* 106, 19940–19945. doi: 10.1073/pnas.0907898106

Bergelson, J. M., Cunningham, J. A., Droguett, G., Kurt-Jones, E. A., Krithivas, A., Hong, J. S., et al. (1997). Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science* 275, 1320–1323.

Bergman, P. J., McKnight, J., Novosad, A., Charney, S., Farrelly, J., Craft, D., et al. (2003). Long-term survival of dogs with advanced malignant melanoma after DNA vaccination with xenogeneic human tyrosinase: a phase I trial. *Clin. Cancer Res.* 9, 1284–1290.

Berthoud, T. K., Hamill, M., Lillie, P. J., Hwenda, L., Collins, K. A., Ewer, K. J., et al. (2011). Potent CD8+ T-cell immunogenicity in humans of a novel heterosubtypic influenza A vaccine, MVA-NP+M1. *Clin. Infect. Dis.* 52, 1–7. doi: 10.1093/cid/ciq015

Bertley, F. M., Kozlowski, P. A., Wang, S. W., Chappelle, J., Patel, J., Sonuyi, O., et al. (2004). Control of simian/human immunodeficiency virus viremia and disease progression after IL-2-augmented DNA-modified vaccinia virus Ankara nasal vaccination in nonhuman primates. *J. Immunol.* 172, 3745–3757. doi: 10.4049/jimmunol.172.6.3745

Bilello, J. P., Manrique, J. M., Shin, Y. C., Lauer, W., Li, W., Lifson, J. D., et al. (2011). Vaccine protection against simian immunodeficiency virus in monkeys using recombinant gamma-2 herpesvirus. *J. Virol.* 85, 12708–12720. doi: 10.1128/JVI.00865-11

Bodin, K., Ellmerich, S., Kahan, M. C., Tennent, G. A., Loesch, A., Gilbertson, J. A., et al. (2010). Antibodies to human serum amyloid P component eliminate visceral amyloid deposits. *Nature* 468, 93–97. doi: 10.1038/nature09494

Bomsel, M., Tudor, D., Drillet, A. S., Alfsen, A., Ganor, Y., Roger, M. G., et al. (2011). Immunization with HIV-1 gp41 subunit virosomes induces mucosal antibodies protecting nonhuman primates against vaginal SHIV challenges. *Immunity* 34, 269–280. doi: 10.1016/j.immuni.2011.01.015

Bonaldo, M. C., Martins, M. A., Rudersdorf, R., Mudd, P. A., Sacha, J. B., Piaskowski, S. M., et al. (2010). Recombinant yellow fever vaccine virus 17D expressing simian immunodeficiency virus SIVmac239 gag induces SIV-specific CD8+ T-cell responses in rhesus macaques. *J. Virol.* 84, 3699–3706. doi: 10.1128/JVI.02255-09

Borthwick, N., Ahmed, T., Ondondo, B., Hayes, P., Rose, A., Ebrahimsa, U., et al. (2014). Vaccine-elicited human T cells recognizing conserved protein regions inhibit HIV-1. *Mol. Ther.* 22, 464–475. doi: 10.1038/mt.2013.248

Boyer, J. D., Robinson, T. M., Kutzler, M. A., Parkinson, R., Calarota, S. A., Sidhu, M. K., et al. (2005). SIV DNA vaccine co-administered with IL-12 expression plasmid enhances CD8 SIV cellular immune responses in cynomolgus macaques. *J. Med. Primatol.* 34, 262–270. doi: 10.1111/j.1600-0684.2005.00124.x

Bradney, C. P., Sempowski, G. D., Liao, H. X., Haynes, B. F., and Staats, H. F. (2002). Cytokines as adjuvants for the induction of anti-human immunodeficiency virus peptide immunoglobulin G (IgG) and IgA antibodies in serum and mucosal secretions after nasal immunization. *J. Virol.* 76, 517–524. doi: 10.1128/JVI.76.2.517-524.2002

Brave, A., Gudmundsdotter, L., Sandstrom, E., Haller, B. K., Hallengard, D., Maltais, A. K., et al. (2010). Biodistribution, persistence and lack of integration of a multigene HIV vaccine delivered by needle-free intradermal injection and electroporation. *Vaccine* 28, 8203–8209. doi: 10.1016/j.vaccine.2010.08.108

Brenchley, J. M., Schacker, T. W., Ruff, L. E., Price, D. A., Taylor, J. H., Beilman, G. J., et al. (2004). CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. *J. Exp. Med.* 200, 749–759. doi: 10.1084/jem.20040874

Bruder, J. T., Chen, P., Semenova, E., Thomas, C. A., Konovalova, S., Ekberg, G., et al. (2013). Identification of a suppressor mutation that improves the yields of hexon-modified adenovirus vectors. *J. Virol.* 87, 9661–9671. doi: 10.1128/JVI.00462-13

Bruder, J. T., Semenova, E., Chen, P., Limbach, K., Patterson, N. B., Stefaniak, M. E., et al. (2012). Modification of Ad5 hexon hypervariable regions circumvents pre-existing Ad5 neutralizing antibodies and induces protective immune responses. *PLoS ONE* 7:e33920. doi: 10.1371/journal.pone.0033920

Buchbinder, S. P., Mehrotra, D. V., Duerr, A., Fitzgerald, D. W., Mogg, R., Li, D., et al. (2008). Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. *Lancet* 372, 1881–1893. doi: 10.1016/S0140-6736(08)61591-3

Caley, I. J., Betts, M. R., Irlbeck, D. M., Davis, N. L., Swanstrom, R., Frelinger, J. A., et al. (1997). Humoral, mucosal, and cellular immunity in response to a human immunodeficiency virus type 1 immunogen expressed by a Venezuelan equine encephalitis virus vaccine vector. *J. Virol.* 71, 3031–3038

Capone, S., Reyes-Sandoval, A., Naddeo, M., Siani, L., Ammendola, V., Rollier, C. S., et al. (2010). Immune responses against a liver-stage malaria antigen induced by simian adenoviral vector AdCh63 and MVA prime-boost immunisation in non-human primates. *Vaccine* 29, 256–265. doi: 10.1016/j.vaccine.2010.10.041

Carroll, M. W., and Moss, B. (1997). Host range and cytopathogenicity of the highly attenuated MVA strain of vaccinia virus: propagation and generation of recombinant viruses in a nonhuman mammalian cell line. *Virology* 238, 198–211. doi: 10.1006/viro.1997.8845

Casimiro, D. R., Bett, A. J., Fu, T. M., Davies, M. E., Tang, A., Wilson, K. A., et al. (2004). Heterologous human immunodeficiency virus type 1 priming-boosting immunization strategies involving replication-defective adenovirus and poxvirus vaccine vectors. *J. Virol.* 78, 11434–11438. doi: 10.1128/JVI.78.20.11434-11438.2004

Casimiro, D. R., Chen, L., Fu, T. M., Evans, R. K., Caulfield, M. J., Davies, M. E., et al. (2003a). Comparative immunogenicity in rhesus monkeys of DNA plasmid, recombinant vaccinia virus, and replication-defective adenovirus vectors expressing a human immunodeficiency virus type 1 gag gene. *J. Virol.* 77, 6305–6313. doi: 10.1128/JVI.77.11.6305-6313.2003

Casimiro, D. R., Tang, A., Chen, L., Fu, T. M., Evans, R. K., Davies, M. E., et al. (2003b). Vaccine-induced immunity in baboons by using DNA and replication-incompetent adenovirus type 5 vectors expressing a human immunodeficiency virus type 1 gag gene. *J. Virol.* 77, 7663–7668. doi: 10.1128/JVI.77.13.7663-7668.2003

Catanzaro, A. T., Koup, R. A., Roederer, M., Bailer, R. T., Enama, M. E., Moodie, Z., et al. (2006). Phase 1 safety and immunogenicity evaluation of a multiclade HIV-1 candidate vaccine delivered by a replication-defective recombinant adenovirus vector. *J. Infect. Dis.* 194, 1638–1649. doi: 10.1086/509258

Cerwenka, A., Morgan, T. M., and Dutton, R. W. (1999). Naive, effector, and memory CD8 T cells in protection against pulmonary influenza virus infection: homing properties rather than initial frequencies are crucial. *J. Immunol.* 163, 5535–5543.

Chanzu, N., and Ondondo, B. (2014). Induction of potent and long-lived antibody and cellular immune responses in the genitorectal mucosa could be the critical determinant of HIV vaccine efficacy. *Front. Immunol.* 5:202. doi: 10.3389/fimmu.2014.00202

Chen, H., Xiang, Z. Q., Li, Y., Kurupati, R. K., Jia, B., Bian, A., et al. (2010). Adenovirus-based vaccines: comparison of vectors from three species of adenoviridae. *J. Virol.* 84, 10522–10532. doi: 10.1128/JVI.00450-10

Chong, S. Y., Egan, M. A., Kutzler, M. A., Megati, S., Masood, A., Roopchard, V., et al. (2007). Comparative ability of plasmid IL-12 and IL-15 to enhance cellular and humoral immune responses elicited by a SIVgag plasmid DNA vaccine and alter disease progression following SHIV(89.6P) challenge in rhesus macaques. *Vaccine* 25, 4967–4982. doi: 10.1016/j.vaccine.2006.11.070

Chuang, I., Sedegah, M., Cicatelli, S., Spring, M., Polhemus, M., Tamminga, C., et al. (2013). DNA prime/Adenovirus boost malaria vaccine encoding *P. falciparum* CSP and AMA1 induces sterile protection associated with cell-mediated immunity. *PLoS ONE* 8:e55571. doi: 10.1371/journal.pone.0055571

Colloca, S., Barnes, E., Folgori, A., Ammendola, V., Capone, S., Cirillo, A., et al. (2012). Vaccine vectors derived from a large collection of simian adenoviruses induce potent cellular immunity across multiple species. *Sci. Transl. Med.* 4, 115ra2. doi: 10.1126/scitranslmed.3002925

Corona Gutierrez, C. M., Tinoco, A., Navarro, T., Contreras, M. L., Cortes, R. R., Calzado, P., et al. (2004). Therapeutic vaccination with MVA E2 can eliminate precancerous lesions (CIN 1, CIN 2, and CIN 3) associated with infection by oncogenic human papillomavirus. *Hum. Gene Ther.* 15, 421–431. doi: 10.1089/10430340460745757

Cristillo, A. D., Ferrari, M. G., Hudacik, L., Lewis, B., Galmin, L., Bowen, B., et al. (2011). Induction of mucosal and systemic antibody and T-cell responses following prime-boost immunization with novel adjuvanted human immunodeficiency virus-1-vaccine formulations. *J. Gen. Virol.* 92(Pt 1), 128–140. doi: 10.1099/vir.0.023242-0

Cristillo, A. D., Weiss, D., Hudacik, L., Restrepo, S., Galmin, L., Suschak, J., et al. (2008). Persistent antibody and T cell responses induced by HIV-1 DNA vaccine delivered by electroporation. *Biochem. Biophys. Res. Commun.* 366, 29–35. doi: 10.1016/j.bbrc.2007.11.052

Czerkinsky, C., and Holmgren, J. (2012). Mucosal delivery routes for optimal immunization: targeting immunity to the right tissues. *Curr. Top. Microbiol. Immunol.* 354, 1–18. doi: 10.1007/82_2010_112

Daftarian, P., Ali, S., Sharan, R., Lacey, S. F., La Rosa, C., Longmate, J., et al. (2003). Immunization with Th-CTL fusion peptide and cytosine-phosphate-guanine DNA in transgenic HLA-A2 mice induces recognition of HIV-infected T cells and clears vaccinia virus challenge. *J. Immunol.* 171, 4028–4039. doi: 10.4049/jimmunol.171.8.4028

Davis, B. S., Chang, G. J., Cropp, B., Roehrig, J. T., Martin, D. A., Mitchell, C. J., et al. (2001). West Nile virus recombinant DNA vaccine protects mouse and horse from virus challenge and expresses *in vitro* a noninfectious recombinant antigen that can be used in enzyme-linked immunosorbent assays. *J. Virol.* 75, 4040–4047. doi: 10.1128/JVI.75.9.4040-4047.2001

Dicks, M. D., Spencer, A. J., Edwards, N. J., Wadell, G., Bojang, K., Gilbert, S. C., et al. (2012). A novel chimpanzee adenovirus vector with low human seroprevalence: improved systems for vector derivation and comparative immunogenicity. *PLoS ONE* 7:e40385. doi: 10.1371/journal.pone.0040385

Dorrell, L., Yang, H., Ondondo, B., Dong, T., di Gleria, K., Suttill, A., et al. (2006). Expansion and diversification of virus-specific T cells following immunization of human immunodeficiency virus type 1 (HIV-1)-infected individuals with a recombinant modified vaccinia virus Ankara/HIV-1 Gag vaccine. *J. Virol.* 80, 4705–4716. doi: 10.1128/JVI.80.10.4705-4716.2006

Drape, R. J., Macklin, M. D., Barr, L. J., Jones, S., Haynes, J. R., and Dean, H. J. (2006). Epidermal DNA vaccine for influenza is immunogenic in humans. *Vaccine* 24, 4475–4481. doi: 10.1016/j.vaccine.2005.08.012

Draper, S. J., Biswas, S., Spencer, A. J., Remarque, E. J., Capone, S., Naddeo, M., et al. (2010). Enhancing blood-stage malaria subunit vaccine immunogenicity in rhesus macaques by combining adenovirus, poxvirus, and protein-in-adjuvant vaccines. *J. Immunol.* 185, 7583–7595. doi: 10.4049/jimmunol.1001760

Draper, S. J., Cottingham, M. G., and Gilbert, S. C. (2013). Utilizing poxviral vectored vaccines for antibody induction-progress and prospects. *Vaccine* 31, 4223–4230. doi: 10.1016/j.vaccine.2013.05.091

Draper, S. J., Moore, A. C., Goodman, A. L., Long, C. A., Holder, A. A., Gilbert, S. C., et al. (2008). Effective induction of high-titer antibodies by viral vector vaccines. *Nat. Med.* 14, 819–821. doi: 10.1038/nm.1850

Dumais, N., Patrick, A., Moss, R. B., Davis, H. L., and Rosenthal, K. L. (2002). Mucosal immunization with inactivated human immunodeficiency virus plus CpG oligodeoxynucleotides induces genital immune responses and protection against intravaginal challenge. *J. Infect. Dis.* 186, 1098–1105. doi: 10.1086/344232

Dunachie, S. J., Walther, M., Epstein, J. E., Keating, S., Berthoud, T., Andrews, L., et al. (2006). A DNA prime-modified vaccinia virus ankara boost vaccine encoding thrombospondin-related adhesion protein but not circumsporozoite protein partially protects healthy malaria-naive adults against *Plasmodium falciparum* sporozoite challenge. *Infect. Immun.* 74, 5933–5942. doi: 10.1128/IAI.00590-06

Egan, M. A., Chong, S. Y., Megati, S., Montefiori, D. C., Rose, N. F., Boyer, J. D., et al. (2005). Priming with plasmid DNAs expressing interleukin-12 and simian immunodeficiency virus gag enhances the immunogenicity and efficacy of an experimental AIDS vaccine based on recombinant vesicular stomatitis virus. *AIDS Res. Hum. Retroviruses* 21, 629–643. doi: 10.1089/aid.2005.21.629

Egan, M. A., Chong, S. Y., Rose, N. F., Megati, S., Lopez, K. J., Schadeck, E. B., et al. (2004). Immunogenicity of attenuated vesicular stomatitis virus vectors expressing HIV type 1 Env and SIV Gag proteins: comparison of intranasal and intramuscular vaccination routes. *AIDS Res. Hum. Retroviruses* 20, 989–1004. doi: 10.1089/0889222042222746

Emini, E. A., and Koff, W. C. (2004). AIDS/HIV. Developing an AIDS vaccine: need, uncertainty, hope. *Science* 304, 1913–1914. doi: 10.1126/science.1100368

Ersching, J., Hernandez, M. I., Cezarotto, F. S., Ferreira, J. D., Martins, A. B., Switzer, W. M., et al. (2010). Neutralizing antibodies to human and simian adenoviruses in humans and New-World monkeys. *Virology* 407, 1–6. doi: 10.1016/j.virol.2010.07.043

Estcourt, M. J., Ramsay, A. J., Brooks, A., Thomson, S. A., Medveckzy, C. J., and Ramshaw, I. A. (2002). Prime-boost immunization generates a high frequency, high-avidity CD8(+) cytotoxic T lymphocyte population. *Int. Immunol.* 14, 31–37. doi: 10.1093/intimm/14.1.31

Evans, T. G., Keefer, M. C., Weinhold, K. J., Wolff, M., Montefiori, D., Gorse, G. J., et al. (1999). A canarypox vaccine expressing multiple human immunodeficiency virus type 1 genes given alone or with rgp120 elicits broad and durable CD8+ cytotoxic T lymphocyte responses in seronegative volunteers. *J. Infect. Dis.* 180, 290–298. doi: 10.1086/314895

Excler, J. L., Parks, C. L., Ackland, J., Rees, H., Gust, I. D., and Koff, W. C. (2010). Replicating viral vectors as HIV vaccines: summary report from the IAVI-sponsored satellite symposium at the AIDS vaccine 2009 conference. *Biologicals* 38, 511–521. doi: 10.1016/j.biologicals.2010.03.005

Faul, E. J., Aye, P. P., Papaneri, A. B., Pahar, B., McGettigan, J. P., Schiro, F., et al. (2009). Rabies virus-based vaccines elicit neutralizing antibodies, poly-functional CD8+ T cell, and protect rhesus macaques from AIDS-like disease after SIV(mac251) challenge. *Vaccine* 28, 299–308. doi: 10.1016/j.vaccine.2009.10.051

Finn, J. D., Bassett, J., Millar, J. B., Grinshtein, N., Yang, T. C., Parsons, R., et al. (2009). Persistence of transgene expression influences CD8+ T-cell expansion and maintenance following immunization with recombinant adenovirus. *J. Virol.* 83, 12027–12036. doi: 10.1128/JVI.00593-09

Fitzgerald, J. C., Gao, G. P., Reyes-Sandoval, A., Pavlakis, G. N., Xiang, Z. Q., Wlazlo, A. P., et al. (2003). A simian replication-defective adenoviral recombinant vaccine to HIV-1 gag. *J. Immunol.* 170, 1416–1422. doi: 10.4049/jimmunol.170.3.1416

Flynn, N. M., Forthal, D. N., Harro, C. D., Judson, F. N., Mayer, K. H., Para, M. F., et al. (2005). Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection. *J. Infect. Dis.* 191, 654–665. doi: 10.1086/428404

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

Fuchs, J., Frank, I., Kochar, N., Elizaga, M., Allen, M., Carter, D., et al. (2012). First-in-human phase I clinical trial of a recombinant vesicular stomatitis virus (rVSV)-based preventive HIV-1 vaccine. *Retrovirology* 9, P134. doi: 10.1186/1742-4690-9-S2-P134

Fuchs, J., Frank, I., Kochar, N., Elizaga, M., Allen, M., Frahm, N., et al. (2013). *A Recombinant Vesicular Stomatitis Virus (rVSV) HIV-1 gag Vaccine is Safe And Immunogenic in Healthy, HIV-1 Uninfected Phase I Trial Participants*. Barcelona: AIDS vaccine.

Gabitzsch, E. S., Xu, Y., Yoshida, L. H., Balint, J., Amalfitano, A., and Jones, F. R. (2009). Novel Adenovirus type 5 vaccine platform induces cellular immunity against HIV-1 Gag, Pol, Nef despite the presence of Ad5 immunity. *Vaccine* 27, 6394–6398. doi: 10.1016/j.vaccine.2009.06.028

Gaggar, A., Shayakhmetov, D. M., and Lieber, A. (2003). CD46 is a cellular receptor for group B adenoviruses. *Nat. Med.* 9, 1408–1412. doi: 10.1038/nm952

Garcia, F., Bernaldo de Quiros, J. C., Gomez, C. E., Perdiguero, B., Najera, J. L., Jimenez, V., et al. (2011). Safety and immunogenicity of a modified pox vector-based HIV/AIDS vaccine candidate expressing Env, Gag, Pol and Nef proteins of HIV-1 subtype B (MVA-B) in healthy HIV-1-uninfected volunteers: a phase I clinical trial (RISVAC02). *Vaccine* 29, 8309–8316. doi: 10.1016/j.vaccine.2011.08.098

Garcia-Arriaza, J., Arnaez, P., Gomez, C. E., Sorzano, C. O., and Esteban, M. (2013). Improving adaptive and memory immune responses of an HIV/AIDS Vaccine Candidate MVA-B by deletion of vaccinia virus genes (C6L and K7R) blocking interferon signaling pathways. *PLoS ONE* 8:e66894. doi: 10.1371/journal.pone.0066894

Garcia-Hernandez, E., Gonzalez-Sanchez, J. L., Andrade-Manzano, A., Contreras, M. L., Padilla, S., Guzman, C. C., et al. (2006). Regression of papilloma high-grade lesions (CIN 2 and CIN 3) is stimulated by therapeutic vaccination with MVA E2 recombinant vaccine. *Cancer Gene Ther.* 13, 592–597. doi: 10.1038/sj.cgt.7700937

Garcia-Sastre, A., and Palese, P. (1995). Influenza virus vectors. *Biologicals* 23, 171–178.

Garver, K. A., LaPatra, S. E., and Kurath, G. (2005). Efficacy of an infectious hematopoietic necrosis (IHN) virus DNA vaccine in Chinook Oncorhynchus tshawytscha and sockeye O. nerka salmon. *Dis. Aquat. Organ.* 64, 13–22. doi: 10.3354/dao064013

Gherardi, M. M., and Esteban, M. (1999). Mucosal and systemic immune responses induced after oral delivery of vaccinia virus recombinants. *Vaccine* 17, 1074–1083.

Gherardi, M. M., and Esteban, M. (2005). Recombinant poxviruses as mucosal vaccine vectors. *J. Gen. Virol.*86(Pt 11), 2925–2936. doi: 10.1099/vir.0.81181-0

Gherardi, M. M., Najera, J. L., Perez-Jimenez, E., Guerra, S., Garcia-Sastre, A., and Esteban, M. (2003). Prime-boost immunization schedules based on influenza virus and vaccinia virus vectors potentiate cellular immune responses against human immunodeficiency virus Env protein systemically and in the genitorectal draining lymph nodes. *J. Virol.* 77, 7048–7057. doi: 10.1128/JVI.77.12.7048-7057.2003

Gherardi, M. M., Perez-Jimenez, E., Najera, J. L., and Esteban, M. (2004). Induction of HIV immunity in the genital tract after intranasal delivery of a MVA vector: enhanced immunogenicity after DNA prime-modified vaccinia virus Ankara boost immunization schedule. *J. Immunol.* 172, 6209–6220. doi: 10.4049/jimmunol.172.10.6209

Gherardi, M. M., Ramirez, J. C., and Esteban, M. (2000). Interleukin-12 (IL-12) enhancement of the cellular immune response against human immunodeficiency virus type 1 env antigen in a DNA prime/vaccinia virus boost vaccine regimen is time and dose dependent: suppressive effects of IL-12 boost are mediated by nitric oxide. *J. Virol.* 74, 6278–6286. doi: 10.1128/JVI.74.14.6278-6286.2000

Gherardi, M. M., Ramirez, J. C., Rodriguez, D., Rodriguez, J. R., Sano, G., Zavala, F., et al. (1999). IL-12 delivery from recombinant vaccinia virus attenuates the vector and enhances the cellular immune response against HIV-1 Env in a dose-dependent manner. *J. Immunol.* 162, 6724–6733.

Gilbert, P. B., Peterson, M. L., Follmann, D., Hudgens, M. G., Francis, D. P., Gurwith, M., et al. (2005). Correlation between immunologic responses to a recombinant glycoprotein 120 vaccine and incidence of HIV-1 infection in a phase 3 HIV-1 preventive vaccine trial. *J. Infect. Dis.* 191, 666–677. doi: 10.1086/428405

Gilbert, S. C., Schneider, J., Hannan, C. M., Hu, J. T., Plebanski, M., Sinden, R., et al. (2002). Enhanced CD8 T cell immunogenicity and protective efficacy in a mouse malaria model using a recombinant adenoviral vaccine in heterologous prime-boost immunisation regimes. *Vaccine* 20, 1039–1045. doi: 10.1016/S0264-410X(01)00450-9

Gilbert, S. C., Schneider, J., Plebanski, M., Hannan, C. M., Blanchard, T. J., Smith, G. L., et al. (1999). Ty virus-like particles, DNA vaccines and Modified Vaccinia Virus Ankara; comparisons and combinations. *Biol. Chem.* 380, 299–303. doi: 10.1515/BC.1999.041

Gillmore, J. D., Tennent, G. A., Hutchinson, W. L., Gallimore, J. R., Lachmann, H. J., Goodman, H. J., et al. (2010). Sustained pharmacological depletion of serum amyloid P component in patients with systemic amyloidosis. *Br. J. Haematol.* 148, 760–767. doi: 10.1111/j.1365-2141.2009.08036.x

Goepfert, P. A., Elizaga, M. L., Sato, A., Qin, L., Cardinali, M., Hay, C. M., et al. (2011). Phase 1 safety and immunogenicity testing of DNA and recombinant modified vaccinia Ankara vaccines expressing HIV-1 virus-like particles. *J. Infect. Dis.* 203, 610–619. doi: 10.1093/infdis/jiq105

Gomez, C. E., Najera, J. L., Perdiguero, B., Garcia-Arriaza, J., Sorzano, C. O., Jimenez, V., et al. (2011). The HIV/AIDS vaccine candidate MVA-B administered as a single immunogen in humans triggers robust, polyfunctional, and selective effector memory T cell responses to HIV-1 antigens. *J. Virol.* 85, 11468–11478. doi: 10.1128/JVI.05165-11

Gomez, C. E., Perdiguero, B., Najera, J. L., Sorzano, C. O., Jimenez, V., Gonzalez-Sanz, R., et al. (2012). Removal of vaccinia virus genes that block interferon type I and II pathways improves adaptive and memory responses of the HIV/AIDS vaccine candidate NYVAC-C in mice. *J. Virol.* 86, 5026–5038. doi: 10.1128/JVI.06684-11

Goonetilleke, N., Moore, S., Dally, L., Winstone, N., Cebere, I., Mahmoud, A., et al. (2006). Induction of multifunctional human immunodeficiency virus type 1 (HIV-1)-specific T cells capable of proliferation in healthy subjects by using a prime-boost regimen of DNA- and modified vaccinia virus Ankara-vectored vaccines expressing HIV-1 Gag coupled to CD8+ T-cell epitopes. *J. Virol.* 80, 4717–4728. doi: 10.1128/JVI.80.10.4717-4728.2006

Graham, B. S., Enama, M. E., Nason, M. C., Gordon, I. J., Peel, S. A., Ledgerwood, J. E., et al. (2013). DNA vaccine delivered by a needle-free injection device improves potency of priming for antibody and CD8+ T-cell responses after rAd5 boost in a randomized clinical trial. *PLoS ONE* 8:e59340. doi: 10.1371/journal.pone.0059340

Graham, B. S., Koup, R. A., Roederer, M., Bailer, R. T., Enama, M. E., Moodie, Z., et al. (2006). Phase 1 safety and immunogenicity evaluation of a multiclade HIV-1 DNA candidate vaccine. *J. Infect. Dis.* 194, 1650–1660. doi: 10.1086/509259

Gray, G. E., Allen, M., Moodie, Z., Churchyard, G., Bekker, L. G., Nchabeleng, M., et al. (2011). Safety and efficacy of the HVTN 503/Phambili study of a clade-B-based HIV-1 vaccine in South Africa: a double-blind, randomised, placebo-controlled test-of-concept phase 2b study. *Lancet Infect. Dis.* 11, 507–515. doi: 10.1016/S1473-3099

Greenough, T. C., Cunningham, C. K., Muresan, P., McManus, M., Persaud, D., Fenton, T., et al. (2008). Safety and immunogenicity of recombinant poxvirus HIV-1 vaccines in young adults on highly active antiretroviral therapy. *Vaccine* 26, 6883–6893. doi: 10.1016/j.vaccine.2008.09.084

Gudmundsdotter, L., Nilsson, C., Brave, A., Hejdeman, B., Earl, P., Moss, B., et al. (2009). Recombinant Modified Vaccinia Ankara (MVA) effectively boosts DNA-primed HIV-specific immune responses in humans despite pre-existing vaccinia immunity. *Vaccine* 27, 4468–4474. doi: 10.1016/j.vaccine.2009.05.018

Guimaraes-Walker, A., Mackie, N., McCormack, S., Hanke, T., Schmidt, C., Gilmour, J., et al. (2008). Lessons from IAVI-006, a phase I clinical trial to evaluate the safety and immunogenicity of the pTHr.HIVA DNA and MVA.HIVA vaccines in a prime-boost strategy to induce HIV-1 specific T-cell responses in healthy volunteers. *Vaccine* 26, 6671–6677. doi: 10.1016/j.vaccine.2008.09.016

Haglund, K., Leiner, I., Kerksiek, K., Buonocore, L., Pamer, E., and Rose, J. K. (2002). Robust recall and long-term memory T-cell responses induced by prime-boost regimens with heterologous live viral vectors expressing human immunodeficiency virus type 1 Gag and Env proteins. *J. Virol.* 76, 7506–7517. doi: 10.1128/JVI.76.15.7506-7517.2002

Hammer, S. M., Sobieszczyk, M. E., Janes, H., Karuna, S. T., Mulligan, M. J., Grove, D., et al. (2013). Efficacy trial of a DNA/rAd5 HIV-1 preventive vaccine. *N. Engl. J. Med.* 369, 2083–2092. doi: 10.1056/NEJMoa1310566

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

Hansen, S. G., Piatak, M. Jr., Ventura, A. B., Hughes, C. M., Gilbride, R. M., Ford, J. C., et al. (2013). Immune clearance of highly pathogenic SIV infection. *Nature* 502, 100–104. doi: 10.1038/nature12519

Hansen, S. G., Vieville, C., Whizin, N., Coyne-Johnson, L., Siess, D. C., Drummond, D. D., et al. (2009). Effector memory T cell responses are associated with protection of rhesus monkeys from mucosal simian immunodeficiency virus challenge. *Nat. Med.* 15, 293–299. doi: 10.1038/nm.1935

Harari, A., Bart, P. A., Stohr, W., Tapia, G., Garcia, M., Medjitna-Rais, E., et al. (2008). An HIV-1 clade C DNA prime, NYVAC boost vaccine regimen induces reliable, polyfunctional, and long-lasting T cell responses. *J. Exp. Med.* 205, 63–77. doi: 10.1084/jem.20071331

Harari, A., Rozot, V., Cavassini, M., Enders, F. B., Vigano, S., Tapia, G., et al. (2012). NYVAC immunization induces polyfunctional HIV-specific T-cell responses in chronically-infected, ART-treated HIV patients. *Eur. J. Immunol.* 42, 3038–3048. doi: 10.1002/eji.201242696

Harrop, R., Connolly, N., Redchenko, I., Valle, J., Saunders, M., Ryan, M. G., et al. (2006). Vaccination of colorectal cancer patients with modified vaccinia Ankara delivering the tumor antigen 5T4 (TroVax) induces immune responses which correlate with disease control: a phase I/II trial. *Clin. Cancer Res.* 12(Pt 1), 3416–3424. doi: 10.1158/1078-0432.CCR-05-2732

Haut, L. H., Lin, S. W., Tatsis, N., DiMenna, L. J., Giles-Davis, W., Pinto, A. R., et al. (2010). Robust genital gag-specific CD8+ T-cell responses in mice upon intramuscular immunization with simian adenoviral vectors expressing HIV-1-gag. *Eur. J. Immunol.* 40, 3426–3438. doi: 10.1002/eji.201040440

He, Z., Wlazlo, A. P., Kowalczyk, D. W., Cheng, J., Xiang, Z. Q., Giles-Davis, W., et al. (2000). Viral recombinant vaccines to the E6 and E7 antigens of HPV-16. *Virology* 270, 146–161. doi: 10.1006/viro.2000.0271

Hel, Z., Tsai, W. P., Thornton, A., Nacsa, J., Giuliani, L., Tryniszewska, E., et al. (2001). Potentiation of simian immunodeficiency virus (SIV)-specific CD4(+) and CD8(+) T cell responses by a DNA-SIV and NYVAC-SIV prime/boost regimen. *J. Immunol.* 167, 7180–7191. doi: 10.4049/jimmunol.167.12.7180

Hemelaar, J., Gouws, E., Ghys, P. D., Osmanov, S., and Isolation W-UNfH, Characterisation. (2011). Global trends in molecular epidemiology of HIV-1 during 2000-2007. *AIDS* 25, 679–689. doi: 10.1097/QAD.0b013e328342ff93

Hensley, S. E., Giles-Davis, W., McCoy, K. C., Weninger, W., and Ertl, H. C. (2005). Dendritic cell maturation, but not CD8+ T cell induction, is dependent on type I IFN signaling during vaccination with adenovirus vectors. *J. Immunol.* 175, 6032–6041. doi: 10.4049/jimmunol.175.9.6032

Hill, A. V., Reyes-Sandoval, A., O'Hara, G., Ewer, K., Lawrie, A., Goodman, A., et al. (2010). Prime-boost vectored malaria vaccines: progress and prospects. *Hum. Vaccin.* 6, 78–83. doi: 10.4161/hv.6.1.10116

Hoke, C. H. Jr., and Snyder, C. E. Jr. (2013). History of the restoration of adenovirus type 4 and type 7 vaccine, live oral (Adenovirus Vaccine) in the context of the Department of Defense acquisition system. *Vaccine* 31, 1623–1632. doi: 10.1016/j.vaccine.2012.12.029

Holmgren, J., and Czerkinsky, C. (2005). Mucosal immunity and vaccines. *Nat. Med.* 11, S45–53. doi: 10.1038/nm1213

Holmgren, J., Czerkinsky, C., Eriksson, K., and Mharandi, A. (2003). Mucosal immunisation and adjuvants: a brief overview of recent advances and challenges. *Vaccine* 21(Suppl. 2), S89–S95. doi: 10.1016/S0264-410X(03)00206-8

Holterman, L., Vogels, R., van der Vlugt, R., Sieuwerts, M., Grimbergen, J., Kaspers, J., et al. (2004). Novel replication-incompetent vector derived from adenovirus type 11 (Ad11) for vaccination and gene therapy: low seroprevalence and non-cross-reactivity with Ad5. *J. Virol.* 78, 13207–13215. doi: 10.1128/JVI.78.23.13207-13215.2004

Hopkins, R., Bridgeman, A., Bourne, C., Mbewe-Mvula, A., Sadoff, J. C., Both, G. W., et al. (2011a). Optimizing HIV-1-specific CD8+ T-cell induction by recombinant BCG in prime-boost regimens with heterologous viral vectors. *Eur. J. Immunol.* 41, 3542–3552. doi: 10.1002/eji.201141962

Hopkins, R., Bridgeman, A., Joseph, J., Gilbert, S. C., McShane, H., and Hanke, T., (2011b). Dual neonate vaccine platform against HIV-1 and M. tuberculosis. *PLoS ONE* 6:e20067. doi: 10.1371/journal.pone.0020067

Horner, A. A., Datta, S. K., Takabayashi, K., Belyakov, I. M., Hayashi, T., Cinman, N., et al. (2001). Immunostimulatory DNA-based vaccines elicit multifaceted immune responses against HIV at systemic and mucosal sites. *J. Immunol.* 167, 1584–1591. doi: 10.4049/jimmunol.167.3.1584

Howles, S., Guimaraes-Walker, A., Yang, H., Hancock, G., di Gleria, K., Tarragona-Fiol, T., et al. (2010). Vaccination with a modified vaccinia virus Ankara (MVA)-vectored HIV-1 immunogen induces modest vector-specific T cell responses in human subjects. *Vaccine* 28, 7306–7312. doi: 10.1016/j.vaccine.2010.08.077

Hu, K., Luo, S., Tong, L., Huang, X., Jin, W., Huang, W., et al. (2013). CCL19 and CCL28 augment mucosal and systemic immune responses to HIV-1 gp140 by mobilizing responsive immunocytes into secondary lymph nodes and mucosal tissue. *J. Immunol.* 191, 1935–1947. doi: 10.4049/jimmunol.1300120

Huster, K. M., Koffler, M., Stemberger, C., Schiemann, M., Wagner, H., and Busch, D. H. (2006). Unidirectional development of CD8+ central memory T cells into protective Listeria-specific effector memory T cells. *Eur. J. Immunol.* 36, 1453–1464. doi: 10.1002/eji.200635874

Hutnick, N. A., Carnathan, D. G., Dubey, S. A., Cox, K. S., Kierstead, L., Makadonas, G., et al. (2010). Vaccination with Ad5 vectors expands Ad5-specific CD8 T cells without altering memory phenotype or functionality. *PLoS ONE* 5:e14385. doi: 10.1371/journal.pone.0014385

Jalah, R., Kulkarni, V., Patel, V., Rosati, M., Alicea, C., Bear, J., et al. (2014). DNA and protein co-immunization improves the magnitude and longevity of humoral immune responses in macaques. *PLoS ONE* 9:e91550. doi: 10.1371/journal.pone.0091550

Jiang, J. Q., Patrick, A., Moss, R. B., and Rosenthal, K. L. (2005). CD8+ T-cell-mediated cross-clade protection in the genital tract following intranasal immunization with inactivated human immunodeficiency virus antigen plus CpG oligodeoxynucleotides. *J. Virol.* 79, 393–400. doi: 10.1128/JVI.79.1.393-400.2005

Kalams, S. A., Parker, S., Jin, X., Elizaga, M., Metch, B., Wang, M., et al. (2012). Safety and immunogenicity of an HIV-1 gag DNA vaccine with or without IL-12 and/or IL-15 plasmid cytokine adjuvant in healthy, HIV-1 uninfected adults. *PLoS ONE* 7:e29231. doi: 10.1371/journal.pone.0029231

Kalams, S. A., Parker, S. D., Elizaga, M., Metch, B., Edupuganti, S., Hural, J., et al. (2013). Safety and comparative immunogenicity of an HIV-1 DNA vaccine in combination with plasmid interleukin 12 and impact of intramuscular electroporation for delivery. *J. Infect. Dis.* 208, 818–829. doi: 10.1093/infdis/jit236

Kaleebu, P., Njai, H. F., Wang, L., Jones, N., Ssewanyana, I., Richardson, P., et al. (2014). Immunogenicity of ALVAC-HIV vCP1521 in infants of HIV-1-infected women in Uganda (HPTN 027): the first pediatric HIV vaccine trial in Africa. *J. Acquir. Immune Defic. Syndr.* 65, 268–277. doi: 10.1097/01.qai.0000435600.65845.31

Kawada, M., Tsukamoto, T., Yamamoto, H., Takeda, A., Igarashi, H., Watkins, D. I., et al. (2007). Long-term control of simian immunodeficiency virus replication with central memory CD4+ T-cell preservation after nonsterile protection by a cytotoxic T-lymphocyte-based vaccine. *J. Virol.* 81, 5202–5211. doi: 10.1128/JVI.02881-06

Keefer, M. C., Frey, S. E., Elizaga, M., Metch, B., De Rosa, S. C., Barroso, P. F., et al. (2011). A phase I trial of preventive HIV vaccination with heterologous poxviral-vectors containing matching HIV-1 inserts in healthy HIV-uninfected subjects. *Vaccine* 29, 1948–1958. doi: 10.1016/j.vaccine.2010.12.104

Keefer, M. C., Gilmour, J., Hayes, P., Gill, D., Kopycinski, J., Cheeseman, H., et al. (2012). A phase I double blind, placebo-controlled, randomized study of a multigenic HIV-1 adenovirus subtype 35 vector vaccine in healthy uninfected adults. *PLoS ONE* 7:e41936. doi: 10.1371/journal.pone.0041936

Kennedy, J. S., Co, M., Green, S., Longtine, K., Longtine, J., O'Neill, M. A., et al. (2008). The safety and tolerability of an HIV-1 DNA prime-protein boost vaccine (DP6-001) in healthy adult volunteers. *Vaccine* 26, 4420–4424. doi: 10.1016/j.vaccine.2008.05.090

Kent, S. J., Zhao, A., Best, S. J., Chandler, J. D., Boyle, D. B., and Ramshaw, I. A. (1998). Enhanced T-cell immunogenicity and protective efficacy of a human immunodeficiency virus type 1 vaccine regimen consisting of consecutive priming with DNA and boosting with recombinant fowlpox virus. *J. Virol.* 72, 10180–10188.

Kibler, K. V., Gomez, C. E., Perdiguero, B., Wong, S., Huynh, T., Holechek, S., et al. (2011). Improved NYVAC-based vaccine vectors. *PLoS ONE* 6:e25674. doi: 10.1371/journal.pone.0025674

Kim, T. S., Hufford, M. M., Sun, J., Fu, Y. X., and Braciale, T. J. (2010). Antigen persistence and the control of local T cell memory by migrant respiratory dendritic cells after acute virus infection. *J. Exp. Med.* 207, 1161–1172. doi: 10.1084/jem.20092017

Kintu, K., Andrew, P., Musoke, P., Richardson, P., Asiimwe-Kateera, B., Nakyanzi, T., et al. (2013). Feasibility and safety of ALVAC-HIV vCP1521 vaccine in HIV-exposed infants in Uganda: results from the first HIV vaccine trial in infants in Africa. *J. Acquir. Immune Defic. Syndr.* 63, 1–8. doi: 10.1097/QAI.0b013e31827f1c2d

Kiyono, H., and Fukuyama, S. (2004). NALT- versus Peyer's-patch-mediated mucosal immunity. *Nat. Rev. Immunol.* 4, 699–710. doi: 10.1038/nri1439

Kong, W. P., Wu, L., Wallstrom, T. C., Fischer, W., Yang, Z. Y., Ko, S. Y., et al. (2009). Expanded breadth of the T-cell response to mosaic human immunodeficiency virus type 1 envelope DNA vaccination. *J. Virol.* 83, 2201–2215. doi: 10.1128/JVI.02256-08

Kopycinski, J., Cheeseman, H., Ashraf, A., Gill, D., Hayes, P., Hannaman, D., et al. (2012). A DNA-based candidate HIV vaccine delivered via *in vivo* electroporation induces CD4 responses toward the alpha4beta7-binding V2 loop of HIV gp120 in healthy volunteers. *Clin. Vaccine Immunol.* 19, 1557–1559. doi: 10.1128/CVI.00327-12

Korber, B. T., Letvin, N. L., and Haynes, B. F. (2009). T-cell vaccine strategies for human immunodeficiency virus, the virus with a thousand faces. *J. Virol.* 83, 8300–8314. doi: 10.1128/JVI.00114-09

Kurath, G., Garver, K. A., Corbeil, S., Elliott, D. G., Anderson, E. D., and LaPatra, S. E. (2006). Protective immunity and lack of histopathological damage two years after DNA vaccination against infectious hematopoietic necrosis virus in trout. *Vaccine* 24, 345–354. doi: 10.1016/j.vaccine.2005.07.068

Lai, L., Kwa, S., Kozlowski, P. A., Montefiori, D. C., Ferrari, G., Johnson, W. E., et al. (2011). Prevention of infection by a granulocyte-macrophage colony-stimulating factor co-expressing DNA/modified vaccinia Ankara simian immunodeficiency virus vaccine. *J. Infect. Dis.* 204, 164–173. doi: 10.1093/infdis/jir199

Lasaro, M. O., and Ertl, H. C. (2009). New insights on adenovirus as vaccine vectors. *Mol. Ther.* 17, 1333–1339. doi: 10.1038/mt.2009.130

Leroux-Roels, G., Maes, C., Clement, F., van Engelenburg, F., van den Dobbelsteen, M., Adler, M., et al. (2013). Randomized phase I: safety, immunogenicity and mucosal antiviral activity in young healthy women vaccinated with HIV-1 Gp41 P1 peptide on virosomes. *PLoS ONE* 8:e55438. doi: 10.1371/journal.pone.0055438

Letourneau, S., Im, E. J., Mashishi, T., Brereton, C., Bridgeman, A., Yang, H., et al. (2007). Design and pre-clinical evaluation of a universal HIV-1 vaccine. *PLoS ONE* 2:e984. doi: 10.1371/journal.pone.0000984

Li, J., Arevalo, M. T., and Zeng, M. (2013). Engineering influenza viral vectors. *Bioengineered* 4, 9–14. doi: 10.4161/bioe.21950

Li, S., Polonis, V., Isobe, H., Zaghouani, H., Guinea, R., Moran, T., et al. (1993a). Chimeric influenza virus induces neutralizing antibodies and cytotoxic T cells against human immunodeficiency virus type 1. *J. Virol.* 67, 6659–6666.

Li, S., Rodrigues, M., Rodriguez, D., Rodriguez, J. R., Esteban, M., Palese, P., et al. (1993b). Priming with recombinant influenza virus followed by administration of recombinant vaccinia virus induces CD8+ T-cell-mediated protective immunity against malaria. *Proc. Natl. Acad. Sci. U.S.A.* 90, 5214–5218.

Lietz, R., Bayer, W., Ontikatze, T., Johrden, L., Tenbusch, M., Storcksdieck Genannt Bonsmann, M., et al. (2012). Codelivery of the chemokine CCL3 by an adenovirus-based vaccine improves protection from retrovirus infection. *J. Virol.* 86, 1706–1716. doi: 10.1128/JVI.06244-11

Lillie, P. J., Berthoud, T. K., Powell, T. J., Lambe, T., Mullarkey, C., Spencer, A. J., et al. (2012). Preliminary assessment of the efficacy of a T-cell-based influenza vaccine, MVA-NP+M1, in humans. *Clin. Infect. Dis.* 55, 19–25. doi: 10.1093/cid/cis327

Liu, C., Du, S., Li, C., Wang, Y., Wang, M., Li, Y., et al. (2013). Immunogenicity analysis following human immunodeficiency virus recombinant DNA and recombinant vaccinia virus Tian Tan prime-boost immunization. *Sci. China Life Sci.* 56, 531–540. doi: 10.1007/s11427-013-4484-2

Liu, J., O'Brien, K. L., Lynch, D. M., Simmons, N. L., La Porte, A., Riggs, A. M., et al. (2009). Immune control of an SIV challenge by a T-cell-based vaccine in rhesus monkeys. *Nature* 457, 87–91. doi: 10.1038/nature07469

Lopez-Gordo, E., Podgorski, I. I., Downes, N., and Alemany, R. (2014). Circumventing antivector immunity: potential use of nonhuman adenoviral vectors. *Hum. Gene Ther.* 25, 285–300. doi: 10.1089/hum.2013.228

Luo, X. M., Maarschalk, E., O'Connell, R. M., Wang, P., Yang, L., and Baltimore, D. (2009). Engineering human hematopoietic stem/progenitor cells to produce a broadly neutralizing anti-HIV antibody after *in vitro* maturation to human B lymphocytes. *Blood* 113, 1422–1431. doi: 10.1182/blood-2008-09-177139

Mahnel, H., and Mayr, A. (1994). [Experiences with immunization against orthopox viruses of humans and animals using vaccine strain MVA]. *Berl. Munch. Tierarztl. Wochenschr.* 107, 253–256.

Makitalo, B., Lundholm, P., Hinkula, J., Nilsson, C., Karlen, K., Morner, A., et al. (2004). Enhanced cellular immunity and systemic control of SHIV infection by combined parenteral and mucosal administration of a DNA prime MVA boost vaccine regimen. *J. Gen. Virol.* 85(Pt 8), 2407–2419. doi: 10.1099/vir.0.79869-0

Mann, J. F., McKay, P. F., Fiserova, A., Klein, K., Cope, A., Rogers, P., et al. (2014). Enhanced immunogenicity of an HIV-1 DNA vaccine delivered with electroporation via combined intramuscular and intradermal routes. *J. Virol.* 88, 6959–6969. doi: 10.1128/JVI.00183-14

Masopust, D., Ha, S. J., Vezys, V., and Ahmed, R. (2006). Stimulation history dictates memory CD8 T cell phenotype: implications for prime-boost vaccination. *J. Immunol.* 177, 831–839. doi: 10.4049/jimmunol.177.2.831

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

Mast, T. C., Kierstead, L., Gupta, S. B., Nikas, A. A., Kallas, E. G., Novitsky, V., et al. (2010). International epidemiology of human pre-existing adenovirus (Ad) type-5, type-6, type-26 and type-36 neutralizing antibodies: correlates of high Ad5 titers and implications for potential HIV vaccine trials. *Vaccine* 28, 950–957. doi: 10.1016/j.vaccine.2009.10.145

Matano, T., Kano, M., Nakamura, H., Takeda, A., and Nagai, Y. (2001). Rapid appearance of secondary immune responses and protection from acute CD4 depletion after a highly pathogenic immunodeficiency virus challenge in macaques vaccinated with a DNA prime/Sendai virus vector boost regimen. *J. Virol.* 75, 11891–11896. doi: 10.1128/JVI.75.23.11891-118l96.2001

Mazumder, S., Maji, M., Das, A., and Ali, N. (2011). Potency, efficacy and durability of DNA/DNA, DNA/protein and protein/protein based vaccination using gp63 against Leishmania donovani in BALB/c mice. *PLoS ONE* 6:e14644. doi: 10.1371/journal.pone.0014644

McConkey, S. J., Reece, W. H., Moorthy, V. S., Webster, D., Dunachie, S., Butcher, G., et al. (2003). Enhanced T-cell immunogenicity of plasmid DNA vaccines boosted by recombinant modified vaccinia virus Ankara in humans. *Nat. Med.* 9, 729–735. doi: 10.1038/nm881

McCormack, S., Stohr, W., Barber, T., Bart, P. A., Harari, A., Moog, C., et al. (2008). EV02: a Phase I trial to compare the safety and immunogenicity of HIV DNA-C prime-NYVAC-C boost to NYVAC-C alone. *Vaccine* 26, 3162–3174. doi: 10.1016/j.vaccine.2008.02.072

McCoy, K., Tatsis, N., Korioth-Schmitz, B., Lasaro, M. O., Hensley, S. E., Lin, S. W., et al. (2007). Effect of preexisting immunity to adenovirus human serotype 5 antigens on the immune responses of nonhuman primates to vaccine regimens based on human- or chimpanzee-derived adenovirus vectors. *J. Virol.* 81, 6594–6604. doi: 10.1128/JVI.02497-06

McElrath, M. J., De Rosa, S. C., Moodie, Z., Dubey, S., Kierstead, L., Janes, H., et al. (2008). HIV-1 vaccine-induced immunity in the test-of-concept Step Study: a case-cohort analysis. *Lancet* 372, 1894–1905. doi: 10.1016/S0140-6736(08)61592-5

McKay, P. F., Cope, A. V., Mann, J. F., Joseph, S., Esteban, M., Tatoud, R., et al. (2014). Glucopyranosyl lipid A adjuvant significantly enhances HIV specific T and B cell responses elicited by a DNA-MVA-protein vaccine regimen. *PLoS ONE* 9:e84707. doi: 10.1371/journal.pone.0084707

McMichael, A. J. (2006). HIV vaccines. *Annu. Rev. Immunol.* 24, 227–255. doi: 10.1146/annurev.immunol.24.021605.090605

McShane, H. (2002). Prime-boost immunization strategies for infectious diseases. *Curr. Opin. Mol. Ther.* 4, 23–27.

Mehandru, S., Poles, M. A., Tenner-Racz, K., Horowitz, A., Hurley, A., Hogan, C., et al. (2004). Primary HIV-1 infection is associated with preferential depletion of CD4+ T lymphocytes from effector sites in the gastrointestinal tract. *J. Exp. Med.* 200, 761–770. doi: 10.1084/jem.20041196

Meyer, H., Sutter, G., and Mayr, A. (1991). Mapping of deletions in the genome of the highly attenuated vaccinia virus MVA and their influence on virulence. *J. Gen. Virol.* 72(Pt 5), 1031–1038.

Mooij, P., Balla-Jhagjhoorsingh, S. S., Koopman, G., Beenhakker, N., van Haaften, P., Baak, I., et al. (2008). Differential CD4+ versus CD8+ T-cell responses elicited by different poxvirus-based human immunodeficiency virus type 1 vaccine candidates provide comparable efficacies in primates. *J. Virol.* 82, 2975–2988. doi: 10.1128/JVI.02216-07

Moser, C., Amacker, M., Kammer, A. R., Rasi, S., Westerfeld, N., and Zurbriggen, R. (2007). Influenza virosomes as a combined vaccine carrier and adjuvant system for prophylactic and therapeutic immunizations. *Expert Rev. Vaccines* 6, 711–721. doi: 10.1586/14760584.6.5.711

Muruve, D. A., Petrilli, V., Zaiss, A. K., White, L. R., Clark, S. A., Ross, P. J., et al. (2008). The inflammasome recognizes cytosolic microbial and host DNA and triggers an innate immune response. *Nature* 452, 103–107. doi: 10.1038/nature06664

Muster, T., Ferko, B., Klima, A., Purtscher, M., Trkola, A., Schulz, P., et al. (1995). Mucosal model of immunization against human immunodeficiency virus type 1 with a chimeric influenza virus. *J. Virol.* 69, 6678–6686.

Muster, T., Guinea, R., Trkola, A., Purtscher, M., Klima, A., Steindl, F., et al. (1994). Cross-neutralizing activity against divergent human immunodeficiency virus type 1 isolates induced by the gp41 sequence ELDKWAS. *J. Virol.* 68, 4031–4034.

Muster, T., Steindl, F., Purtscher, M., Trkola, A., Klima, A., Himmler, G., et al. (1993). A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. *J. Virol.* 67, 6642–6647.

Mwau, M., Cebere, I., Sutton, J., Chikoti, P., Winstone, N., Wee, E. G., et al. (2004). A human immunodeficiency virus 1 (HIV-1) clade A vaccine in clinical trials: stimulation of HIV-specific T-cell responses by DNA and recombinant modified vaccinia virus Ankara (MVA) vaccines in humans. *J. Gen. Virol.* 85, 911–919. doi: 10.1099/vir.0.19701-0

Nazir, S. A., and Metcalf, J. P. (2005). Innate immune response to adenovirus. *J. Investig. Med.* 53, 292–304. doi: 10.2310/6650.2005.53605

Newman, M. J. (2002). Heterologous prime-boost vaccination strategies for HIV-1: augmenting cellular immune responses. *Curr. Opin. Investig. Drugs* 3, 374–378.

Nolz, J. C., and Harty, J. T. (2011). Strategies and implications for prime-boost vaccination to generate memory CD8 T cells. *Adv. Exp. Med. Biol.* 780:69–83. doi: 10.1007/978-1-4419-5632-3_7

O'Hara, G. A., Duncan, C. J., Ewer, K. J., Collins, K. A., Elias, S. C., Halstead, F. D., et al. (2012). Clinical assessment of a recombinant simian adenovirus ChAd63: a potent new vaccine vector. *J. Infect. Dis.* 205, 772–781. doi: 10.1093/infdis/jir850

Ohs, I., Windmann, S., Wildner, O., Dittmer, U., and Bayer, W. (2013). Interleukin-encoding adenoviral vectors as genetic adjuvant for vaccination against retroviral infection. *PLoS ONE* 8:e82528. doi: 10.1371/journal.pone.0082528

Olive, M., Eisenlohr, L., Flomenberg, N., Hsu, S., and Flomenberg, P. (2002). The adenovirus capsid protein hexon contains a highly conserved human CD4+ T-cell epitope. *Hum. Gene Ther.* 13, 1167–1178. doi: 10.1089/104303402320138952

Ondondo, B. O., Yang, H., Dong, T., di Gleria, K., Suttill, A., Conlon, C., et al. (2006). Immunisation with recombinant modified vaccinia virus Ankara expressing HIV-1 gag in HIV-1-infected subjects stimulates broad functional CD4+ T cell responses. *Eur. J. Immunol.* 36, 2585–2594. doi: 10.1002/eji.200636508

Palese, P., Zavala, F., Muster, T., Nussenzweig, R. S., and Garcia-Sastre, A. (1997). Development of novel influenza virus vaccines and vectors. *J. Infect. Dis.* 176(Suppl. 1), S45–S49.

Patel, V., Jalah, R., Kulkarni, V., Valentin, A., Rosati, M., Alicea, C., et al. (2013). DNA and virus particle vaccination protects against acquisition and confers control of viremia upon heterologous simian immunodeficiency virus challenge. *Proc. Natl. Acad. Sci. U.S.A.* 110, 2975–2980. doi: 10.1073/pnas.1215393110

Patel, V., Valentin, A., Kulkarni, V., Rosati, M., Bergamaschi, C., Jalah, R., et al. (2010). Long-lasting humoral and cellular immune responses and mucosal dissemination after intramuscular DNA immunization. *Vaccine* 28, 4827–4836. doi: 10.1016/j.vaccine.2010.04.064

Patterson, L. J., and Robert-Guroff, M. (2008). Replicating adenovirus vector prime/protein boost strategies for HIV vaccine development. *Expert Opin. Biol. Ther.* 8, 1347–1363. doi: 10.1517/14712598.8.9.1347

Perreau, M., Pantaleo, G., and Kremer, E. J. (2008). Activation of a dendritic cell-T cell axis by Ad5 immune complexes creates an improved environment for replication of HIV in T cells. *J. Exp. Med.* 205, 2717–2725. doi: 10.1084/jem.20081786

Pichla-Gollon, S. L., Lin, S. W., Hensley, S. E., Lasaro, M. O., Herkenhoff-Haut, L., Drinker, M., et al. (2009). Effect of preexisting immunity on an adenovirus vaccine vector: *in vitro* neutralization assays fail to predict inhibition by antiviral antibody *in vivo*. *J. Virol.* 83, 5567–5573. doi: 10.1128/JVI.00405-09

Pitisuttithum, P., Gilbert, P., Gurwith, M., Heyward, W., Martin, M., van Griensven, F., et al. (2006). Randomized, double-blind, placebo-controlled efficacy trial of a bivalent recombinant glycoprotein 120 HIV-1 vaccine among injection drug users in Bangkok, Thailand. *J. Infect. Dis.* 194, 1661–1671. doi: 10.1086/508748

Price, P. J., Torres-Dominguez, L. E., Brandmuller, C., Sutter, G., and Lehmann, M. H. (2013). Modified Vaccinia virus Ankara: innate immune activation and induction of cellular signalling. *Vaccine* 31, 4231–4234. doi: 10.1016/j.vaccine.2013.03.017

Prodger, J. L., Gray, R., Kigozi, G., Nalugoda, F., Galiwango, R., Hirbod, T., et al. (2012). Foreskin T-cell subsets differ substantially from blood with respect to HIV co-receptor expression, inflammatory profile, and memory status. *Mucosal Immunol.* 5, 121–128. doi: 10.1038/mi.2011.56

Qureshi, H., Ma, Z. M., Huang, Y., Hodge, G., Thomas, M. A., DiPasquale, J., et al. (2012). Low-dose penile SIVmac251 exposure of rhesus macaques infected with adenovirus type 5 (Ad5) and then immunized with a replication-defective Ad5-based SIV gag/pol/nef vaccine recapitulates the results of the phase IIb step trial of a similar HIV-1 vaccine. *J. Virol.* 86, 2239–2250. doi: 10.1128/JVI.06175-11

Ramshaw, I. A., and Ramsay, A. J. (2000). The prime-boost strategy: exciting prospects for improved vaccination. *Immunol. Today* 21, 163–165. doi: 10.1016/S0167-5699(00)01612-1

Ranasinghe, C., Turner, S. J., McArthur, C., Sutherland, D. B., Kim, J. H., Doherty, P. C., et al. (2007). Mucosal HIV-1 pox virus prime-boost immunization induces high-avidity CD8+ T cells with regime-dependent cytokine/granzyme B profiles. *J. Immunol.* 178, 2370–2379. doi: 10.4049/jimmunol.178.4.2370

Ratto-Kim, S., Currier, J. R., Cox, J. H., Excler, J. L., Valencia-Micolta, A., Thelian, D., et al. (2012). Heterologous prime-boost regimens using rAd35 and rMVA vectors elicit stronger cellular immune responses to HIV proteins than homologous regimens. *PLoS ONE* 7:e45840. doi: 10.1371/journal.pone.0045840

Rerks-Ngarm, S., Paris, R. M., Chunsutthiwat, S., Premsri, N., Namwat, C., Bowonwatanuwong, C., et al. (2013). Extended evaluation of the virologic, immunologic, and clinical course of volunteers who acquired HIV-1 infection in a phase III vaccine trial of ALVAC-HIV and AIDSVAX B/E. *J. Infect. Dis.* 207, 1195–1205. doi: 10.1093/infdis/jis478

Rerks-Ngarm, S., Pitisuttithum, P., Nitayaphan, S., Kaewkungwal, J., Chiu, J., Paris, R., et al. (2009). Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. *N. Engl. J. Med.* 361, 2209–2220. doi: 10.1056/NEJMoa0908492

Reyes-Sandoval, A., Berthoud, T., Alder, N., Siani, L., Gilbert, S. C., Nicosia, A., et al. (2010). Prime-boost immunization with adenoviral and modified vaccinia virus Ankara vectors enhances the durability and polyfunctionality of protective malaria CD8+ T-cell responses. *Infect. Immun.* 78, 145–153. doi: 10.1128/IAI.00740-09

Reyes-Sandoval, A., Fitzgerald, J. C., Grant, R., Roy, S., Xiang, Z. Q., Li, Y., et al. (2004). Human immunodeficiency virus type 1-specific immune responses in primates upon sequential immunization with adenoviral vaccine carriers of human and simian serotypes. *J. Virol.* 78, 7392–7399. doi: 10.1128/JVI.78.14.7392-7399.2004

Reyes-Sandoval, A., Sridhar, S., Berthoud, T., Moore, A. C., Harty, J. T., Gilbert, S. C., et al. (2008). Single-dose immunogenicity and protective efficacy of simian adenoviral vectors against Plasmodium berghei. *Eur. J. Immunol.* 38, 732–741. doi: 10.1002/eji.200737672

Reynolds, M. R., Weiler, A. M., Piaskowski, S. M., Kolar, H. L., Hessell, A. J., Weiker, M., et al. (2010). Macaques vaccinated with simian immunodeficiency virus SIVmac239Delta nef delay acquisition and control replication after repeated low-dose heterologous SIV challenge. *J. Virol.* 84, 9190–9199. doi: 10.1128/JVI.00041-10

Reynolds, M. R., Weiler, A. M., Weisgrau, K. L., Piaskowski, S. M., Furlott, J. R., Weinfurter, J. T., et al. (2008). Macaques vaccinated with live-attenuated SIV control replication of heterologous virus. *J. Exp. Med.* 205, 2537–2550. doi: 10.1084/jem.20081524

Roberts, D. M., Nanda, A., Havenga, M. J., Abbink, P., Lynch, D. M., Ewald, B. A., et al. (2006). Hexon-chimaeric adenovirus serotype 5 vectors circumvent pre-existing anti-vector immunity. *Nature* 441, 239–243. doi: 10.1038/nature04721

Robinson, H. L., and Amara, R. R. (2005). T cell vaccines for microbial infections. *Nat. Med.* 11, S25–S32. doi: 10.1038/nm1212

Rodriguez, D., Rodriguez, J. R., Llorente, M., Vazquez, I., Lucas, P., Esteban, M., et al. (1999). A human immunodeficiency virus type 1 Env-granulocyte-macrophage colony-stimulating factor fusion protein enhances the cellular immune response to Env in a vaccinia virus-based vaccine. *J. Gen. Virol.* 80 (Pt 1), 217–223.

Roederer, M., Keele, B. F., Schmidt, S. D., Mason, R. D., Welles, H. C., Fischer, W., et al. (2014). Immunological and virological mechanisms of vaccine-mediated protection against SIV and HIV. *Nature* 505, 502–508. doi: 10.1038/nature12893

Rosati, M., Bergamaschi, C., Valentin, A., Kulkarni, V., Jalah, R., Alicea, C., et al. (2009). DNA vaccination in rhesus macaques induces potent immune responses and decreases acute and chronic viremia after SIVmac251 challenge. *Proc. Natl. Acad. Sci. U.S.A.* 106, 15831–15836. doi: 10.1073/pnas.0902628106

Rosati, M., von Gegerfelt, A., Roth, P., Alicea, C., Valentin, A., Robert-Guroff, M., et al. (2005). DNA vaccines expressing different forms of simian immunodeficiency virus antigens decrease viremia upon SIVmac251 challenge. *J. Virol.* 79, 8480–8492. doi: 10.1128/JVI.79.13.8480-8492.2005

Rose, N. F., Marx, P. A., Luckay, A., Nixon, D. F., Moretto, W. J., Donahoe, S. M., et al. (2001). An effective AIDS vaccine based on live attenuated vesicular stomatitis virus recombinants. *Cell* 106, 539–549. doi: 10.1016/S0092-8674(01)00482-2

Roshorm, Y., Cottingham, M. G., Potash, M. J., Volsky, D. J., and Hanke, T. (2012). T cells induced by recombinant chimpanzee adenovirus alone and in prime-boost regimens decrease chimeric EcoHIV/NDK challenge virus load. *Eur. J. Immunol.* 42, 3243–3255. doi: 10.1002/eji.201242624

Sallusto, F., Geginat, J., and Lanzavecchia, A. (2004). Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu. Rev. Immunol.* 22, 745–763. doi: 10.1146/annurev.immunol.22.012703.104702

Sandstrom, E., Nilsson, C., Hejdeman, B., Brave, A., Bratt, G., Robb, M., et al. (2008). Broad immunogenicity of a multigene, multiclade HIV-1 DNA vaccine boosted with heterologous HIV-1 recombinant modified vaccinia virus Ankara. *J. Infect. Dis.* 198, 1482–1490. doi: 10.1086/592507

Santra, S., Liao, H. X., Zhang, R., Muldoon, M., Watson, S., Fischer, W., et al. (2010). Mosaic vaccines elicit CD8+ T lymphocyte responses that confer enhanced immune coverage of diverse HIV strains in monkeys. *Nat. Med.* 16, 324–328. doi: 10.1038/nm.2108

Santra, S., Muldoon, M., Watson, S., Buzby, A., Balachandran, H., Carlson, K. R., et al. (2012). Breadth of cellular and humoral immune responses elicited in rhesus monkeys by multi-valent mosaic and consensus immunogens. *Virology* 428, 121–127. doi: 10.1016/j.virol.2012.03.012

Saubi, N., Im, E. J., Fernandez-Lloris, R., Gil, O., Cardona, P. J., Gatell, J. M., et al. (2011). Newborn mice vaccination with BCG.HIVA(2)(2)(2) + MVA.HIVA enhances HIV-1-specific immune responses: influence of age and immunization routes. *Clin. Dev. Immunol.* 2011:516219. doi: 10.1155/2011/516219

Saubi, N., Mbewe-Mvula, A., Gea-Mallorqui, E., Rosario, M., Gatell, J. M., Hanke, T., et al. (2012). Pre-clinical development of BCG.HIVA(CAT), an antibiotic-free selection strain, for HIV-TB pediatric vaccine vectored by lysine auxotroph of BCG. *PLoS ONE* 7:e42559. doi: 10.1371/journal.pone.0042559

Schell, J., Rose, N. F., Fazo, N., Marx, P. A., Hunter, M., Ramsburg, E., et al. (2009). Long-term vaccine protection from AIDS and clearance of viral DNA following SHIV89.6P challenge. *Vaccine* 27, 979–986. doi: 10.1016/j.vaccine.2008.12.017

Schell, J. B., Rose, N. F., Bahl, K., Diller, K., Buonocore, L., Hunter, M., et al. (2011). Significant protection against high-dose simian immunodeficiency virus challenge conferred by a new prime-boost vaccine regimen. *J. Virol.* 85, 5764–5772. doi: 10.1128/JVI.00342-11

Schneider, J., Gilbert, S. C., Blanchard, T. J., Hanke, T., Robson, K. J., Hannan, C. M., et al. (1998). Enhanced immunogenicity for CD8+ T cell induction and complete protective efficacy of malaria DNA vaccination by boosting with modified vaccinia virus Ankara. *Nat. Med.* 4, 397–402.

Schneider, J., Gilbert, S. C., Hannan, C. M., Degano, P., Prieur, E., Sheu, E. G., et al. (1999). Induction of CD8+ T cells using heterologous prime-boost immunisation strategies. *Immunol. Rev.* 170, 29–38.

Scriba, T. J., Tameris, M., Smit, E., van der Merwe, L., Hughes, E. J., Kadira, B., et al. (2012). A phase IIa trial of the new tuberculosis vaccine, MVA85A, in HIV- and/or Mycobacterium tuberculosis-infected adults. *Am. J. Respir. Crit. Care Med.* 185, 769–778. doi: 10.1164/rccm.201108-1548OC

Sedegah, M., Tamminga, C., McGrath, S., House, B., Ganeshan, H., Lejano, J., et al. (2011). Adenovirus 5-vectored P. falciparum vaccine expressing CSP and AMA1. Part A: safety and immunogenicity in seronegative adults. *PLoS ONE* 6:e24586. doi: 10.1371/journal.pone.0024586

Sekaly, R. P. (2008). The failed HIV Merck vaccine study: a step back or a launching point for future vaccine development? *J. Exp. Med.* 205, 7–12. doi: 10.1084/jem.20072681

Selby, M., Goldbeck, C., Pertile, T., Walsh, R., and Ulmer, J. (2000). Enhancement of DNA vaccine potency by electroporation *in vivo*. *J. Biotechnol.* 83, 147–152. doi: 10.1016/S0168-1656(00)00308-4

Sexton, A., De Rose, R., Reece, J. C., Alcantara, S., Loh, L., Moffat, J. M., et al. (2009). Evaluation of recombinant influenza virus-simian immunodeficiency virus vaccines in macaques. *J. Virol.* 83, 7619–7628. doi: 10.1128/JVI.00470-09

Sharpe, S., Fooks, A., Lee, J., Hayes, K., Clegg, C., and Cranage, M. (2002). Single oral immunization with replication deficient recombinant adenovirus elicits long-lived transgene-specific cellular and humoral immune responses. *Virology* 293, 210–216. doi: 10.1006/viro.2001.1281

Sheehy, S. H., Duncan, C. J., Elias, S. C., Biswas, S., Collins, K. A., O'Hara, G. A., et al. (2012). Phase Ia clinical evaluation of the safety and immunogenicity of the *Plasmodium falciparum* blood-stage antigen AMA1 in ChAd63 and MVA vaccine vectors. *PLoS ONE* 7:e31208. doi: 10.1371/journal.pone.0031208

Sheehy, S. H., Duncan, C. J., Elias, S. C., Collins, K. A., Ewer, K. J., Spencer, A. J., et al. (2011). Phase Ia clinical evaluation of the *Plasmodium falciparum* blood-stage antigen MSP1 in ChAd63 and MVA vaccine vectors. *Mol. Ther.* 19, 2269–2276. doi: 10.1038/mt.2011.176

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

Shiver, J. W., Fu, T. M., Chen, L., Casimiro, D. R., Davies, M. E., Evans, R. K., et al. (2002). Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. *Nature* 415, 331–335. doi: 10.1038/415331a

Smaill, F., Jeyanathan, M., Smieja, M., Medina, M. F., Thanthrige-Don, N., Zganiacz, A., et al. (2013). A human type 5 adenovirus-based tuberculosis vaccine induces robust T cell responses in humans despite preexisting anti-adenovirus immunity. *Sci. Transl. Med.* 5, 205ra134. doi: 10.1126/scitranslmed.3006843

Smith, G. L., and Moss, B. (1983). Infectious poxvirus vectors have capacity for at least 25 000 base pairs of foreign DNA. *Gene* 25, 21–28.

Spearman, P., Lally, M. A., Elizaga, M., Montefiori, D., Tomaras, G. D., McElrath, M. J., et al. (2011). A trimeric, V2-deleted HIV-1 envelope glycoprotein vaccine elicits potent neutralizing antibodies but limited breadth of neutralization in human volunteers. *J. Infect. Dis.* 203, 1165–1173. doi: 10.1093/infdis/jiq175

Stevceva, L., Alvarez, X., Lackner, A. A., Tryniszewska, E., Kelsall, B., Nacsa, J., et al. (2002). Both mucosal and systemic routes of immunization with the live, attenuated NYVAC/simian immunodeficiency virus SIV(gpe) recombinant vaccine result in gag-specific CD8(+) T-cell responses in mucosal tissues of macaques. *J. Virol.* 76, 11659–11676. doi: 10.1128/JVI.76.22.11659-11676.2002

Suleman, M., Galea, S., Gavard, F., Merillon, N., Klonjkowski, B., Tartour, E., et al. (2011). Antigen encoded by vaccine vectors derived from human adenovirus serotype 5 is preferentially presented to CD8+ T lymphocytes by the CD8alpha+ dendritic cell subset. *Vaccine* 29, 5892–5903. doi: 10.1016/j.vaccine.2011.06.071

Takeda, A., Igarashi, H., Nakamura, H., Kano, M., Iida, A., Hirata, T., et al. (2003). Protective efficacy of an AIDS vaccine, a single DNA priming followed by a single booster with a recombinant replication-defective Sendai virus vector, in a macaque AIDS model. *J. Virol.* 77, 9710–9715. doi: 10.1128/JVI.77.17.9710-9715.2003

Tameris, M., Geldenhuys, H., Luabeya, A. K., Smit, E., Hughes, J. E., Vermaak, S., et al. (2014). The candidate TB vaccine, MVA85A, induces highly durable Th1 responses. *PLoS ONE* 9:e87340. doi: 10.1371/journal.pone.0087340

Tameris, M. D., Hatherill, M., Landry, B. S., Scriba, T. J., Snowden, M. A., Lockhart, S., et al. (2013). Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: a randomised, placebo-controlled phase 2b trial. *Lancet* 381, 1021–1028. doi: 10.1016/S0140-6736

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

Tatsis, N., and Ertl, H. C. (2004). Adenoviruses as vaccine vectors. *Mol. Ther.* 10, 616–629. doi: 10.1016/j.ymthe.2004.07.013

Tatsis, N., Fitzgerald, J. C., Reyes-Sandoval, A., Harris-McCoy, K. C., Hensley, S. E., Zhou, D., et al. (2007a). Adenoviral vectors persist *in vivo* and maintain activated CD8+ T cells: implications for their use as vaccines. *Blood* 110, 1916–1923. doi: 10.1182/blood-2007-02-062117

Tatsis, N., Lasaro, M. O., Lin, S. W., Haut, L. H., Xiang, Z. Q., Zhou, D., et al. (2009). Adenovirus vector-induced immune responses in nonhuman primates: responses to prime boost regimens. *J. Immunol.* 182, 6587–6599. doi: 10.4049/jimmunol.0900317

Tatsis, N., Lin, S. W., Harris-McCoy, K., Garber, D. A., Feinberg, M. B., and Ertl, H. C., (2007b). Multiple immunizations with adenovirus and MVA vectors improve CD8+ T cell functionality and mucosal homing. *Virology* 367, 156–167. doi: 10.1016/j.virol.2007.05.028

Tenbusch, M., Ignatius, R., Temchura, V., Nabi, G., Tippler, B., Stewart-Jones, G., et al. (2012). Risk of immunodeficiency virus infection may increase with vaccine-induced immune response. *J. Virol.* 86, 10533–10539. doi: 10.1128/JVI.00796-12

Thorner, A. R., and Barouch, D. H. (2007). HIV-1 vaccine development: progress and prospects. *Curr. Infect. Dis. Rep.* 9, 71–75. doi: 10.1007/s11908-007-0025-0

Tregoning, J. S., Buffa, V., Oszmiana, A., Klein, K., Walters, A. A., and Shattock, R. J. (2013). A "prime-pull" vaccine strategy has a modest effect on local and systemic antibody responses to HIV gp140 in mice. *PLoS ONE* 8:e80559. doi: 10.1371/journal.pone.0080559

Vaine, M., Wang, S., Liu, Q., Arthos, J., Montefiori, D., Goepfert, P., et al. (2010). Profiles of human serum antibody responses elicited by three leading HIV vaccines focusing on the induction of Env-specific antibodies. *PLoS ONE* 5:e13916. doi: 10.1371/journal.pone.0013916

Valentin, A., von Gegerfelt, A., Rosati, M., Miteloudis, G., Alicea, C., Bergamaschi, C., et al. (2010). Repeated DNA therapeutic vaccination of chronically SIV-infected macaques provides additional virological benefit. *Vaccine* 28, 1962–1974. doi: 10.1016/j.vaccine.2009.10.099

van Gils, M. J., and Sanders, R. W. (2013). Broadly neutralizing antibodies against HIV-1: templates for a vaccine. *Virology* 435, 46–56. doi: 10.1016/j.virol.2012.10.004

Van Rompay, K. K., Abel, K., Earl, P., Kozlowski, P. A., Easlick, J., Moore, J., et al. (2010). Immunogenicity of viral vector, prime-boost SIV vaccine regimens in infant rhesus macaques: attenuated vesicular stomatitis virus (VSV) and modified vaccinia Ankara (MVA) recombinant SIV vaccines compared to live-attenuated SIV. *Vaccine* 28, 1481–1492. doi: 10.1016/j.vaccine.2009.11.061

Van Rompay, K. K., Abel, K., Lawson, J. R., Singh, R. P., Schmidt, K. A., Evans, T., et al. (2005). Attenuated poxvirus-based simian immunodeficiency virus (SIV) vaccines given in infancy partially protect infant and juvenile macaques against repeated oral challenge with virulent SIV. *J. Acquir. Immune Defic. Syndr.* 38, 124–134. doi: 10.1097/00126334-200502010-00002

Vasan, S., Hurley, A., Schlesinger, S. J., Hannaman, D., Gardiner, D. F., Dugin, D. P., et al. (2011). *In vivo* electroporation enhances the immunogenicity of an HIV-1 DNA vaccine candidate in healthy volunteers. *PLoS ONE* 6:e19252. doi: 10.1371/journal.pone.0019252

von Gegerfelt, A. S., Rosati, M., Alicea, C., Valentin, A., Roth, P., Bear, J., et al. (2007). Long-lasting decrease in viremia in macaques chronically infected with simian immunodeficiency virus SIVmac251 after therapeutic DNA immunization. *J. Virol.* 81, 1972–1979. doi: 10.1128/JVI.01990-06

Wallace, A., West, K., Rothman, A. L., Ennis, F. A., Lu, S., and Wang, S. (2013). Post-translational intracellular trafficking determines the type of immune response elicited by DNA vaccines expressing Gag antigen of Human Immunodeficiency Virus Type 1 (HIV-1). *Hum. Vaccin. Immunother.* 9, 2095–2102. doi: 10.4161/hv.26009

Wang, S., Farfan-Arribas, D. J., Shen, S., Chou, T. H., Hirsch, A., He, F., et al. (2006a). Relative contributions of codon usage, promoter efficiency and leader sequence to the antigen expression and immunogenicity of HIV-1 Env DNA vaccine. *Vaccine* 24, 4531–4540. doi: 10.1016/j.vaccine.2005.08.023

Wang, S., Kennedy, J. S., West, K., Montefiori, D. C., Coley, S., Lawrence, J., et al. (2008b). Cross-subtype antibody and cellular immune responses induced by a polyvalent DNA prime-protein boost HIV-1 vaccine in healthy human volunteers. *Vaccine* 26, 1098–1110. doi: 10.1016/j.vaccine.2007.12.024

Wang, S., Pal, R., Mascola, J. R., Chou, T. H., Mboudjeka, I., Shen, S., et al. (2006b). Polyvalent HIV-1 Env vaccine formulations delivered by the DNA priming plus protein boosting approach are effective in generating neutralizing antibodies against primary human immunodeficiency virus type 1 isolates from subtypes A, B, C, D and E. *Virology* 350, 34–47. doi: 10.1016/j.virol.2006.02.032

Wang, S., Zhang, C., Zhang, L., Li, J., Huang, Z., and Lu, S., (2008a). The relative immunogenicity of DNA vaccines delivered by the intramuscular needle injection, electroporation and gene gun methods. *Vaccine* 26, 2100–2110. doi: 10.1016/j.vaccine.2008.02.033

Wang, S. W., Bertley, F. M., Kozlowski, P. A., Herrmann, L., Manson, K., Mazzara, G., et al. (2004). An SHIV DNA/MVA rectal vaccination in macaques provides systemic and mucosal virus-specific responses and protection against AIDS. *AIDS Res. Hum. Retroviruses* 20, 846–859. doi: 10.1089/0889222041725253

Wang, Y., Guo, Y., Wang, X., Huang, J., Shang, J., and Sun, S. (2011). Human serum amyloid P functions as a negative regulator of the innate and adaptive immune responses to DNA vaccines. *J. Immunol.* 186, 2860–2870. doi: 10.4049/jimmunol.1003641

Wang, Y., Guo, Y., Wang, X., Huang, J., Shang, J., and Sun, S. (2012). Serum amyloid P component facilitates DNA clearance and inhibits plasmid transfection: implications for human DNA vaccine. *Gene Ther.* 19, 70–77. doi: 10.1038/gt.2011.67

Webster, D. P., Dunachie, S., Vuola, J. M., Berthoud, T., Keating, S., Laidlaw, S. M., et al. (2005). Enhanced T cell-mediated protection against malaria in human challenges by using the recombinant poxviruses FP9 and modified vaccinia virus Ankara. *Proc. Natl. Acad. Sci. U.S.A.* 102, 4836–4841. doi: 10.1073/pnas.0406381102

Whelan, K. T., Pathan, A. A., Sander, C. R., Fletcher, H. A., Poulton, I., Alder, N. C., et al. (2009). Safety and immunogenicity of boosting BCG vaccinated subjects with BCG: comparison with boosting with a new TB vaccine, MVA85A. *PLoS ONE* 4:e5934. doi: 10.1371/journal.pone.0005934

Widera, G., Austin, M., Rabussay, D., Goldbeck, C., Barnett, S. W., Chen, M., et al. (2000). Increased DNA vaccine delivery and immunogenicity by electroporation *in vivo*. *J. Immunol.* 164, 4635–4640. doi: 10.4049/jimmunol.164.9.4635

Winstone, N., Wilson, A. J., Morrow, G., Boggiano, C., Chiuchiolo, M. J., Lopez, M., et al. (2011). Enhanced control of pathogenic Simian immunodeficiency virus SIVmac239 replication in macaques immunized with an interleukin-12 plasmid and a DNA prime-viral vector boost vaccine regimen. *J. Virol.* 85, 9578–9587. doi: 10.1128/JVI.05060-11

Xiang, Z., Gao, G., Reyes-Sandoval, A., Cohen, C. J., Li, Y., Bergelson, J. M., et al. (2002). Novel, chimpanzee serotype 68-based adenoviral vaccine carrier for induction of antibodies to a transgene product. *J. Virol.* 76, 2667–2675. doi: 10.1128/JVI.76.6.2667-2675.2002

Xiang, Z., Li, Y., Cun, A., Yang, W., Ellenberg, S., Switzer, W. M., et al. (2006). Chimpanzee adenovirus antibodies in humans, sub-Saharan Africa. *Emerging Infect. Dis.* 12, 1596–1599. doi: 10.3201/eid1210.060078

Xiang, Z. Q., Gao, G. P., Reyes-Sandoval, A., Li, Y., Wilson, J. M., and Ertl, H. C. (2003). Oral vaccination of mice with adenoviral vectors is not impaired by preexisting immunity to the vaccine carrier. *J. Virol.* 77, 10780–10789. doi: 10.1128/JVI.77.20.10780-10789.2003

Xiang, Z. Q., Knowles, B. B., McCarrick, J. W., and Ertl, H. C. (1995). Immune effector mechanisms required for protection to rabies virus. *Virology* 214, 398–404. doi: 10.1006/viro.1995.0049

Xiang, Z. Q., Pasquini, S., and Ertl, H. C. (1999). Induction of genital immunity by DNA priming and intranasal booster immunization with a replication-defective adenoviral recombinant. *J. Immunol.* 162, 6716–6723.

Xiang, Z. Q., Yang, Y., Wilson, J. M., and Ertl, H. C. (1996). A replication-defective human adenovirus recombinant serves as a highly efficacious vaccine carrier. *Virology* 219, 220–227. doi: 10.1006/viro.1996.0239

Yan, J., Yoon, H., Kumar, S., Ramanathan, M. P., Corbitt, N., Kutzler, M., et al. (2007). Enhanced cellular immune responses elicited by an engineered HIV-1 subtype B consensus-based envelope DNA vaccine. *Mol. Ther.* 15, 411–421. doi: 10.1038/sj.mt.6300036

Yang, H., Dong, T., Turnbull, E., Ranasinghe, S., Ondondo, B., Goonetilleke, N., et al. (2007c). Broad TCR usage in functional HIV-1-specific CD8+ T cell expansions driven by vaccination during highly active antiretroviral therapy. *J. Immunol.* 179, 597–606. doi: 10.4049/jimmunol.179.1.597

Yang, T. C., Dayball, K., Wan, Y. H., and Bramson, J. (2003a). Detailed analysis of the CD8+ T-cell response following adenovirus vaccination. *J. Virol.* 77, 13407–13411. doi: 10.1128/JVI.77.24.13407-13411.2003

Yang, T. C., Millar, J. B., Grinshtein, N., Bassett, J., Finn, J., and Bramson, J. L., (2007a). T-cell immunity generated by recombinant adenovirus vaccines. *Expert Rev. Vaccines* 6, 347–356. doi: 10.1586/14760584.6.3.347

Yang, T. C., Millar, J., Groves, T., Grinshtein, N., Parsons, R., Takenaka, S., et al. (2006). The CD8+ T cell population elicited by recombinant adenovirus displays a novel partially exhausted phenotype associated with prolonged antigen presentation that nonetheless provides long-term immunity. *J. Immunol.* 176, 200–210. doi: 10.4049/jimmunol.176.1.200

Yang, T. C., Millar, J., Groves, T., Zhou, W., Grinshtein, N., Parsons, R., et al. (2007b). On the role of CD4+ T cells in the CD8+ T-cell response elicited by recombinant adenovirus vaccines. *Mol. Ther.* 15, 997–1006. doi: 10.1038/sj.mt.6300130

Yang, Z. Y., Wyatt, L. S., Kong, W. P., Moodie, Z., Moss, B., and Nabel, G. J., (2003b). Overcoming immunity to a viral vaccine by DNA priming before vector boosting. *J. Virol.* 77, 799–803. doi: 10.1128/JVI.77.1.799-803.2003

Yu, Q., Jones, B., Hu, N., Chang, H., Ahmad, S., Liu, J., et al. (2006). Comparative analysis of tropism between canarypox (ALVAC) and vaccinia viruses reveals a more restricted and preferential tropism of ALVAC for human cells of the monocytic lineage. *Vaccine* 24, 6376–6391. doi: 10.1016/j.vaccine.2006.06.011

Yuki, Y., and Kiyono, H. (2003). New generation of mucosal adjuvants for the induction of protective immunity. *Rev. Med. Virol.* 13, 293–310. doi: 10.1002/rmv.398

Zhang, S., Huang, W., Zhou, X., Zhao, Q., Wang, Q., and Jia, B. (2013). Seroprevalence of neutralizing antibodies to human adenoviruses type-5 and type-26 and chimpanzee adenovirus type-68 in healthy Chinese adults. *J. Med. Virol.* 85, 1077–1084. doi: 10.1002/jmv.23546

Zhang, X., Cassis-Ghavami, F., Eller, M., Currier, J., Slike, B. M., Chen, X., et al. (2007). Direct comparison of antigen production and induction of apoptosis by canarypox virus- and modified vaccinia virus ankara-human immunodeficiency virus vaccine vectors. *J. Virol.* 81, 7022–7033. doi: 10.1128/JVI.02654-06

Zhang, Y., Chirmule, N., Gao, G. P., Qian, R., Croyle, M., Joshi, B., et al. (2001). Acute cytokine response to systemic adenoviral vectors in mice is mediated by dendritic cells and macrophages. *Mol. Ther.* 697–707. doi: 10.1006/mthe.2001.0329

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 30 June 2014; accepted: 03 August 2014; published online: 22 August 2014.
Citation: Ondondo BO (2014) The influence of delivery vectors on HIV vaccine efficacy. Front. Microbiol. ***5****:439. doi: 10.3389/fmicb.2014.00439*
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Microbiology.
Copyright © 2014 Ondondo. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 01 May 2014
doi: 10.3389/fmicb.2014.00200

Plasmodium falciparum induces Foxp3hi CD4T cells independent of surface *Pf*EMP1 expression via small soluble parasite components

Anja Scholzen[1,2†], Brian M. Cooke[3] and Magdalena Plebanski[1]*

[1] *Department of Immunology, Monash University, Melbourne, VIC, Australia*
[2] *Department of Medical Microbiology, Radboud university medical center, Nijmegen, Netherlands*
[3] *Department of Microbiology, Monash University, Clayton, VIC, Australia*

Edited by:
Shuo Li, Macfarlane Burnet Institute for Medical Research and Public Health, Australia

Reviewed by:
Hajime Hisaeda, Gunma University, Japan
Diana Silvia Hansen, The Walter and Eliza Hall Institute of Medical Research, Australia

***Correspondence:**
Magdalena Plebanski, Department of Immunology, Monash University, Commercial Road, Melbourne, VIC 3004, Australia
e-mail: magdalena.plebanski@monash.edu

[†]***Present address:***
Anja Scholzen, Department of Medical Microbiology, Radboud university medical center, Nijmegen, Netherlands

Elevated levels of regulatory T cells following *Plasmodium* infection are a well-reported phenomenon that can influence both protective and pathological anti-parasite responses, and might additionally impact on vaccine responses in acutely malaria infected individuals. The mechanisms underlying their induction or expansion by the parasite, however, are incompletely understood. In a previous study, *Plasmodium falciparum* infected red blood cells (iRBCs) were shown to induce effector-cytokine producing Foxp3int CD4+ T cells, as well as regulatory Foxp3hi CD4+ T cells *in vitro*. The aim of the present study was to determine the contribution of parasite components to the induction of Foxp3 expression in human CD4+ T cells. Using the surface *Pf*EMP1-deficient parasite line 1G8, we demonstrate that induction of Foxp3hi and Foxp3int CD4+T cells is independent of *Pf*EMP1 expression on iRBCs. We further demonstrate that integrity of iRBCs is no requirement for the induction of Foxp3 expression. Finally, transwell experiments showed that induction of Foxp3 expression, and specifically the generation of Foxp3hi as opposed to Foxp3int CD4T cells, can be mediated by soluble parasite components smaller than 20 nm and thus likely distinct from the malaria pigment hemozoin. These results suggest that the induction of Foxp3hi T cells by *P. falciparum* is largely independent of two key immune modulatory parasite components, and warrant future studies into the nature of the Foxp3hi inducing parasite components to potentially allow their exclusion from vaccine formulations.

Keywords: Malaria, *Plasmodium falciparum*, regulatory T cell, Foxp3, *Pf*EMP-1, hemozoin

INTRODUCTION

Malaria caused by infection with protozoan *Plasmodium* parasites is a life threatening disease that is at least partially immune mediated. Disease-contributing factors include excessive inflammatory responses and overwhelming parasite replication insufficiently controlled by anti-parasite immune responses. Regulatory T cells (Tregs) can suppress both protective as well as pathological adaptive immune responses, and are elevated in both human *falciparum* and *vivax* malaria as well as in murine malaria models (Scholzen et al., 2010). Although the consequences of elevated Treg levels during malaria are yet to be determined, several studies indicate that depending on the stage of infection, increased Treg levels can be protective or detrimental to the host (Finney et al., 2010; Hansen and Schofield, 2010; Scholzen et al., 2010). In addition to potentially limiting responses to parasite antigens (Ho et al., 1986; Bejon et al., 2007), elevated Treg levels during acute blood-stage malaria infection might also contribute to the reduced acquisition of immune responses to heterologous antigens, such as standard childhood vaccines (Greenwood et al., 1972; Williamson and Greenwood, 1978; Whittle et al., 1984). The mechanisms underlying the elevated levels of this important cell type during malaria, however, are incompletely understood.

We have previously dissected the host immune mechanisms contributing to the induction of effector-cytokine producing CD25+Foxp3int CD4+ T cells, as well as regulatory CD25+Foxp3hi CD4+ T cells that inhibited Foxp3int effector cytokine production, following *P. falciparum*-infected red blood cells (iRBCs) exposure *in vitro* (Scholzen et al., 2009). The parasite factors responsible for Treg induction during malaria are yet unknown. This is especially relevant as the identification of parasite-specific components relevant for Treg induction may allow the development of intervention strategies directly targeted at the parasite, and the specific exclusion or inclusion of parasite components in therapeutic or vaccine formulations (Casares and Richie, 2009; Higgins et al., 2011).

Two key parasite components that have attracted attention as modulators of immune response in both human and murine malaria are the virulence factor and variant surface antigen *P. falciparum* erythrocyte membrane protein (*Pf*EMP)-1, and the heme degradation product and malaria pigment hemozoin. Whilst findings for *Pf*EMP-1 are controversial, both have been shown in a number of studies to interfere with the activation and maturation of antigen-presenting cells such as monocytes and dendritic cells (Millington et al., 2006; Wykes and Good, 2008; Stevenson et al., 2011). This is particularly relevant as both in

human *in vitro* studies (Scholzen et al., 2009; Finney et al., 2012; Clemente et al., 2013) and in murine models (Hisaeda et al., 2008), antigen-presenting cells are crucial mediators of Treg induction and activation by malaria parasites. The aim of the present study was therefore to determine the contribution of parasite components to the induction of Foxp3 expression in human CD4+ T cells.

MATERIALS AND METHODS

P. falciparum CULTURE AND TROPHOZOITE ISOLATION

Mycoplasma-free blood-stage parasites of *P. falciparum* (strain 3D7) were maintained in O+ erythrocytes in RPMI-1640 medium (JRH, Lenexa, KS, USA) supplemented with 1 mM glutamine, 11 mM glucose, 25 mM HEPES, 0.2% (w/v) sodium bicarbonate, 200 mM hypoxanthine, 40 mg/ml gentamycin (all Sigma–Aldrich, St. Louis, MO, USA), and 0.5% (w/v) AlbuMAX II (GIBCO, Invitrogen, Carlsbad, CA, USA) at 37°C in an atmosphere of 5% CO2 and 1% O2 in N2. Knob-expressing parasites were enriched weekly using gelofusine solution (Braun Melsungen, Germany). The 3D7-derived SBP-1 knock-out parasite line [clone 1G8 (Cooke et al., 2006)] was grown under drug-pressure (2.5 nM WR99210 and 4 μM Ganciclovir). Trophozoite stage parasites were isolated by density gradient centrifugation following layering onto a gradient of 40/60/80% isotonic Percoll (Amersham Biosciences, Uppsala, Sweden). The percentage of infected erythrocytes was typically 90–100%.

PBMC ISOLATION AND IRBC:PBMC CO-CULTURE

To examine the induction of Foxp3 expression by iRBCs, we employed the *in vitro* co-culture system previously validated in our laboratory (Scholzen et al., 2009). Peripheral blood mononuclear cells (PBMCs) were recovered by Ficoll–Hypaque (Amersham Biosciences) density gradient centrifugation from buffy coats [Australian Red Cross Blood Service (ARCBS), Melbourne, VIC, Australia]. The ARCBS received informed consent from all donors to use their donation for research purposes and the Monash University Human Research Ethics Committee approved the research purpose for which buffy coats were used. Autologous human serum (HS) was obtained by coagulating platelet-rich plasma from buffy coats with 0.3% (w/v) CaCl2, followed by heat inactivation at 56uC for 30 min. PBMCs were cultured in AIM-V medium (GIBCO, Invitrogen) supplemented with 5% autologous HS alone (untreated controls), with non-infected control erythrocytes or trophozoite-stage iRBCs. An iRBC:PBMC ratio of 2:1 was chosen (Scholzen et al., 2009), calculated to reflect a clinically relevant parasitemia found in natural infections (0.1% parasitemia or 5000 iRBC/μl blood) (Minigo et al., 2009; Walther et al., 2009). To obtain iRBC lysate, the integrity of iRBCs was disrupted by five rounds of freeze-thawing. In some experiments, tissue culture inserts (Anopore, NUNC, Naperville, IL, USA) were used to separate iRBCs (inside) from PBMCs (outside the transwell). A pore size of 20 nm was chosen to prohibit transfer of hemozoin crystals, which are on all sides larger than this cut-off (Noland et al., 2003).

CELL PHENOTYPING BY FLOW CYTOMETRY

Cells were washed with PBS and incubated with antibodies diluted in PBS/10% HS/0.01% NaN3 (sodium azide) for 30 min on ice. Surface antibodies were anti-CD4 PerCp (clone SK3), CD3 FITC (clone UCHT1), and CD25 PE (clone M-A251, all BD Biosciences). Intracellular staining with anti-Foxp3 APC (clone PCH101, eBiosciences) was performed using the eBioscience fixation/permeabilization buffer kit. A minimum of 10^5 events in the lymphocyte gate was acquired using a FACScalibur flow cytometer for 4-color analysis and analyzed using WEASEL software (WEHI, Melbourne, VIC, Australia). Cells were gated first based on forward and side scatter to excluded dead cells and cell debris. T cells in the lymphocyte gate were identified based on CD3 expression, further sub-gated on CD4+ T cells (**Figure 1A**) and CD25+ cells then subdivided into Foxp3hi and Foxp3int cells (**Figure 1B**).

DATA PRESENTATION AND STATISTICAL ANALYSIS

We employed normalization onto control conditions for each donor, to be able to analyze changes in Foxp3hi or Foxp3int proportions (measured as percentage of CD4 T cells). Normalized values are referred to as fold change compared to control conditions (value 1).

Statistical analysis was carried out using GraphPad Prism software v4 (San Diego, CA, USA). Due to the small power of non-parametric tests to detect differences in small sample sizes, all tests were chosen to be parametric. *P* values between two groups were determined by two-tailed paired Student's *t*-test. Three or more groups were compared by repeated-measures one-way ANOVA, followed by Tukey's multiple comparison post test. A $p < 0.05$ was considered significant.

RESULTS

We firstly addressed the question, whether the induction of Foxp3 expression in CD4+ T cells was dependent on interactions between iRBC surface-expressed *Pf*EMP-1 and corresponding receptors on peripheral blood mononuclear cells such as monocytes using a recently established 3D7-derived surface *Pf*EMP1 deficient parasite line. This parasite line lacks, due to targeted gene disruption, expression of skeleton-binding protein 1 (SBP-1), a Maurer's cleft-associated protein essential for the transport of *Pf*EMP-1 to the iRBC surface (Cooke et al., 2006). When co-cultured with PBMC for 6 days, surface *Pf*EMP-1 deficient 1G8 parasites induced proportions of CD25+Foxp3hi and CD25+Foxp3int cells that were not significantly different from those induced by wild type 3D7 iRBCs (**Figure 1**, one-way ANOVA with Tukey's post-test). This indicates that induction of Foxp3 expression in CD4+ T cells is independent of surface *Pf*EMP-1 expression on iRBCs and therefore surface *Pf*EMP-1-host immune receptor interaction.

Since two previous studies have used iRBC lysate or soluble extract for Foxp3 induction *in vitro* (Finney et al., 2012; Clemente et al., 2013), we first assessed there was a difference between intact and lysed iRBC in their ability to induce CD25+Foxp3 expressing CD4+ T cells. When comparing Foxp3 induction in PBMC co-cultures with either intact iRBCs or iRBC lysate, iRBC lysate was nearly as effective in inducing both CD25+Foxp3hi and CD25+Foxp3int CD4+ T cells (**Figure 2**), with no significant difference between the two for neither absolute percentages or

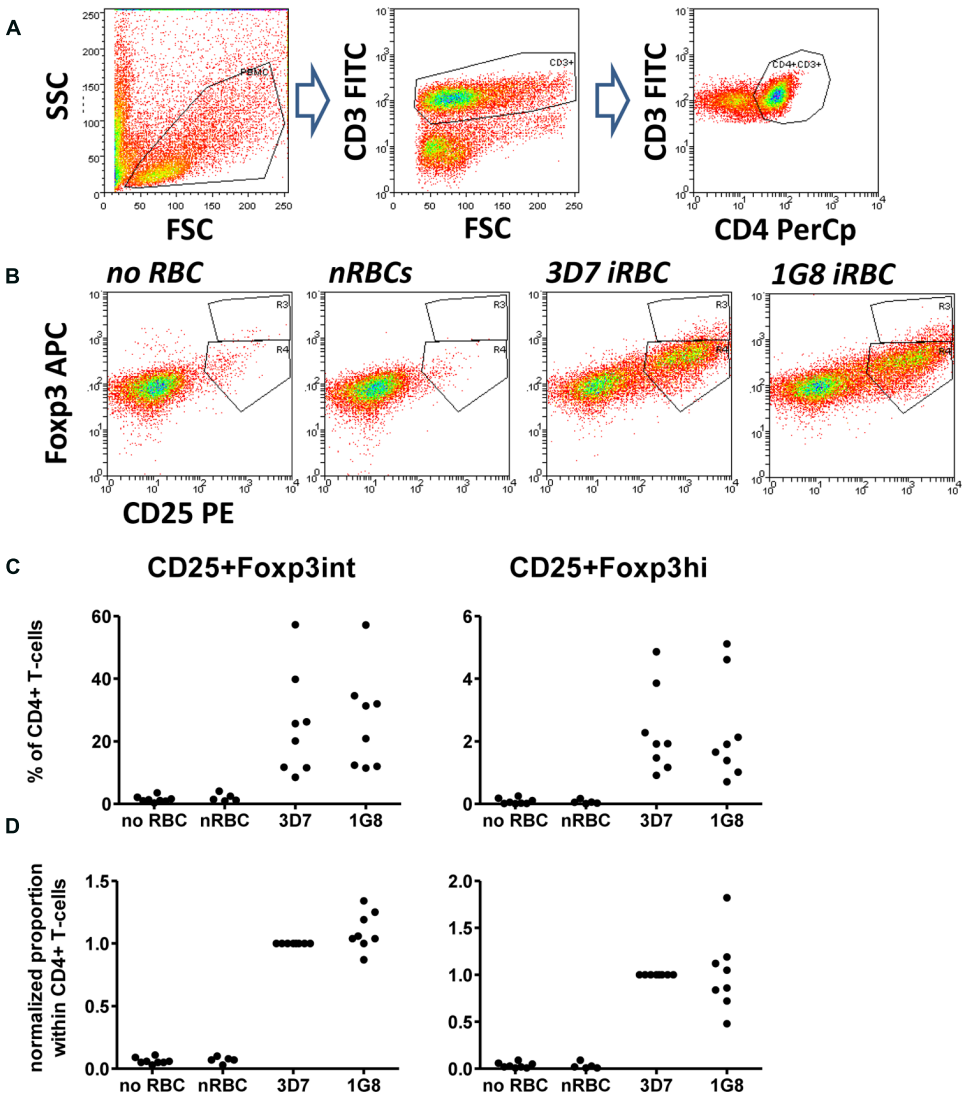

FIGURE 1 | Infected red blood cell (iRBC)-mediated Foxp3 expression is independent of surface *Pf*EMP-1 expression. PBMC were cultured alone or in the presence of normal (nRBC) or infected RBCs (iRBC) at an RBC:PBMC ratio of 2:1. RBCs were infected with either the parental strain 3D7 or the SBP-1 knock-out parasite line 1G8. On day 6, cells were harvested and analyzed by flow cytometry. **(A)** Lymphocytes were selected by FSC/SSC gating and further gated based on CD3 and CD4 staining. CD4+ T cells were then analyzed based on CD25 and Foxp3 expression. **(B)** Representative dots plots for PBMC from one donor stimulated with 3D7 and 1G8 parasites. **(C)** Proportions of Foxp3hi and Foxp3int CD4+CD25+ T cells determined as a percentage of CD3+CD4+ T cells for eight donors in six independent experiments. **(D)** Values of all conditions were normalized for each individual donor on proportions induced by 3D7 iRBCs. Vertical bars represent median values.

after normalization. Therefore, iRBC integrity is indeed not a prerequisite for the Foxp3 induction in CD4+ T cells upon parasite exposure.

To further investigate the possibility that large intracellular components such as hemozoin crystals interacting with monocytes were contributing to Foxp3 induction, we employed tissue culture inserts to separate iRBCs (inside) and PBMCs (outside the transwell). We specifically chose a pore size of 20 nm to prohibit transfer of large parasite components, including membrane fragments and intact hemozoin crystals, which are larger than 20 nm in diameter on either side of their brick-like cuboidal body (Noland et al., 2003), between the two chambers. Accordingly, in this transwell setting we found no light-microscopic evidence of hemozoin incorporation into monocytes (which in direct co-cultures are typically filled with dark hemozoin material, data not shown). As shown in **Figure 3**, similar to direct co-cultures, iRBC separated from PBMC by a transwell were also capable of inducing Foxp3 expression in CD4+ T cells. Importantly, in four out of five donors Foxp3hi CD4+ T cells were still induced at levels comparable to direct co-culture (**Figure 3**), while induction of Foxp3int CD4+ T cells was reduced in all five donors tested (**Figures 3B,C**; 20–31% compared to direct co-culture; $p < 0.001$, one-way ANOVA with Tukey's post-test). As a result, there was a trend that exposure of PBMC to soluble iRBC components

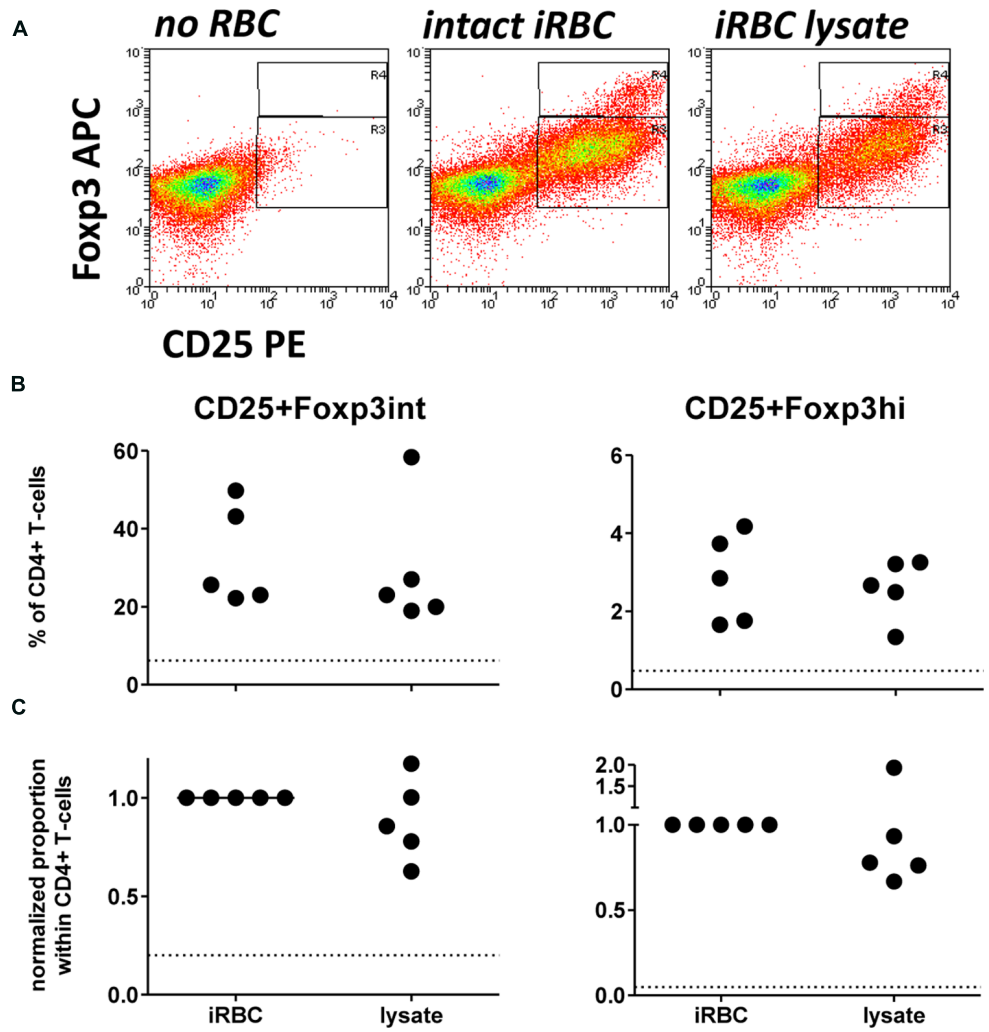

FIGURE 2 | iRBC-mediated Foxp3 expression does not require intact iRBCs. PBMC were cultured alone or in the presence of normal (nRBC) or infected RBCs (iRBC) at an RBC:PBMC ratio of 2:1. PBMC were co-cultured with equal amounts of either intact 3D7 iRBCs, or 3D7 iRBC lysate resulting from five rounds of freezing and thawing. **(A)** Representative dots plots for PBMCs from one donor stimulated with intact iRBCs versus iRBC lysate. **(B)** Proportions of Foxp3hi and Foxp3int CD4+CD25+ T cells were determined on day 6 as a percentage of CD3+CD4+ T cells for five donors in two independent experiments. **(C)** Values of all conditions were normalized for each individual donor on proportions induced by intact iRBCs. Vertical bars represent median values. Dotted lines show the upper 95% confidence interval of the mean of uRBC-stimulated control cultures.

smaller than 20 nm instead of complete iRBC enhanced the ratio of Foxp3hi:Foxp3int cells within the CD4+CD25+ population (**Figure 3D**).

DISCUSSION

In this study, we show that *P. falciparum* iRBCs can induce Foxp3hi CD4 T cells independent of surface-expressed PfEMP-1 via soluble parasite components smaller than 20 nm.

Similar to our finding that induction of Foxp3 expression is independent of iRBC surface *Pf*EMP-1 expression and contact with intact iRBCs, a recent study demonstrated that human monocyte-derived DC maturation can be inhibited by *P. falciparum* independent of surface *Pf*EMP1 expression and also across a transwell (Elliott et al., 2007). Using a murine malaria model, Orengo et al. (2008) reported that inhibition of murine DC maturation following *P. yoelii* infection was also mediated by a yet unidentified soluble factor. Future studies are now needed to further identify the soluble factor mediating these immunomodulatory effects of the parasite. Importantly, monocytes or monocytes-derived DCs were previously shown to be required to drive the induction of Foxp3 expression in human CD4 T cells upon *P. falciparum* exposure *in vitro* (Scholzen et al., 2009; Clemente et al., 2013) and DCs activated Tregs in *P. yoelii* infected mice (Hisaeda et al., 2008). Future studies may therefore address the question whether it may even be the same mechanism by which the parasite modulates not only with monocyte and DC function, but also initiates the induction of Tregs.

Previous studies have used either intact iRBCs (Scholzen et al., 2009), iRBC lysate (Finney et al., 2012), or the soluble fraction

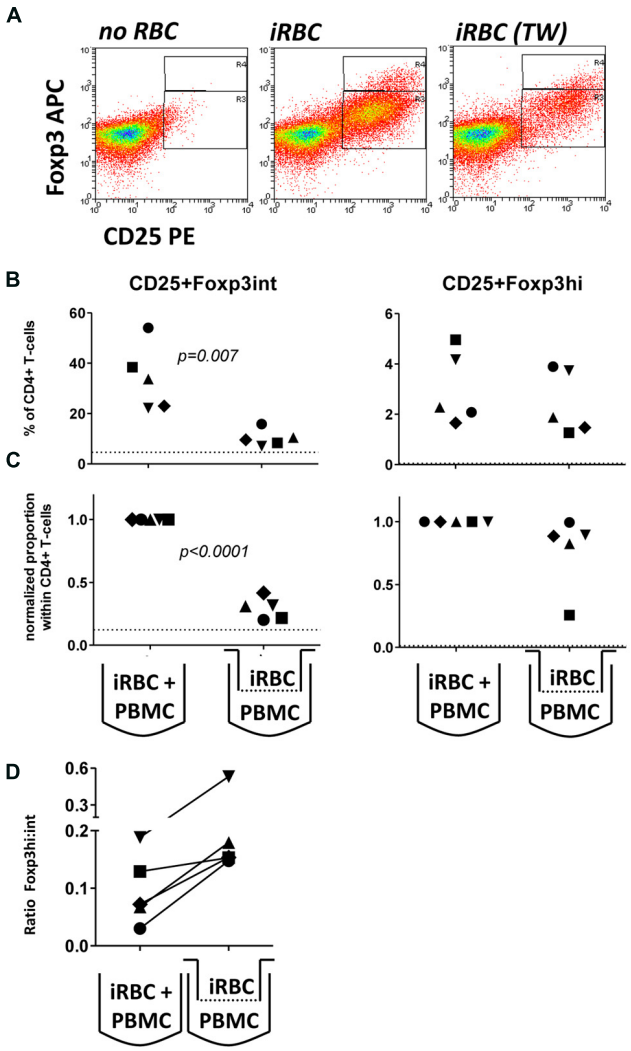

FIGURE 3 | Foxp3hi and to a lesser degree Foxp3int induction is mediated by soluble iRBC components smaller than 20 nm. To exclude large membrane fragments and intact hemozoin crystals and to examine the contribution of small soluble molecules derived from iRBCs to Foxp3 induction, intact iRBC were separated from PBMC through a transwell membrane (pore size 20 nm). **(A)** Representative dots plots for PBMCs from one donor cultured in direct contact with iRBCs compared to transwell-separated iRBCs. **(B)** Proportions of Foxp3hi and Foxp3int CD4+CD25+ T cells were determined on day 6 as a percentage of CD4+ T cells for five donors in two independent experiments. **(C)** Values of all conditions were normalized for proportions induced by direct co-culture with iRBCs for each donor. Vertical bars represent median values. **(D)** The ratio of Foxp3hi:Foxp3int cells within the CD4+CD25+ T cell population was calculated for each individual donor in direct or transwell separated co-cultures. Vertical bars represent median values. Individual donors are identified by unique symbols. Dotted lines show the upper 95% confidence interval of the mean of uRBC-stimulated control cultures.

of iRBC lysate (Clemente et al., 2011) as a stimulus for Foxp3 expression in human CD4 T cells *in vitro*. In this direct side-by-side comparison we show that Foxp3 induction by iRBC lysate is indeed comparable to intact iRBC. Therefore, if direct cell–cell interaction between immune cells and iRBCs are not a pre-requisite for Treg induction, which is instead mediated by soluble factors resulting from schizont rupture, then Treg induction can also occur at distant sites clear of measurable parasitemia.

For instance, while mature trophozoite-stage *P. falciparum* parasites are typically sequestered in the microvasculature (to avoid clearance in the spleen), soluble parasite components released upon parasite rupture would have access to monocytes and T cells in the spleen. Moreover, soluble parasite components might also mediate Treg induction in more distant sites outside the circulation, such as in lymph nodes. In as how far this occurs, and whether this would affect vaccination-induced immune responses during acute blood-stage malaria infection (Greenwood et al., 1972; Williamson and Greenwood, 1978; Whittle et al., 1984), remains to be determined. Finally, such small parasite components may be able to cross the placental barrier and thus explain and contribute to the induction of Tregs in cord blood even in the absence of direct cord blood parasitemia. Indeed, in some studies levels of Tregs have been found to be elevated in neonates born to mothers who had experienced malaria episodes during pregnancy (Brustoski et al., 2006; Mackroth et al., 2011), while in other studies, *ex vivo* cord blood Treg frequencies were unaffected by placental malaria and only increased only upon *in vitro* stimulation with iRBC extract (Flanagan et al., 2010; Soulard et al., 2011). It is yet unclear whether Treg induction during a single *Plasmodium* infection predisposes the immune system to heightened regulatory responses at the next encounter with the parasite, but *in utero* exposure to Treg-inducing parasite components may prime the fetus's immune system to respond with a less inflammatory response upon re-exposure to malaria-antigens (Malhotra et al., 2009; Flanagan et al., 2010). Moreover, such malaria-induced immune modulation might also be one explanation for observations of reduced vaccination-responses in children born to women with placental-malaria (Labeaud et al., 2009; Walther et al., 2012).

Hemozoin has been shown in several studies to activate TLR9 (Shio et al., 2010). Moreover, DCs activated via TLR9 [either by iRBCs in mice (Hisaeda et al., 2008) or CpG DNA in human (Moseman et al., 2004)] can induce/activate Tregs. These findings indicate a potential role for hemozoin in Treg induction. It remains to be formally shown whether iRBC-purified or synthetic hemozoin contributes to the induction of Tregs by *P. falciparum* iRBCs. Our results from experiments using transwell inserts with a 20 nm pore size to separate PBMCs and iRBCs, however, suggest that hemozoin crystals do not have a major contribution to the generation of Foxp3hi T cells: Intact hemozoin crystals are larger than 20 nm (Noland et al., 2003) and therefore unlikely to have crossed the 20 nm sized pores of the transwell inserts used in this study. We adoped a transwell approach to prohibit the transfer of intact hemozoin crystals after schizont rupture, since it is currently technically not possible to deplete iRBC extracts of hemozoin without denaturing other lysate components and antigens (Coban et al., 2010). And while we cannot rule out that smaller hemozoin crystal fragments might have crossed the transwell border, the TLR9 binding capacity of hemozoin has recently been shown to be restricted to a crystal size range of 50–200 nm, while hemin molecules smaller than 50 nm were ineffective (Coban et al., 2010).

A curious finding was the trend towards a favored induction of Foxp3hi over Foxp3int T cells when components larger than 20 nm were excluded from the co-culture using transwell membranes. Whilst these data require further analysis in future studies, it is tempting to speculate that small soluble parasite components are preferentially driving Foxp3hi induction, whilst Foxp3int effector T cells may rely on a greater pool of membrane associated antigens. We have previously shown that although iRBC-mediated induction of Foxp3hi T cells does rely on T effector-produced IL-2 and is further driven by cytokines such as IL-10 and TGFβ, in itself it is not dependent on MHC class II antigen-presentation and those cells therefore not necessarily malaria antigen-specific (Scholzen et al., 2009). The current data are in line with this finding, suggesting that activation of T cells in an environment of direct contact of antigen-presenting cells with intact iRBCs might result in a more efficient induction of (Foxp3int) effector T cells in a parasite antigen-specific manner. In contrast, induction of Foxp3hi T cells with a T regulatory phenotype may also occur independent of membrane-associated antigens at a distant site, mediated by cytokines acting in concert with circulating soluble parasite molecules. Further research is now necessary to determine the nature and mechanism of action of these soluble parasite components.

To conclude, our results indicate that the induction of Foxp3hi regulatory T cells by *P. falciparum* may be largely independent of two key immunomodulatory parasite components, namely the surface protein *Pf*EMP1 and the malaria pigment hemozoin and warrant future studies into the nature of the Foxp3hi inducing parasite components. Furthermore, similar to their distinct cytokine requirements (Scholzen et al., 2009), Foxp3int effector-like and Foxp3hi regulatory-like CD4+ T cells appear to rely on different parasite components for their induction. These findings merit future in-depth studies to identify the parasite components responsible for regulatory versus effector T cell induction. Identification of such parasite components will be important to ensure appropriate exclusion or inclusion of such parasite components from vaccine formulations.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: Anja Scholzen, Magdalena Plebanski. Performed the experiments and analyzed the data: Anja Scholzen. Contributed essential reagents/materials/analysis tools: Brian M. Cooke, Magdalena Plebanski. Interpreted data and wrote the paper: Anja Scholzen, Magdalena Plebanski.

ACKNOWLEDGMENTS

This work was supported by the Australian NHMRC (Brian M. Cooke and Magdalena Plebanski), the Howard Hughes Medical Institute International Scholar's Program (support to Magdalena Plebanski). AS was a recipient of an Endeavour International Postgraduate Research Scholarship and a Melbourne International Research Scholarship at the University of Melbourne, Department of Medicine. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Red blood cells for *P. falciparum* culture and buffy coats were kindly provided by the Australian Red Blood Cross Service, Melbourne, Australia.

REFERENCES

Bejon, P., Mwacharo, J., Kai, O., Todryk, S., Keating, S., Lowe, B., et al. (2007). The induction and persistence of T cell IFN-gamma responses after vaccination or natural exposure is suppressed by *Plasmodium falciparum*. *J. Immunol.* 179, 4193–4201.

Brustoski, K., Moller, U., Kramer, M., Hartgers, F. C., Kremsner, P. G., Krzych, U., et al. (2006). Reduced cord blood immune effector-cell responsiveness mediated by CD4+ cells induced in utero as a consequence of placental *Plasmodium falciparum* infection. *J. Infect. Dis.* 193, 146–154. doi: 10.1086/498578

Casares, S., and Richie, T. L. (2009). Immune evasion by malaria parasites: a challenge for vaccine develop. *Curr. Opin. Immunol.* 21, 321–330. doi: 10.1016/j.coi.2009.05.015

Clemente, A., Caporale, R., Sannella, A. R., Majori, G., Severini, C., Fadigati, G., et al. (2011). *Plasmodium falciparum* soluble extracts potentiate the suppressive function of polyclonal T regulatory cells through activation of TGFbeta-mediated signals. *Cell. Microbiol.* 13, 1328–1338. doi: 10.1111/j.1462-5822.2011.01622.x

Clemente, A. M., Fadigati, G., Caporale, R., Marchese, D. G., Castronovo, G., Sannella, A. R., et al. (2013). Modulation of the immune and inflammatory responses by *Plasmodium falciparum* schizont extracts: role of myeloid dendritic cells in effector and regulatory functions of CD4+ lymphocytes. *Infect. Immun.* 81, 1842–1851. doi: 10.1128/IAI.01226-12

Coban, C., Igari, Y., Yagi, M., Reimer, T., Koyama, S., Aoshi, T., et al. (2010). Immunogenicity of whole-parasite vaccines against *Plasmodium falciparum* involves malarial hemozoin and host TLR9. *Cell Host Microbe* 7, 50–61. doi: 10.1016/j.chom.2009.12.003

Cooke, B. M., Buckingham, D. W., Glenister, F. K., Fernandez, K. M., Bannister, L. H., Marti, M., et al. (2006). A Maurer's cleft-associated protein is essential for expression of the major malaria virulence antigen on the surface of infected red blood cells. *J. Cell Biol.* 172, 899–908. doi: 10.1083/jcb.200509122

Elliott, S. R., Spurck, T. P., Dodin, J. M., Maier, A. G., Voss, T. S., Yosaatmadja, F., et al. (2007). Inhibition of dendritic cell maturation by malaria is dose dependent and does not require *Plasmodium falciparum* erythrocyte membrane protein 1. *Infect. Immun.* 75, 3621–3632. doi: 10.1128/IAI.00095-07

Finney, O. C., Lawrence, E., Gray, A. P., Njie, M., Riley, E. M., and Walther, M. (2012). Freeze-thaw lysates of *Plasmodium falciparum*-infected red blood cells induce differentiation of functionally competent regulatory T cells from memory T cells. *Eur. J. Immunol.* 42, 1767–1777. doi: 10.1002/eji.201142164

Finney, O. C., Riley, E. M., and Walther, M. (2010). Regulatory T cells in malaria - friend or foe? *Trends Immunol.* 31, 63–70. doi: 10.1016/j.it.2009.12.002

Flanagan, K. L., Halliday, A., Burl, S., Landgraf, K., Jagne, Y. J., Noho-Konteh, F., et al. (2010). The effect of placental malaria infection on cord blood and maternal immunoregulatory responses at birth. *Eur. J. Immunol.* 40, 1062–1072. doi: 10.1002/eji.200939638

Greenwood, B. M., Bradley-Moore, A. M., Bryceson, A. D., and Palit, A. (1972). Immunosuppression in children with malaria. *Lancet* 1, 169–172. doi: 10.1016/S0140-6736(72)90569-7

Hansen, D. S., and Schofield, L. (2010). Natural regulatory T cells in malaria: host or parasite allies? *PLoS Pathog.* 6:e1000771. doi: 10.1371/journal.ppat.1000771

Higgins, S. J., Kain, K. C., and Liles, W. C. (2011). Immunopathogenesis of falciparum malaria: implications for adjunctive therapy in the management of severe and cerebral malaria. *Expert Rev. Anti. Infect. Ther.* 9, 803–819. doi: 10.1586/eri.11.96

Hisaeda, H., Tetsutani, K., Imai, T., Moriya, C., Tu, L., Hamano, S., et al. (2008). Malaria parasites require TLR9 signaling for immune evasion by activating regulatory T cells. *J. Immunol.* 180, 2496–2503.

Ho, M., Webster, H. K., Looareesuwan, S., Supanaranond, W., Phillips, R. E., Chanthavanich, P., et al. (1986). Antigen-specific immunosuppression in human malaria due to *Plasmodium falciparum*. *J. Infect. Dis.* 153, 763–771. doi: 10.1093/infdis/153.4.763

Labeaud, A. D., Malhotra, I., King, M. J., King, C. L., and King, C. H. (2009). Do antenatal parasite infections devalue childhood vaccination? *PLoS Negl. Trop. Dis.* 3:e442. doi: 10.1371/journal.pntd.0000442

Mackroth, M. S., Malhotra, I., Mungai, P., Koech, D., Muchiri, E., and King, C. L. (2011). Human cord blood CD4+CD25hi regulatory T cells suppress prenatally acquired T cell responses to *Plasmodium falciparum* antigens. *J. Immunol.* 186, 2780–2791. doi: 10.4049/jimmunol.1001188

Malhotra, I., Dent, A., Mungai, P., Wamachi, A., Ouma, J. H., Narum, D. L., et al. (2009). Can prenatal malaria exposure produce an immune tolerant phenotype? A prospective birth cohort study in Kenya. *PLoS Med.* 6:e1000116. doi: 10.1371/journal.pmed.1000116

Millington, O. R., Di Lorenzo, C., Phillips, R. S., Garside, P., and Brewer, J. M. (2006). Suppression of adaptive immunity to heterologous antigens during *Plasmodium* infection through hemozoin-induced failure of dendritic cell function. *J. Biol.* 5, 1–22. doi: 10.1186/jbiol34

Minigo, G., Woodberry, T., Piera, K. A., Salwati, E., Tjitra, E., Kenangalem, E., et al. (2009). Parasite-dependent expansion of TNF receptor II-positive regulatory T cells with enhanced suppressive activity in adults with severe malaria. *PLoS Pathog.* 5:e1000402. doi: 10.1371/journal.ppat.1000402

Moseman, E. A., Liang, X., Dawson, A. J., Panoskaltsis-Mortari, A., Krieg, A. M., Liu, Y. J., et al. (2004). Human plasmacytoid dendritic cells activated by CpG oligodeoxynucleotides induce the generation of CD4+CD25+ regulatory T cells. *J. Immunol.* 173, 4433–4442.

Noland, G. S., Briones, N., and Sullivan, D. J. Jr. (2003). The shape and size of hemozoin crystals distinguishes diverse *Plasmodium* species. *Mol. Biochem. Parasitol.* 130, 91–99. doi: 10.1016/S0166-6851(03)00163-4

Orengo, J. M., Wong, K. A., Ocana-Morgner, C., and Rodriguez, A. (2008). A *Plasmodium yoelii* soluble factor inhibits the phenotypic maturation of dendritic cells. *Malar. J.* 7:254. doi: 10.1186/1475-2875-7-254

Scholzen, A., Minigo, G., and Plebanski, M. (2010). Heroes or villains? T regulatory cells in malaria infection. *Trends Parasitol.* 26, 16–25. doi: 10.1016/j.pt.2009.10.004

Scholzen, A., Mittag, D., Rogerson, S. J., Cooke, B. M., and Plebanski, M. (2009). *Plasmodium falciparum*-mediated induction of human CD25hiFoxp3hi CD4 T cells is independent of direct TCR stimulation and requires IL-2, IL-10 and TGFbeta. *PLoS Pathog.* 5:e1000543. doi: 10.1371/journal.ppat.1000543

Shio, M. T., Kassa, F. A., Bellemare, M. J., and Olivier, M. (2010). Innate inflammatory response to the malarial pigment hemozoin. *Microbes Infect.* 12, 889–899. doi: 10.1016/j.micinf.2010.07.001

Soulard, V., Amadoudji Zin, M., Fitting, C., Ibitokou, S., Oesterholt, M., Luty, A. J., et al. (2011). Placental malaria-associated suppression of parasite-specific immune response in neonates has no major impact on systemic CD4 T cell homeostasis. *Infect. Immun.* 79, 2801–2809. doi: 10.1128/IAI.00203-11

Stevenson, M. M., Ing, R., Berretta, F., and Miu, J. (2011). Regulating the adaptive immune response to blood-stage malaria: role of dendritic cells and CD4(+)Foxp3(+) regulatory T cells. *Int. J. Biol. Sci.* 7, 1311–1322. doi: 10.7150/ijbs.7.1311

Walther, B., Miles, D. J., Waight, P., Palmero, M. S., Ojuola, O., Touray, E. S., et al. (2012). Placental malaria is associated with attenuated CD4 T-cell responses to tuberculin PPD 12 months after BCG vaccination. *BMC Infect. Dis.* 12:6. doi: 10.1186/1471-2334-12-6

Walther, M., Jeffries, D., Finney, O. C., Njie, M., Ebonyi, A., Deininger, S., et al. (2009). Distinct roles for FOXP3+ and FOXP3- CD4+ T cells in regulating cellular immunity to uncomplicated and severe *Plasmodium falciparum* malaria. *PLoS Pathog.* 5:e1000364. doi: 10.1371/journal.ppat.1000364

Whittle, H. C., Brown, J., Marsh, K., Greenwood, B. M., Seidelin, P., Tighe, H., et al. (1984). T-cell control of Epstein-Barr virus-infected B cells is lost during *P. falciparum* malaria. *Nature* 312, 449–450. doi: 10.1038/312449a0

Williamson, W. A., and Greenwood, B. M. (1978). Impairment of the immune response to vaccination after acute malaria. *Lancet* 1, 1328–1329. doi: 10.1016/S0140-6736(78)92403-0

Wykes, M. N., and Good, M. F. (2008). What really happens to dendritic cells during malaria? *Nat. Rev. Microbiol.* 6, 864–870. doi: 10.1038/nrmicro1988

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.

Received: 20 February 2014; accepted: 15 April 2014; published online: 01 May 2014.
*Citation: Scholzen A, Cooke BM and Plebanski M (2014) Plasmodium falciparum induces Foxp3hi CD4 T cells independent of surface PfEMP1 expression via small soluble parasite components. Front. Microbiol. **5**:200. doi: 10.3389/fmicb.2014.00200*
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Microbiology.

Copyright © 2014 Scholzen, Cooke and Plebanski. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in MICROBIOLOGY

REVIEW ARTICLE
published: 12 June 2014
doi: 10.3389/fmicb.2014.00285

Universal immunity to influenza must outwit immune evasion

Sergio Quiñones-Parra[1], Liyen Loh[1], Lorena E. Brown[1], Katherine Kedzierska[1]*† and Sophie A. Valkenburg[2]*†

[1] Department of Microbiology and Immunology, The University of Melbourne at the Peter Doherty Institute for Infection and Immunity, Parkville, VIC, Australia
[2] Centre for Influenza Research and School of Public Health, The University of Hong Kong, Hong Kong, China

Edited by:
Magdalena Plebanski, Monash University, Australia

Reviewed by:
Birgit Strobl, University of Veterinary Medicine Vienna, Austria
Shuo Li, Burnet Institute, Australia

***Correspondence:**
Katherine Kedzierska, Department of Microbiology and Immunology, The University of Melbourne, Parkville, Melbourne, VIC 3010, Australia
e-mail: kkedz@unimelb.edu.au;
Sophie A. Valkenburg, Center of Influenza Research and School of Public Health, The University of Hong Kong, L5-25 Laboratory Block, 21 Sassoon rd., Pokfulam, Hong Kong, China
e-mail: sophiev@hku.hk
† These authors have contributed equally to this work.

Although an influenza vaccine has been available for 70 years, influenza virus still causes seasonal epidemics and worldwide pandemics. Currently available vaccines elicit strain-specific antibody (Ab) responses to the surface haemagglutinin (HA) and neuraminidase (NA) proteins, but these can be ineffective against serologically-distinct viral variants and novel subtypes. Thus, there is a great need for cross-protective or "universal" influenza vaccines to overcome the necessity for annual immunization against seasonal influenza and to provide immunity to reduce the severity of infection with pandemic or outbreak viruses. It is well established that natural influenza infection can provide cross-reactive immunity that can reduce the impact of infection with distinct influenza type A strains and subtypes, including H1N1, H3N2, H2N2, H5N1, and H7N9. The key to generating universal influenza immunity through vaccination is to target functionally-conserved regions of the virus, which include epitopes on the internal proteins for cross-reactive T cell immunity or on the HA stem for broadly reactive Ab responses. In the wake of the 2009 H1N1 pandemic, broadly neutralizing antibodies (bnAbs) have been characterized and isolated from convalescent and vaccinated individuals, inspiring development of new vaccination techniques to elicit such responses. Induction of influenza-specific T cell responses through vaccination has also been recently examined in clinical trials. Strong evidence is available from human and animal models of influenza to show that established influenza-specific T cell memory can reduce viral shedding and symptom severity. However, the published evidence also shows that $CD8^+$ T cells can efficiently select immune escape mutants early after influenza virus infection. Here, we discuss universal immunity to influenza viruses mediated by both cross-reactive T cells and Abs, the mechanisms of immune evasion in influenza, and propose how to counteract commonly occurring immune-escape variants.

Keywords: influenza viruses, T cells memory, antibodies, viral escape mechanisms, vaccine design

INTRODUCTION

Immunization is the most cost effective public health measure to prevent the spread of infectious diseases. Influenza causes seasonal epidemics as well as periodic global pandemics due to the introduction of novel strains and sporadic outbreaks from animal reservoirs. During the 2009 H1N1 pandemic, the overall infection rate was 10%, although the infection rate rose to 43% in school-aged children (Wu et al., 2010). Yet, an influenza vaccine has been available since the 1940's.

Current influenza vaccines predominantly mediate protection by eliciting neutralizing Ab responses to epitopes on the head region of the virion surface glycoprotein, HA, and also to the NA. The traditional trivalent influenza vaccine (TIV) is based on either inactivated whole/subvirion virus or detergent-disrupted virions, and is administered as an intramuscular injection to elicit systemic Ab responses. Alternatively, the live attenuated influenza vaccine (LAIV), is given intranasally. The LAIV vaccine does not boost T cell immunity in adults (He et al., 2006), but does improve protection by eliciting Ab responses locally in the respiratory tract. Notably, LAIV in children is able to establish influenza-specific T cell responses, possibly due to their naïve infection status (He et al., 2006). Whilst LAIV has increased protection compared to TIV (Monto et al., 2009), TIV has much wider use due to a greater number of manufacturers and constrained use of LAIV in the elderly and very young.

Originally, the influenza vaccine was a monovalent preparation, based on the common circulating strain of influenza A virus (IAV) and was swiftly updated to include an influenza B virus when these were recognized in the 1940's. Since the 1970's, a trivalent vaccine has been used to provide coverage for H1N1, H3N2, and influenza B virus, which now co-circulate. Even more recently, in 2013, the vaccine has become available in a quadrivalent form incorporating both Victoria and Yamagata lineages of influenza B. Thus, the number of vaccine strains has progressively been increased from a single strain to four, to provide broader protection and overcome the diversity of multiple influenza virus

subtypes and lineages. Nevertheless, within each subtype or lineage, constant antigenic drift gives rise to new and unpredictable antigenic variants that are not necessarily represented in the vaccine. Mismatch between the vaccine strain, predicted on the basis of antigenically novel isolates circulating in the previous winter in the opposite hemisphere, and the actual strain that emerges in the current winter can result in significant loss of vaccine effectiveness.

The mechanism of action of current vaccines, which mediate protection by induction of neutralizing Ab responses to the rapidly changing head of the HA protein, renders it ineffective after a few years at best, once all the antigenic regions on the head of the HA have mutated in response to pre-existing Ab. Inactivated vaccines are also very poor inducers of $CD8^+$ T cell responses, presumably because of inefficient uptake and priming by appropriate antigen presenting cells (APCs). There is evidence from animal models that TIV vaccination can actually inhibit the induction of cross-reactive T cell responses (Bodewes et al., 2011), which require active virus replication, resulting in a greater susceptibility to subsequent infection by novel viruses such as H5N1 (Bodewes et al., 2010). With an increased appreciation of the immune response and its induction, it is time to consider new vaccine approaches for seasonal influenza that additionally or exclusively target functionally conserved regions of the influenza virus, and may therefore provide some level of disease reduction against serologically distinct emergent strains, even in a pandemic context.

IAVs, whose ancestral host is aquatic birds, have spread to many other species including domestic poultry, horses, swine, humans, and even fruit bats. Although there are 17 different distinct HA subtypes and 10 NA subtypes thus far identified for IAVs, only H1N1 and H3N2, and previously H2N2, subtypes have become endemic in humans causing continual human transmission and seasonal epidemics. The introduction of other novel subtypes, as exemplified by the H5N1, H7N9, and H10N8 strains, cause sporadic human infections and are not yet fully adapted for efficient human-to-human transmission. The segmented nature of the influenza virus genome facilitates reassortment to generate novel hybrid viruses between influenza viruses from different species, some of pandemic potential. Furthermore, the error-prone viral RNA-dependent RNA polymerase, which enables the generation of viral mutants, facilitates selection of influenza viruses resistant to anti-viral drugs and immune effectors. Thus, influenza is continually evolving and novel influenza viruses from animal reservoirs can cause unpredictable outbreaks, such as the most recent outbreaks from swine in the US (variant H3N2), poultry markets of China (H7N9) and Hong Kong (H5N1), leaving us unprepared and unprotected. Furthermore, H7N9 and H5N1 infections are highly lethal, with around 30 and 60% hospitalization-associated mortality, respectively. Therefore, there is a dire need for a vaccine that is effective against a "moving target," influenza viruses.

CROSS-REACTIVE ANTIBODIES LEAD TO RENEWED INTEREST IN B CELL VACCINES

The novel 2009 H1N1 pandemic virus (pH1N1-09) spread worldwide within 4 months due to minimal specific Ab immunity across the population. However, due to the novelty of the HA protein in some cases infection or vaccination resulted in the induction of novel broadly "neutralizing" antibody responses (reviewed in Corti and Lanzavecchia, 2013), leading to a renewed interest in developing the targets of theses Abs (summarized in **Table 1**) for universal vaccines.

The HA glycoprotein on the influenza virion exists as a trimer and dominates the surface of the virus. The amino acid sequence shows 40–70% conservation between different HA subtypes (e.g. H1 vs. H7) and greater than 80% between strains within a single subtype (e.g. H1 strains). Subtype variation underlies the classification of influenza viruses into two phylogenetic groups: group 1 (H5, H2, H1) and 2 (H3, H7, H10). Each HA monomer consists of two disulphide-linked polypeptide chains HA1 and HA2 (**Figures 1A,B**). The majority of the HA1 chain goes to make up the globular head of the molecule, which contains the receptor-binding domain (RBD). The RBD is the primary target for nAb responses elicited by current vaccines, and therefore random mutations that lead to single amino acid changes in Ab binding sites in this domain are selected under pressure to avoid further such Abs, resulting in the process of antigenic drift. In some instances, amino acid changes that alter the glycosylation pattern in the head region can also influence Ab binding. Some bnAbs have been isolated that recognize epitopes on the HA head region, e.g. FE17 (Corti et al., 2010), S139/1 (Yoshida

Table 1 | Potential targets for a universal influenza vaccine and their limitations.

Protein	Location	Site targetted	Function of target	Immune effector	Possible role in influenza protection	Escape possible
HA	Virion surface	HAl_ (head)	Virus binding	nAb	Block HA binding	Yes
	Virion and cell surface	HA2 (stem)	Viral fusion	Non-nAb	Block HA maturation, Fc-mediated lysis	No
NA	Virion surface	Sialidase	Virus release	Non-nAb	Block NA cleavage and virus release	Yes
M2	Infected cell surface	M2 Ectodomain	Ion channel	Non-nAb	Block virus entry	No
NP	Infected cell surface	Unknown	RNP structure	Non-nAb	ADCC and complement mediated lysis	Unknown
	Infected cell	Conserved pMHC	RNP structure	T cells (CD4+ and CD8+)	T cell cytotoxicity reduces viral load	Yes (limited)
All	Infected cell	Conserved pMHC	Various	T cells (CD4+ and CD8+)	T cell cytotoxicity reduces viral load	Yes (limited)

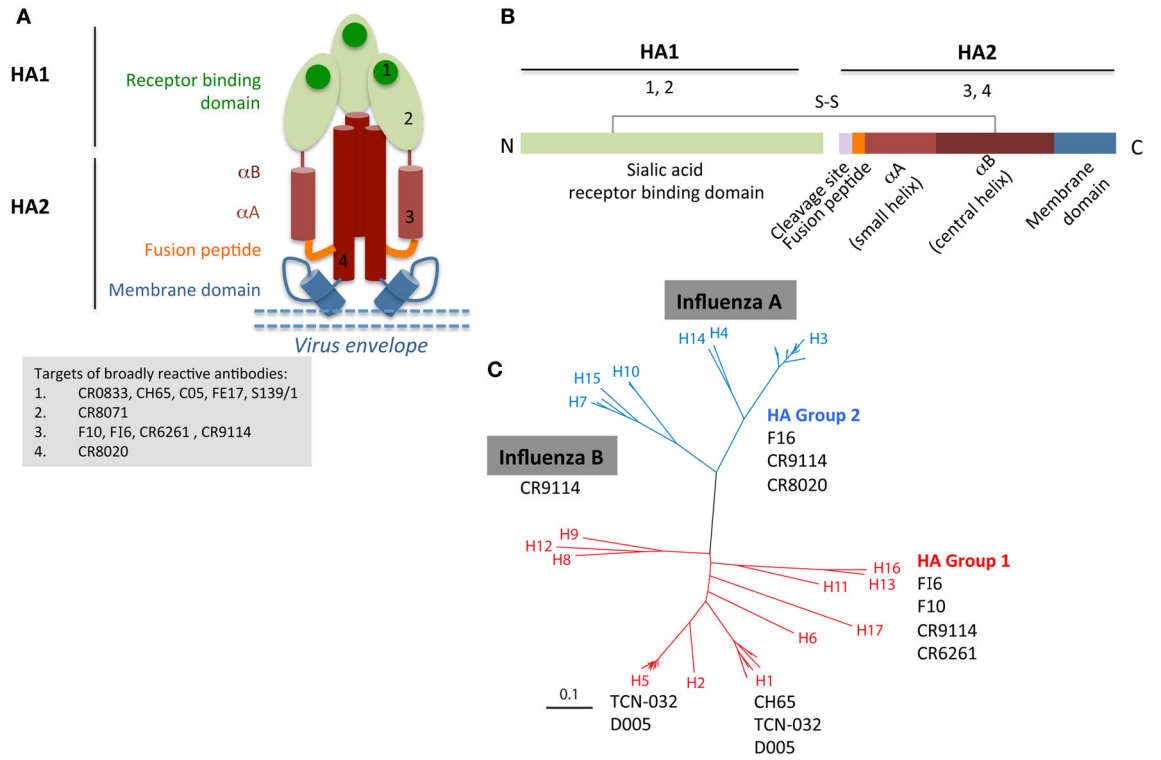

FIGURE 1 | Broadly reactive HA-specific Abs can bind different regions of the HA (A,B), which based on the sequence or structural conservation of the region targeted by Abs can lead to reactivity against otherwise highly divergent influenza viruses (C), such as influenza A group 1 and group 2 and even influenza B viruses. The cartoon depiction of the HA protein **(A,B)** is not to scale. Group 1 and 2 phylogeny of influenza A HA **(C)** was adapted from Corti and Lanzavecchia (2013).

et al., 2009), CH65 (Whittle et al., 2011), C05 (Ekiert et al., 2012) (**Figure 1**). However, escape mutations in the HA1 can be generated either at Ab binding sites or flanking residues after several passages *in vitro,* thus limiting the use of head-specific bnAbs.

The stem of the HA, which supports the globular head, contains a hydrophobic fusion region which is situated at the N-terminus of the HA2 chain. This region plays a crucial role during viral entry into cells, allowing endosome escape of the viral genome, and is functionally and structurally conserved across HA subtypes and therefore not susceptible to drift. The stem contains a number of epitopes spanning the fusion region, which are conserved across different influenza subtypes (Okuno et al., 1993), enabling the isolation of influenza-specific broadly cross-reactive Abs capable of recognizing either group 1 HA (from Crucell CR6261) (Ekiert et al., 2009), group 2 HA (CR8020) (Ekiert et al., 2011), both group 1 and 2 HAs (FI6) (Corti et al., 2011), or even recognize different influenza A and B strains (CR9114) (Dreyfus et al., 2012) (reviewed in Corti and Lanzavecchia, 2013) (**Figure 1**). One hypothesis for the induction of post-pandemic group 1-specific stem Abs is that the pH1N1-09 virus displayed a sufficiently distinct HA1 head domain when compared to the pre-pandemic H1N1 viruses, that pre-existing memory B cells specific for the HA head could not be recruited stem-specific responses. Thus, H1N1-2009 virus exposure generated a primary HA1-specific H1 Ab response but in addition was able to boost the low frequency group 1 stem specific Abs (Corti et al., 2010; Margine et al., 2013).

Importantly, HA2 stem-specific Abs prevent the conformational changes required for viral entry and membrane fusion, thus mutational escape is not possible due to the critical function and conserved helical structure of the stem. However, very high concentrations of stem-specific Abs are often required to mediate virus neutralizing activity, as they are 100–1000 times less potent than HA head-specific Abs (Corti et al., 2010). Typically, stem-specific Abs do not inhibit sialic acid binding by the receptor-binding site, as do classical head-specific Abs. For this reason they are unable to be identified by standard haemaglutination inhibition (HAI) assays, and are measured by virus neutralization or modified ELISA type assays. It should also be noted that bnAbs often represent highly edited B cell receptor sequences from germline requiring affinity maturation and co-ordination with T follicular helper cells (reviewed in Corti and Lanzavecchia, 2013), and thus they are often very rare and of low frequency. Advanced B cell cloning techniques have enabled the identification and isolation of unique bnAbs (Corti et al., 2011) for prophylactic and therapeutic use.

Vaccination with the conserved HA2 stem was first explored as an Abs target nearly 30 years ago (Graves et al., 1983), and is now receiving renewed attention in the wake of the 2009

pandemic. Various methods have been employed by different research groups to elicit bnAbs following exposure to influenza, such as headless HA protein or virus exposing the stem (Steel et al., 2010; Wang et al., 2010), prime-boost with a chimeric HA (Krammer et al., 2013) or by sequential infection with different influenza subtypes (Krammer et al., 2012) and DNA-prime heterologous-boost vaccination (Wei et al., 2010). Furthermore, bnAbs (CR6261, CH65, and scF10) have even been incorporated directly into self-assembling nanoparticles, providing long-term passive immunity in animal models (Kanekiyo et al., 2013), a strategy that could potentially be beneficial for the elderly or immunocompromised. Importantly, using the murine model it has been recently elucidated that the protective effect of some broadly-neutralizing stem-specific Abs is dependent on FcγR interactions (DiLillo et al., 2014) so that their main activity is not by classical neutralization of virus particles.

Other broadly reactive Abs that work in this way are those directed to the highly conserved surface M2 ion channel ectodomain (M2e). M2 is only expressed in very small amounts on the virion surface but is present on the infected cell surface where it can form a target for cross-reactive lytic responses. M2e-specific Abs are induced only at very low frequency by influenza infection, and are not elicited at all by the standard TIV vaccination, although they can be induced by M2e-specific vaccines (Neirynck et al., 1999; Mozdzanowska et al., 2003). Unexpectedly, Abs specific for the viral nucleoprotein (NP), which surrounds the genome, have also shown passive protection in mouse models at very high doses (Carragher et al., 2008; Lamere et al., 2011) and can be found in human serum (Sukeno et al., 1979). *In vitro* studies demonstrated that influenza-infected cells express low levels of NP on their surface (Virelizier et al., 1977; Yewdell et al., 1981), which may enable NP recognition by immune effectors, or alternatively, it is possible that NP-specific Abs are internalized and interrupt virus replication. Utilization of non-neutralizing NP and M2e Abs might be beneficial when combined with additional protective immune mechanisms.

Abs that are not virus neutralizing may also function in Ab-dependent cellular cytotoxicity (ADCC). pH1N1-09, H5N1-specifc and NP-specific ADCC Abs have been found in the absence of nAb responses in healthy individuals (Jegaskanda et al., 2013b). Influenza infection, but not standard TIV vaccination of macaques, elicited H1N1-specific ADCC Ab responses (Jegaskanda et al., 2013a), thus future vaccines would need to be optimized to elicit ADCC responses.

However, a forewarning comes from recent evidence in mouse models, which showed that influenza virus was able to specifically infect influenza-specific B cell receptor (BCR)-expressing B cells leading to BCR editing, thus allowing establishment of viral infection despite pre-existing Ab responses (Dougan et al., 2013). There is also evidence from a swine vaccination model that stem-specific HA2 Abs can enhance viral fusion and increase immunopathology upon H1N1pdm infection (Khurana et al., 2013). Therefore, while broadly cross reactive Abs are an increasingly promising area for combating influenza infections of distinct strains, their use should not be without investigation and should be used in conjunction with additional immune mechanisms.

HETEROSUBTYPIC T CELL RESPONSES FOR INFLUENZA

$CD8^+$ T cells recognize virus-derived peptides in the context of class I major histocompatibility antigens (MHC-I). pMHC-I is displayed on the surface of APCs enabling $CD8^+$ T cell priming and on virus-infected cells for $CD8^+$ T cell effector function, thus infected cells can be killed before virus progeny is released. The cytotoxic function is mediated mainly via the delivery of perforin and granzymes into the infected cell (Topham et al., 1997) as well as by cytokine release (Marshall et al., 2005). Thus, $CD8^+$ T cell recognition of influenza viruses is only possible for an established infection, in contrast to sterilizing nAb responses. However, $CD8^+$ T cells are critical in the elimination of influenza viruses, expediting viral clearance, and reducing pathology. Seminal work from influenza challenge of healthy human volunteers showed that increased T cell cytotoxicity was associated with reduced virus shedding (McMichael et al., 1983b), even in volunteers lacking nAbs against the infecting virus. Moreover, high levels of influenza-specific pre-existing memory T cells have been associated with milder symptoms during pH1N1 infection (Sridhar et al., 2013). There is no doubt that the current Ab-based approach should be maintained, but the incorporation of an even far-from-perfect T-cell-inducing vaccine (or vaccine component) could still save millions of lives during a pandemic, as T cells have the potential for much broader protection than bnAbs across diverse subtypes of influenza A.

The phenomenon of heterosubtypic immunity refers to memory T cells generated by one subtype that can cross-react against different IAV subtypes, despite wide differences in surface glycoproteins (Braciale, 1977; Kees and Krammer, 1984; Yewdell et al., 1985; Askonas et al., 1988; Wahl et al., 2009). T cell heterosubtypic immunity is mainly due to the majority of T cells recognizing immunogenic peptides derived primarily from highly conserved internal influenza proteins (Elhefnawi et al., 2011). For $CD8^+$ T cells, 193 immunogenic peptides presented by 51 different Human Leukocyte Antigen Class I (HLA-I) molecules have been described to date for influenza viruses (www.iedb.org). The majority of $CD8^+$ T cell antigenic peptides are derived from well-conserved internal proteins NP, M1, and PB1 (Assarsson et al., 2008; Lee et al., 2008; Wu et al., 2011; Grant et al., 2013). T cell cross-reactivity between different IAVs has been demonstrated between H1N1, H3N2, H2N2, H5N1, H3N2v, and H7N9 subtypes (**Table 2**) (Epstein, 2006; Kreijtz et al., 2008; Greenbaum et al., 2009; Hillaire et al., 2013; Quinones-Parra et al., 2014; Van De Sandt et al., 2014). Thus, influenza-specific T cells can provide universal protection against influenza disease and are of significant interest for the design of next generation vaccines.

The viral clearing role of heterosubtypic killer $CD8^+$ T cells is well established in animal models and human studies (**Table 2**). In a primary infection of $CD8^+$ T cell-deficient mice, influenza virus clearance is delayed and mortality is increased (Bender et al., 1992), while in the absence of B cells or Abs, $CD8^+$ T cells can provide protection against otherwise lethal influenza (Graham and Braciale, 1997; Epstein et al., 1998). Furthermore, transfer of influenza-specific $CD8^+$ T cells provides heterosubtypic protection (Yap et al., 1978; Taylor and Askonas, 1986). Secondary recall responses from pre-existing memory $CD8^+$ T cells, established by either influenza virus infection or vaccination (Flynn et al.,

Table 2 | Key human studies on cell-mediated immunity against IAV.

References	Virus(es)	Description
McMichael et al., 1983b	H1N1	Lymphocyte cytotoxic activity was associated with lower virus shedding in individuals challenged with H1N1
Gotch et al., 1988	H3N2→H1N1	$CD8^+$ T cell lines generated with H3N2 virus lyse target cells infected with Vaccinia viruses encoding NP, M1, or PB2 proteins derived from H1N1
Epstein, 2006	H1N1→H2N2	Adults that contracted H1N1 influenza prior to the emergence of pH2N2-57 were pronouncedly less susceptible to the pandemic virus
Kreijtz et al., 2008	H3N2→H5N1	$CD8^+$T cell lines established with H3N2 cross-react with immunogenic peptides derived from H5N1
Lee et al., 2008	Seasonal (s) IAV→H5N1	$CD4^+$ and $CD8^+$ T cells from H5 seronegative donors respond to peptides spanning the H5N1 proteome
Assarsson et al., 2008	H1N1, H3N2, H2N2, H5N1, H7N7, H6N1, H7N7, and H9N2	$CD4^+$ and $CD8^+$ T cells from healthy donors respond to substantially conserved immunogenic peptides
Tu et al., 2010	sH1N1/sH3N2→pH1N1-09	Purified, influenza-specific memory $CD8^+$ T cells expanded with sH1N1 and sH3N2 recognize target cells infected with pH1N1-09
Gras et al., 2010	pH1N1-09→pH1N1-1918	B7-NP_{418}-specific $CD8^+$ T cells elicited by pH1N1-09 infection cross react with the pH1N1-1918-NP_{418} variant
Wilkinson et al., 2012	H3N2	Pre-existing $CD4^+$ T cell responses correlated with lower virus shedding and disease severity upon challenge with H3N2 in seronegative volunteers
Fox et al., 2012	pH1N1-09	$CD8^+$ T cell activation is delayed in patients severely infected with pH1N1-09 and are lymphopenic for $CD4^+$, $CD8^+$ T cells, and NK cells
Zhao et al., 2012	pH1N1-09	Influenza-specific $CD4^+$ T cells responses are associated with progression to severe pH1N1-09 infection
Sridhar et al., 2013	pH1N1-09	Pre-existing memory $CD8^+$ T cell responses from seronegative patients naturally exposed to pH1N1-09 correlate with reduced illness severity
Hillaire et al., 2013	sH1N1/sH3N2→pH1N1-09/H3N2v	$CD8^+$ T cells lines generated with sH1N1, sH3N2 viruses or peptides derived from these strains respond to target cells infected with pH1N1 virus
Van De Sandt et al., 2014	pH1N1/sH1N1/sH3N2→H7N9	$CD8^+$ T cells stimulated with sH1N1, sH3N2, or pH1N1 recognize and lyse target cells infected with H7N9
Quinones-Parra et al., 2014	Any human IAV including H7N9	$CD8^+$ T cells from healthy donors expressing the HLA-A*0201, -A*0301, -B*5701, -B*1801 allele, and/or B*0801 allele(s) respond to universally conserved immunogenic peptides

1999) resulted in superior viral clearance and reduced pathology (Flynn et al., 1998). Moreover, tertiary challenge of mice with highly lethal H7N7 resulted in recall of heterosubtypic memory $CD8^+$ T cell responses (established from priming with H1N1 and then H3N2) that provided exceptionally enhanced virus control (within 3 days post-infection) (Christensen et al., 2000).

In comparison to $CD8^+$ T cells, the role of $CD4^+$ T cells in influenza is less well understood, partly due to their heterogeneity and the lack of epitope-specific systems (Sant and McMichael, 2012). The traditionally accepted role of influenza-specific $CD4^+$ T cells is in providing help to B cells for the production of high-quality Abs (Topham and Doherty, 1998), as their activation is dependent on recognition of peptide in the context of MHC-II on professional APCs but also have a major role in providing help for the establishment of $CD8^+$ T cell memory, critical for a robust recall response (Sun et al., 2004). Transfer of influenza-specific effector $CD4^+$ T cells into T cell-deficient mice accelerates production of neutralizing Abs, thus cross-reactive memory $CD4^+$ T cells can potentially enhance B cell responses during infection with a novel influenza virus (Scherle and Gerhard, 1986). Furthermore, depletion of $CD4^+$ T cells prior to influenza challenge results in a dramatic drop of Ab titres (Eichelberger et al., 1991), accompanied by only a small delay in virus elimination (Allan et al., 1990), driven by the remaining $CD8^+$ T cell response (Topham et al., 1996; Belz et al., 2002). More recently, a comprehensive transgenic mouse study by McKinstry et al. (2012) illustrated the direct protective role of influenza-specific $CD4^+$ T cells using a series of transfer experiments into immune knockout mice (lacking FcRγ, functional IFNγ, or B cells). The authors showed that $CD4^+$ T cells provide protection by interacting with B cells and $CD8^+$ T cells in an IFN-γ-dependent manner. The mechanism by which $CD4^+$ T cells are able to recognize virus-infected cells given their MHC-II restriction is yet to be deciphered. Nevertheless, in a vaccination setting of individuals receiving a split vaccine, a subset ($ICOS^+CXCR3^+CXCR5^+$) of circulating influenza-specific $CD4^+$ T follicular helper (T_{FH}) cells correlated with more effective B-cell responses and greater Ab titres, suggesting that eliciting this type of cells could be important in inducing more effective Ab-based vaccines (Bentebibel et al., 2013).

The debate of which T cell subtype is more protective for influenza has been reinvigorated from recent human studies that show contrasting results (Wilkinson et al., 2012; Zhao et al., 2012; Sridhar et al., 2013). However, as evidenced from mouse studies

outlined above, both subsets are necessary for a complete and coordinated response against influenza infection. Interestingly, in a challenge study (Wilkinson et al., 2012) with volunteers deliberately infected with H3N2, the numbers of pre-existing influenza-specific $CD4^+$, but not $CD8^+$, T cells were found to correlate with lower virus shedding and less severe, shorter illness. These investigators favored $CD4^+$ T cell cytotoxicity as the possible underlying mechanism. However, in a later study investigating T cell immunity in H1N1pdm virus-infected patients, $CD4^+$ T cell responses were associated with more severe infection (Zhao et al., 2012). A more recent study (Sridhar et al., 2013) followed natural infection of a large cohort over the 2009 pandemic and determined pre-immune correlates with the outcome of influenza disease over the pandemic. The study showed that those individuals with established influenza-specific $CD8^+$ T cell memory experienced milder illness following infection with the newly emerged virus. Although this study did not find a correlation with $CD4^+$ T cell responses and disease outcome, it cannot rule out their importance. Further studies are needed to better understand immune mechanisms underlying T cell-mediated protection against influenza viruses.

Some experimental vaccination protocols have effectively induced protective heterosubtypic T cell immunity, including non-replicative, cold adapted influenza vaccine (Powell et al., 2007), and virus-like particles (Hamada et al., 2013). In addition, DNA vaccines (Ulmer et al., 1993, 1998; Fu et al., 1999), prime-boost protocols (Epstein et al., 2005) and the use of adjuvants can provide and enhance T cell-mediated heterosubtypic protection (Chua et al., 2011, 2014). A live non-replicating vaccinia vaccine encoding the NP and M1 proteins, MVA-NP/M1, is currently being evaluated in human efficacy trials (Berthoud et al., 2011). The vaccine proved effective for human influenza challenge in a limited number of individuals, showing higher levels of influenza-specific $CD8^+$ T cells in vaccinees, especially those displaying the HLA-A*0201 allele, compared to placebo controls correlating with reduced infection and viral shedding (Antrobus et al., 2012; Lillie et al., 2012). Furthermore, the vaccine was able to boost T cell immunity in those aged 65 and over, a promising result for those who need an effective vaccine the most. However, it may be difficult to convince regulatory authorities to license a T cell-based vaccine that still allows individuals to become infected and shed virus. If such a vaccine were to replace the current TIV, a large-scale study would need to be performed in many individuals of distinct HLAs across different ethnicities to prove effectiveness.

Thus, manipulating existing style vaccines to induce or boost T cell immunity could potentially lead toward development of broadly protective influenza vaccines. However, there are still considerable challenges in the development of broadly cross-reactive T cell-inducing vaccines, which include persistence of T cell memory after influenza immunization (Valkenburg et al., 2012), population protective coverage across different HLAs, vaccine-mediated immune escape and immunopathology. Firstly, it is still far from clear for how long functional influenza-specific memory $CD8^+$ T cells persist in humans. Studies from yellow fever and smallpox vaccination suggest that memory T cells can be detected from 10 years (Akondy et al., 2009) to 50 years (Miller et al., 2008) following vaccination, respectively. Yet many adults fail to control influenza infection. A vaccine study of young children found that a threshold level (of >100 SFU/10^6 PBMCs) was required for effective T cell-mediated clinical protection (Forrest et al., 2008). Therefore, the varying levels of T cell immunity that are likely to exist in the wider population, due to different histories of exposure to natural infection, may contribute to the spectrum of disease severity. Early studies on cytotoxic T cells in humans suggested that influenza CTL memory declines rapidly with a half life of 2–3 years (McMichael et al., 1983a). The main purpose of a T cell-inducing vaccine may therefore be to maintain memory $CD8^+$ T cells at levels capable of achieving clinical protection, which may require booster doses every few years. The presence of co-morbidities, age-related differences of innate responses in the young and immunological decline in the elderly (reviewed by Oshansky and Thomas, 2012) could also impair T cell recall responses. Furthermore, an influenza T-cell based vaccine would have to address the issue HLA coverage in a diverse population and be appropriate for ethnic minorities with rare MHC alleles. This could be achieved either by utilization of peptide epitopes representing HLA-super families (Assarsson et al., 2008), or the inclusion of full-length influenza-derived proteins in a form that enables endogenous antigen processing.

INFLUENZA CAN ESCAPE T CELL IMMUNITY

If such a T cell-inducing vaccine could be produced, the issue of vaccine-mediated T cell escape, resonant of Ab-mediated antigenic drift, could theoretically become significant. RNA viruses, such as influenza, are characterized by poor fidelity of replication of their genomes, leading to the emergence of viral variants capable of rapidly adapting in response to immune selective pressure, as seen with seasonal antigenic drift. Subversion of T cell control is well documented for chronic viral infections, like HIV and HCV (Pircher et al., 1990; Moore et al., 2002; Fernandez et al., 2005) and represents one of the major obstacles for viral control and vaccine design.

Within an individual, T cells can select influenza escape variants as the virus replicates. We have recently described the emergence of influenza variants within $CD8^+$ T cell target regions in a persistently infected, immunocompromised child, (Valkenburg et al., 2013). Additionally, $CD8^+$ T cell immune escape viruses could be readily isolated from immunodeficient (B cell knockout) and immune intact wild-type mice. Surprisingly, we observed that these CTL escape variants arise early during infection by day 5, and increase in frequency and variety over the time-course of infection. The selection of $CD8^+$ T cell escape mutants was clearly driven by selective pressure as these variants revert in the absence of immune pressure in MHC-mismatched mice (Valkenburg et al., 2013). Further experiments suggested that influenza-specific escape from T cell responses is heavily dependent on the particular epitope and potentially the underlying characteristics of the T cell receptor repertoire. Interestingly, influenza viruses favored escape at the residues that anchor epitope peptides to MHC (Valkenburg et al., 2013).

The emergence of specific $CD8^+$ T cell mutations in influenza can also be detected at a population level. Factors such as immunodominance, HLA frequency and viral fitness can impact the likelihood of emergence and selection of escape viruses (Berkhoff

et al., 2005). Indeed, there are documented examples of naturally occurring mutations within critical T cell antigenic peptides leading to immune escape in individuals bearing certain HLA alleles. The Rimmelzwann group has pioneered and enhanced our knowledge of $CD8^+$ T cell-mediated immune escape in human influenza. Boon et al. (2004) identified substitutions within the HLA-B*0702- and B*3501-restricted $NP_{418–426}$ epitope that could either result in cross-reactivity, or in some cases, in immune escape. Further immunological and structural characterization of the $NP_{418–426}$ variants from 1918 to 2009 revealed that mutations in solvent-exposed, potentially TCR contact residues, result in immune escape (Gras et al., 2010). At the same time, we confirmed the presence of cross-reactive CTL populations that reacted against a wider spectrum of $NP_{418–426}$ variants (Gras et al., 2010). The identification of epitopes recognized by such populations, and the key residues for TCR recognition within them, may be critical information for producing a vaccine capable of eliciting cross-reactive T cells to provide coverage against the wide spectrum of influenza antigenic variation, an idea that we have pioneered using the B6 mouse model of influenza infections (Valkenburg et al., 2013). Conversely, $CD4^+$ T cell-mediated viral escape in influenza has received considerably less attention and the selection of $CD4^+$ T cell escape peptide variants has not currently been demonstrated, either within an individual or across a population.

In earlier work examining drift in the viral NP at a population level, Voeten et al. (2000) identified mutations in HLA-B*2705-restricted $NP_{383–391}$ epitope and HLA-B*08:01-$NP_{380–388}$ resulting in immune escape. The NP-R384K mutation at an MHC-I anchor residue, which resulted in a loss of $CD8^+$ T cell recognition, was initially detected in 1990 and later in 1993 as R384G. The escape mutant quickly replaced the wild-type sequence in H3N2 viruses (Gog et al., 2003), resulting in a loss of immunogenicity in the population expressing the HLA-B27 allele. Further characterization of this mutant indicated that the escape was driven by CTL selective pressure as the mutation imposed a fitness cost that had to be compensated for by additional mutations in flanking regions (Rimmelzwaan et al., 2005). More recently, a CTL escape variant within the HLA-A*0101-restricted $NP_{44–52}$ in the novel H7N9 virus has been reported (Quinones-Parra et al., 2014; Van De Sandt et al., 2014), with structural data indicating that substitution in an MHC-I anchor residue of the peptide epitope compromised peptide-MHC-I complex stability and thus accessibility to CTLs (Quinones-Parra et al., 2014).

Although immunodominant T cells may generate escape variants, they remain a hugely valuable tool in the arsenal for combating influenza infection. It may be possible to pre-empt escape by priming the T cell repertoire against a variety of dominant mutants at TCR contact sites (Valkenburg et al., 2013) or possibly stabilizing the MHC for low affinity anchor mutants. The NP protein, though capable of harboring T cell escape mutations, is one of the most immunogenic influenza proteins for T cells (Grant et al., 2013). Fortunately, the NP of LAIV can be substituted to represent the current NP or the NP of escape variants without affecting the viral growth or vaccine immunogenicity (Isakova-Sivak et al., 2011).

INNATE T CELLS FOR INFLUENZA VIRUS INFECTION

Until now this review has discussed adaptive immunity to influenza, however another component, innate T cells, have potential use for subverting infection due to innate receptors recognizing conserved universal motifs. The non-conventional or innate T cell compartment comprises of γδ T cells, CD1d-restricted invariant natural killer T cells (iNKT), and MR1-restricted Mucosal-associated invariant T (MAIT) cells. This compartment constitutively expresses high levels of the C-type lectin, CD161. Innate T cells can be activated by a diverse range of ligands, either endogenous (β-GlcCer and iGB3 for NKT; MICA/B for γδ T cells) and exogenous (phosphoantigens and bisphosphonates for γδ T cells, α-GalCer-for iNKT, bacterial lipids for NKT and metabolites for MAIT cells) (Kjer-Nielsen et al., 2012; Rossjohn et al., 2012; Born et al., 2013). Upon TCR recognition of their cognate antigen or by cytokine driven signals, innate T cells can rapidly produce an array of inflammatory and effector molecules (IFNγ, TNF, IL-17, IL-4, IL-22, perforin, granzyme B, MIP-1β). In humans, innate T cells can comprise up to 30% of the peripheral blood $CD3^+$ T cell compartment, and are also enriched at mucosal sites including lung, intestine and liver. Given their location, potent inflammatory and cytolytic function, innate T cells are potentially important players during IAV infection. Although their role is well studied in autoimmunity, cancer, and chronic viral infections such as HIV-1, (Berzins et al., 2011; Cosgrove et al., 2013; Leeansyah et al., 2013; Vantourout and Hayday, 2013), there is a paucity of data on how innate T cells contribute to combating influenza infection.

Several studies in humans have demonstrated that a major subset of human γδ T cells (Vγ9Vδ2) can directly kill human and avian origin influenza-infected macrophages and lung alveolar epithelial cells *in vitro* (Qin et al., 2009; Tu et al., 2011; Li et al., 2013). Further studies in humanized mice have shown that vaccination with aminobisphosphonate pamidronate (PAM) can activate the Vγ9Vδ2 subset, and that Vγ9Vδ2 T cells can inhibit viral replication and dampen inflammatory responses in the lung (Tu et al., 2011). Thus, pre-arming γδ T cells may also be beneficial in human IAV infection as an alternative antiviral strategy (Tu et al., 2011).

Similarly, endogenous iNKT cells have been shown to have an immunoregulatory role in IAV. From adoptive transfer studies in C57BL/6 mice, iNKT cells have been demonstrated to alleviate bronchopneumonia and its associated pathology in $J\alpha18^{-/-}$ (iNKT deficient) mice infected with highly virulent H3N2 (Paget et al., 2011; Kok et al., 2012). Both endogenous and exogenous (α-GalCer) activation of iNKT aids in the development of influenza-specific $CD8^+$ T cells by promoting their survival (Guillonneau et al., 2009) and the maturation of APCs that present epitopes to influenza-specific $CD8^+$ T cells (Paget et al., 2011). Additionally, iNKT-derived cytokines, such as IL-33 and IL-22, produced during IAV infection have been associated with regulation of eosinophil maturation in an IL-5-dependent manner and protection of the airway epithelium, respectively (Paget et al., 2012; Gorski et al., 2013). These studies demonstrate that iNKT can aid in the maturation of both the influenza-specific adaptive and the innate response, and therefore may be important subsets to induce in a universal IAV vaccine.

Although the majority of these studies highlight the immunoregulatory role of murine innate T cells in models of IAV, more studies are needed in humans to investigate whether these and other non-conventional innate T cells may contribute to influenza-specific adaptive immune effectors and thus be of benefit to induce in a universal IAV vaccine.

CONCLUSIONS

Although the current Ab-mediated vaccines are the most cost effective way to combat the yearly influenza epidemics, they are strain-specific and thus need to be updated annually while providing little or no protection from novel outbreak strains. Furthermore, during the 2009 pandemic, it took several months to produce and test the newly made H1N1pdm-specific influenza vaccine, which meant it was only available after the peak of influenza activity. Thus, there is an urgent need to develop novel approaches for a universal influenza vaccine that has broad reactivity across a diversity of influenza strains and subtypes. Ideally, this vaccine would elicit both broadly cross-reactive Abs directed at highly conserved yet sub-dominant targets such as the HA stalk, which are currently proving highly effective in mouse studies. Furthermore, the ideal vaccine would also elicit a robust T cell response with long-term memory potential, recognizing epitopes derived from conserved and immunogenic internal proteins. Recent data on both universal Abs and T cell responses against influenza are promising, but more research still needs to be done to provide insights into the longevity and effectiveness of this type of immunity. The possibility of generating escape mutants by widespread use of vaccines designed to elicit such cross-reactive responses needs to be understood as well as the potential impact on virus evolution.

ACKNOWLEDGMENTS

Sergio Quiñones-Parra is a recipient of the University of Melbourne International Research Scholarship and a CONACyT Scholar. Sophie A. Valkenburg and Liyen Loh are National Health and Medical Research Council of Australia (NHMRC) CJ Martin Fellows, Katherine Kedzierska is an NHMRC Level 2 Career Development Fellow. The work was supported by an NHMRC Program Grant to LEB (AI APP567122).

REFERENCES

Akondy, R. S., Monson, N. D., Miller, J. D., Edupuganti, S., Teuwen, D., Wu, H., et al. (2009). The yellow fever virus vaccine induces a broad and polyfunctional human memory CD8+ T cell response. *J. Immunol.* 183, 7919–7930. doi: 10.4049/jimmunol.0803903

Allan, W., Tabi, Z., Cleary, A., and Doherty, P. C. (1990). Cellular events in the lymph node and lung of mice with influenza. Consequences of depleting CD4+ T cells. *J. Immunol.* 144, 3980–3986.

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

Askonas, B. A., Taylor, P. M., and Esquivel, F. (1988). Cytotoxic T cells in influenza infection. *Ann. N.Y. Acad. Sci.* 532, 230–237. doi: 10.1111/j.1749-6632.1988.tb36342.x

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

Belz, G. T., Wodarz, D., Diaz, G., Nowak, M. A., and Doherty, P. C. (2002). Compromised influenza virus-specific CD8(+)-T-cell memory in CD4(+)-T-cell-deficient mice. *J. Virol.* 76, 12388–12393. doi: 10.1128/JVI.76.23.12388-12393.2002

Bender, B. S., Croghan, T., Zhang, L., and Small, P. A. Jr. (1992). Transgenic mice lacking class I major histocompatibility complex-restricted T cells have delayed viral clearance and increased mortality after influenza virus challenge. *J. Exp. Med.* 175, 1143–1145. doi: 10.1084/jem.175.4.1143

Bentebibel, S. E., Lopez, S., Obermoser, G., Schmitt, N., Mueller, C., Harrod, C., et al. (2013). Induction of ICOS+CXCR3+CXCR5+ TH cells correlates with antibody responses to influenza vaccination. *Sci. Transl. Med.* 5:176ra132. doi: 10.1126/scitranslmed.3005191

Berkhoff, E. G., De Wit, E., Geelhoed-Mieras, M. M., Boon, A. C., Symons, J., Fouchier, R. A., et al. (2005). Functional constraints of influenza A virus epitopes limit escape from cytotoxic T lymphocytes. *J. Virol.* 79, 11239–11246. doi: 10.1128/JVI.79.17.11239-11246.2005

Berthoud, T. K., Hamill, M., Lillie, P. J., Hwenda, L., Collins, K. A., Ewer, K. J., et al. (2011). Potent CD8+ T-cell immunogenicity in humans of a novel heterosubtypic influenza A vaccine, MVA-NP+M1. *Clin. Infect. Dis.* 52, 1–7. doi: 10.1093/cid/ciq015

Berzins, S. P., Smyth, M. J., and Baxter, A. G. (2011). Presumed guilty: natural killer T cell defects and human disease. *Nat. Rev. Immunol.* 11, 131–142. doi: 10.1038/nri2904

Bodewes, R., Fraaij, P. L., Geelhoed-Mieras, M. M., Van Baalen, C. A., Tiddens, H. A., Van Rossum, A. M., et al. (2011). 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., Hillaire, M. L., Geelhoed-Mieras, M. M., Fouchier, R. A., Osterhaus, A. D., et al. (2010). Vaccination with whole inactivated virus vaccine affects the induction of heterosubtypic immunity against influenza virus A/H5N1 and immunodominance of virus-specific CD8+ T-cell responses in mice. *J. Gen. Virol.* 91, 1743–1753. doi: 10.1099/vir.0.020784-0

Boon, A. C., De Mutsert, G., Van Baarle, D., Smith, D. J., Lapedes, A. S., Fouchier, R. A., et al. (2004). Recognition of homo- and heterosubtypic variants of influenza A viruses by human CD8+ T lymphocytes. *J. Immunol.* 172, 2453–2460. doi: 10.4049/jimmunol.172.4.2453

Born, W. K., Kemal Aydintug, M., and O'Brien, R. L. (2013). Diversity of gammadelta T-cell antigens. *Cell. Mol. Immunol.* 10, 13–20. doi: 10.1038/cmi.2012.45

Braciale, T. J. (1977). Immunologic recognition of influenza virus-infected cells. II. Expression of influenza A matrix protein on the infected cell surface and its role in recognition by cross-reactive cytotoxic T cells. *J. Exp. Med.* 146, 673–689. doi: 10.1084/jem.146.3.673

Carragher, D. M., Kaminski, D. A., Moquin, A., Hartson, L., and Randall, T. D. (2008). A novel role for non-neutralizing antibodies against nucleoprotein in facilitating resistance to influenza virus. *J. Immunol.* 181, 4168–4176. doi: 10.4049/jimmunol.181.6.4168

Christensen, J. P., Doherty, P. C., Branum, K. C., and Riberdy, J. M. (2000). Profound protection against respiratory challenge with a lethal H7N7 influenza A virus by increasing the magnitude of CD8(+) T-cell memory. *J. Virol.* 74, 11690–11696. doi: 10.1128/JVI.74.24.11690-11696.2000

Chua, B. Y., Olson, M. R., Bedoui, S., Sekiya, T., Wong, C. Y., Turner, S. J., et al. (2014). The use of a TLR2 agonist-based adjuvant for enhancing effector and memory CD8 T-cell responses. *Immunol. Cell Biol.* 92, 377–383. doi: 10.1038/icb.2013.102

Chua, B. Y., Pejoski, D., Turner, S. J., Zeng, W., and Jackson, D. C. (2011). Soluble proteins induce strong CD8+ T cell and antibody responses through electrostatic association with simple cationic or anionic lipopeptides that target TLR2. *J. Immunol.* 187, 1692–1701. doi: 10.4049/jimmunol.1100486

Corti, D., and Lanzavecchia, A. (2013). Broadly neutralizing antiviral antibodies. *Annu. Rev. Immunol.* 31, 705–742. doi: 10.1146/annurev-immunol-032712-095916

Corti, D., Suguitan, A. L. Jr., Pinna, D., Silacci, C., Fernandez-Rodriguez, B. M., Vanzetta, F., et al. (2010). Heterosubtypic neutralizing antibodies are produced by individuals immunized with a seasonal influenza vaccine. *J. Clin. Invest.* 120, 1663–1673. doi: 10.1172/JCI41902

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

Cosgrove, C., Ussher, J. E., Rauch, A., Gartner, K., Kurioka, A., Huhn, M. H., et al. (2013). Early and nonreversible decrease of CD161++ /MAIT cells in HIV infection. *Blood* 121, 951–961. doi: 10.1182/blood-2012-06-436436

DiLillo, D. J., Tan, G. S., Palese, P., and Ravetch, J. V. (2014). Broadly neutralizing hemagglutinin stalk-specific antibodies require FcgammaR interactions for protection against influenza virus *in vivo*. *Nat. Med.* 20, 143–151. doi: 10.1038/nm.3443

Dougan, S. K., Ashour, J., Karssemeijer, R. A., Popp, M. W., Avalos, A. M., Barisa, M., et al. (2013). Antigen-specific B-cell receptor sensitizes B cells to infection by influenza virus. *Nature* 503, 406–409. doi: 10.1038/nature12637

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

Eichelberger, M. C., Wang, M. L., Allan, W., Webster, R. G., and Doherty, P. C. (1991). Influenza virus RNA in the lung and lymphoid tissue of immunologically intact and CD4-depleted mice. *J. Gen. Virol.* 72(pt 7), 1695–1698. doi: 10.1099/0022-1317-72-7-1695

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

Elhefnawi, M., Alaidi, O., Mohamed, N., Kamar, M., El-Azab, I., Zada, S., et al. (2011). Identification of novel conserved functional motifs across most Influenza A viral strains. *Virol. J.* 8:44. doi: 10.1186/1743-422X-8-44

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

Epstein, S. L., Kong, W. P., Misplon, J. A., Lo, C. Y., Tumpey, T. M., Xu, L., et al. (2005). Protection against multiple influenza A subtypes by vaccination with highly conserved nucleoprotein. *Vaccine* 23, 5404–5410. doi: 10.1016/j.vaccine.2005.04.047

Epstein, S. L., Lo, C. Y., Misplon, J. A., and Bennink, J. R. (1998). Mechanism of protective immunity against influenza virus infection in mice without antibodies. *J. Immunol.* 160, 322–327.

Fernandez, C., Stratov, I., De Rose, R., Walsh, K., Dale, C., Smith, M., et al. (2005). Rapid viral escape at an immunodominant simian-human immunodeficiency virus cytotoxic T-lymphocyte epitope exacts a dramatic fitness cost. *J. Virol.* 79, 5721–5731. doi: 10.1128/JVI.79.9.5721-5731.2005

Flynn, K. J., Belz, G. T., Altman, J. D., Ahmed, R., Woodland, D. L., and Doherty, P. C. (1998). Virus-specific CD8+ T cells in primary and secondary influenza pneumonia. *Immunity* 8, 683–691. doi: 10.1016/S1074-7613(00)80573-7

Flynn, K. J., Riberdy, J. M., Christensen, J. P., Altman, J. D., and Doherty, P. C. (1999). *In vivo* proliferation of naive and memory influenza-specific CD8(+) T cells. *Proc. Natl. Acad. Sci. U.S.A.* 96, 8597–8602. doi: 10.1073/pnas.96.15.8597

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

Fox, A., Le, N. M., Horby, P., Van Doorn, H. R., Nguyen, V. T., Nguyen, H. H., et al. (2012). Severe pandemic H1N1 2009 infection is associated with transient NK and T deficiency and aberrant CD8 responses. *PLoS One* 7:e31535. doi: 10.1371/journal.pone.0031535

Fu, T. M., Guan, L., Friedman, A., Schofield, T. L., Ulmer, J. B., Liu, M. A., et al. (1999). Dose dependence of CTL precursor frequency induced by a DNA vaccine and correlation with protective immunity against influenza virus challenge. *J. Immunol.* 162, 4163–4170.

Gog, J., Rimmelzwaan, G., Osterhaus, A., and Grenfell, B. (2003). Population dynamics of rapid fixation in cytotoxic T lymphocyte escape mutants of influenza A. *Proc. Natl. Acad. Sci. U.S.A.* 100, 11143–11147. doi: 10.1073/pnas.1830296100

Gorski, S. A., Hahn, Y. S., and Braciale, T. J. (2013). Group 2 innate lymphoid cell production of IL-5 is regulated by NKT cells during influenza virus infection. *PLoS Pathog.* 9:e1003615. doi: 10.1371/journal.ppat.1003615

Gotch, F., Mcmichael, A., and Rothbard, J. (1988). Recognition of influenza A matrix protein by HLA-A2-restricted cytotoxic T lymphocytes. Use of analogues to orientate the matrix peptide in the HLA-A2 binding site. *J. Exp. Med.* 168, 2045–2057. doi: 10.1084/jem.168.6.2045

Graham, M. B., and Braciale, T. J. (1997). Resistance to and recovery from lethal influenza virus infection in B lymphocyte-deficient mice. *J. Exp. Med.* 186, 2063–2068. doi: 10.1084/jem.186.12.2063

Grant, E., Wu, C., Chan, K. F., Eckle, S., Bharadwaj, M., Zou, Q. M., et al. (2013). Nucleoprotein of influenza A virus is a major target of immunodominant CD8+ T-cell responses. *Immunol. Cell Biol.* 91, 184–194. doi: 10.1038/icb.2012.78

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

Greenbaum, J. A., Kotturi, M. F., Kim, Y., Oseroff, C., Vaughan, K., Salimi, N., et al. (2009). Pre-existing immunity against swine-origin H1N1 influenza viruses in the general human population. *Proc. Natl. Acad. Sci. U.S.A.* 106, 20365–20370. doi: 10.1073/pnas.0911580106

Guillonneau, C., Mintern, J. D., Hubert, F. X., Hurt, A. C., Besra, G. S., Porcelli, S., et al. (2009). Combined NKT cell activation and influenza virus vaccination boosts memory CTL generation and protective immunity. *Proc. Natl. Acad. Sci. U.S.A.* 106, 3330–3335. doi: 10.1073/pnas.0813309106

Hamada, H., Bassity, E., Flies, A., Strutt, T. M., Garcia-Hernandez Mde, L., McKinstry, K. K., et al. (2013). Multiple redundant effector mechanisms of CD8+ T cells protect against influenza infection. *J. Immunol.* 190, 296–306. doi: 10.4049/jimmunol.1200571

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

Hillaire, M. L., Vogelzang-Van Trierum, S. E., Kreijtz, J. H., De Mutsert, G., Fouchier, R. A., Osterhaus, A. D., et al. (2013). Human T-cells directed to seasonal influenza A virus cross-react with 2009 pandemic influenza A (H1N1) and swine-origin triple-reassortant H3N2 influenza viruses. *J. Gen. Virol.* 94, 583–592. doi: 10.1099/vir.0.048652-0

Isakova-Sivak, I., Chen, L. M., Matsuoka, Y., Voeten, J. T., Kiseleva, I., Heldens, J. G., et al. (2011). Genetic bases of the temperature-sensitive phenotype of a master donor virus used in live attenuated influenza vaccines: A/Leningrad/134/17/57 (H2N2). *Virology* 412, 297–305. doi: 10.1016/j.virol.2011.01.004

Jegaskanda, S., Amarasena, T. H., Laurie, K. L., Tan, H. X., Butler, J., Parsons, M. S., et al. (2013a). Standard trivalent influenza virus protein vaccination does not prime antibody-dependent cellular cytotoxicity in macaques. *J. Virol.* 87, 13706–13718. doi: 10.1128/JVI.01666-13

Jegaskanda, S., Job, E. R., Kramski, M., Laurie, K., Isitman, G., De Rose, R., et al. (2013b). Cross-reactive influenza-specific antibody-dependent cellular cytotoxicity antibodies in the absence of neutralizing antibodies. *J. Immunol.* 190, 1837–1848. doi: 10.4049/jimmunol.1201574

Kanekiyo, M., Wei, C. J., Yassine, H. M., McTamney, P. M., Boyington, J. C., Whittle, J. R., et al. (2013). Self-assembling influenza nanoparticle vaccines elicit broadly neutralizing H1N1 antibodies. *Nature* 499, 102–106. doi: 10.1038/nature12202

Kees, U., and Krammer, P. H. (1984). Most influenza A virus-specific memory cytotoxic T lymphocytes react with antigenic epitopes associated with internal virus determinants. *J. Exp. Med.* 159, 365–377. doi: 10.1084/jem.159.2.365

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

Kjer-Nielsen, L., Patel, O., Corbett, A. J., Le Nours, J., Meehan, B., Liu, L., et al. (2012). MR1 presents microbial vitamin B metabolites to MAIT cells. *Nature* 491, 717–723. doi: 10.1038/nature11605

Kok, W. L., Denney, L., Benam, K., Cole, S., Clelland, C., McMichael, A. J., et al. (2012). Pivotal Advance: Invariant NKT cells reduce accumulation of

inflammatory monocytes in the lungs and decrease immune-pathology during severe influenza A virus infection. *J. Leukoc. Biol.* 91, 357–368. doi: 10.1189/jlb.0411184

Krammer, F., Margine, I., Hai, R., Flood, A., Hirsh, A., Tsvetnitsky, V., et al. (2013). H3 stalk-based chimeric hemagglutinin influenza virus constructs protect mice from H7N9 challenge. *J. Virol.* 88, 2340–2343. doi: 10.1128/JVI.00641-13

Krammer, F., Pica, N., Hai, R., Tan, G. S., and Palese, P. (2012). Hemagglutinin stalk-reactive antibodies are boosted following sequential infection with seasonal and pandemic H1N1 Influenza virus in mice. *J. Virol.* 86, 10302–10307. doi: 10.1128/JVI.01336-12

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

Lamere, M. W., Lam, H. T., Moquin, A., Haynes, L., Lund, F. E., Randall, T. D., et al. (2011). Contributions of antinucleoprotein IgG to heterosubtypic immunity against influenza virus. *J. Immunol.* 186, 4331–4339. doi: 10.4049/jimmunol.1003057

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

Leeansyah, E., Ganesh, A., Quigley, M. F., Sonnerborg, A., Andersson, J., Hunt, P. W., et al. (2013). Activation, exhaustion, and persistent decline of the antimicrobial MR1-restricted MAIT-cell population in chronic HIV-1 infection. *Blood* 121, 1124–1135. doi: 10.1182/blood-2012-07-445429

Li, H., Xiang, Z., Feng, T., Li, J., Liu, Y., Fan, Y., et al. (2013). Human Vgamma9Vdelta2-T cells efficiently kill influenza virus-infected lung alveolar epithelial cells. *Cell. Mol. Immunol.* 10, 159–164. doi: 10.1038/cmi.2012.70

Lillie, P. J., Berthoud, T. K., Powell, T. J., Lambe, T., Mullarkey, C., Spencer, A. J., et al. (2012). Preliminary assessment of the efficacy of a T-cell-based influenza vaccine, MVA-NP+M1, in humans. *Clin. Infect. Dis.* 55, 19–25. doi: 10.1093/cid/cis327

Margine, I., Hai, R., Albrecht, R. A., Obermoser, G., Harrod, A. C., Banchereau, J., et al. (2013). H3N2 influenza virus infection induces broadly reactive hemagglutinin stalk antibodies in humans and mice. *J. Virol.* 87, 4728–4737. doi: 10.1128/JVI.03509-12

Marshall, D. R., Olivas, E., Andreansky, S., La Gruta, N. L., Neale, G. A., Gutierrez, A., et al. (2005). Effector CD8+ T cells recovered from an influenza pneumonia differentiate to a state of focused gene expression. *Proc. Natl. Acad. Sci. U.S.A.* 102, 6074–6079. doi: 10.1073/pnas.0501960102

McKinstry, K. K., Strutt, T. M., Kuang, Y., Brown, D. M., Sell, S., Dutton, R. W., et al. (2012). Memory CD4+ T cells protect against influenza through multiple synergizing mechanisms. *J. Clin. Invest.* 122, 2847–2856. doi: 10.1172/JCI 63689

McMichael, A. J., Gotch, F. M., Dongworth, D. W., Clark, A., and Potter, C. W. (1983a). Declining T-cell immunity to influenza, 1977-82. *Lancet* 2, 762–764. doi: 10.1016/S0140-6736(83)92297-3

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

Miller, J. D., Van Der Most, R. G., Akondy, R. S., Glidewell, J. T., Albott, S., Masopust, D., et al. (2008). Human effector and memory CD8+ T cell responses to smallpox and yellow fever vaccines. *Immunity* 28, 710–722. doi: 10.1016/j.immuni.2008.02.020

Monto, A. S., Ohmit, S. E., Petrie, J. G., Johnson, E., Truscon, R., Teich, E., et al. (2009). Comparative efficacy of inactivated and live attenuated influenza vaccines. *N. Engl. J. Med.* 361, 1260–1267. doi: 10.1056/NEJMoa0808652

Moore, C., John, M., James, I., Christiansen, F., Witt, C., and Mallal, S. (2002). Evidence of HIV-1 adaptation to HLA-restricted immune responses at a population level. *Science* 296, 1439–1443. doi: 10.1126/science.1069660

Mozdzanowska, K., Feng, J., Eid, M., Kragol, G., Cudic, M., Otvos, L., 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

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

Okuno, Y., Isegawa, Y., Sasao, F., and Ueda, S. (1993). A common neutralizing epitope conserved between the hemagglutinins of influenza A virus H1 and H2 strains. *J. Virol.* 67, 2552–2558.

Oshansky, C. M., and Thomas, P. G. (2012). The human side of influenza. *J. Leukoc. Biol.* 92, 83–96. doi: 10.1189/jlb.1011506

Paget, C., Ivanov, S., Fontaine, J., Blanc, F., Pichavant, M., Renneson, J., et al. (2011). Potential role of invariant NKT cells in the control of pulmonary inflammation and CD8+ T cell response during acute influenza A virus H3N2 pneumonia. *J. Immunol.* 186, 5590–5602. doi: 10.4049/jimmunol.1002348

Paget, C., Ivanov, S., Fontaine, J., Renneson, J., Blanc, F., Pichavant, M., et al. (2012). Interleukin-22 is produced by invariant natural killer T lymphocytes during influenza A virus infection: potential role in protection against lung epithelial damages. *J. Biol. Chem.* 287, 8816–8829. doi: 10.1074/jbc.M111.304758

Pircher, H., Moskophidis, D., Rohrer, U., Burki, K., Hengartner, H., and Zinkernagel, R. (1990). Viral escape by selection of cytotoxic T cell-resistant virus variants *in vivo*. *Nature* 346, 629–633. doi: 10.1038/346629a0

Powell, T. J., Strutt, T., Reome, J., Hollenbaugh, J. A., Roberts, A. D., Woodland, D. L., et al. (2007). Priming with cold-adapted influenza A does not prevent infection but elicits long-lived protection against supralethal challenge with heterosubtypic virus. *J. Immunol.* 178, 1030–1038. doi: 10.4049/jimmunol.178.2.1030

Qin, G., Mao, H., Zheng, J., Sia, S. F., Liu, Y., Chan, P. L., et al. (2009). Phosphoantigen-expanded human gammadelta T cells display potent cytotoxicity against monocyte-derived macrophages infected with human and avian influenza viruses. *J. Infect. Dis.* 200, 858–865. doi: 10.1086/605413

Quinones-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

Rimmelzwaan, G. F., Berkhoff, E. G., Nieuwkoop, N. J., Smith, D. J., Fouchier, R. A., and Osterhaus, A. D. (2005). Full restoration of viral fitness by multiple compensatory co-mutations in the nucleoprotein of influenza A virus cytotoxic T-lymphocyte escape mutants. *J. Gen. Virol.* 86, 1801–1805. doi: 10.1099/vir.0.80867-0

Rossjohn, J., Pellicci, D. G., Patel, O., Gapin, L., and Godfrey, D. I. (2012). Recognition of CD1d-restricted antigens by natural killer T cells. *Nat. Rev. Immunol.* 12, 845–857. doi: 10.1038/nri3328

Sant, A. J., and McMichael, A. (2012). Revealing the role of CD4(+) T cells in viral immunity. *J. Exp. Med.* 209, 1391–1395. doi: 10.1084/jem.20121517

Scherle, P. A., and Gerhard, W. (1986). Functional analysis of influenza-specific helper T cell clones *in vivo*. T cells specific for internal viral proteins provide cognate help for B cell responses to hemagglutinin. *J. Exp. Med.* 164, 1114–1128. doi: 10.1084/jem.164.4.1114

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

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, 1–9. doi: 10.1128/mBio.00018-10

Sukeno, N., Otsuki, Y., Konno, J., Yamane, N., Odagiri, T., Arikawa, J., et al. (1979). Anti-nucleoprotein antibody response in influenza A infection. *Tohoku J. Exp. Med.* 128, 241–249. doi: 10.1620/tjem.128.241

Sun, J. C., Williams, M. A., and Bevan, M. J. (2004). CD4+ T cells are required for the maintenance, not programming, of memory CD8+ T cells after acute infection. *Nat. Immunol.* 5, 927–933. doi: 10.1038/ni1105

Taylor, P. M., and Askonas, B. A. (1986). Influenza nucleoprotein-specific cytotoxic T-cell clones are protective *in vivo*. *Immunology* 58, 417–420.

Topham, D. J., and Doherty, P. C. (1998). Clearance of an influenza A virus by CD4+ T cells is inefficient in the absence of B cells. *J. Virol.* 72, 882–885.

Topham, D. J., Tripp, R. A., and Doherty, P. C. (1997). CD8+ T cells clear influenza virus by perforin or Fas-dependent processes. *J. Immunol.* 159, 5197–5200.

Topham, D. J., Tripp, R. A., Sarawar, S. R., Sangster, M. Y., and Doherty, P. C. (1996). Immune CD4+ T cells promote the clearance of influenza virus from major histocompatibility complex class II -/- respiratory epithelium. *J. Virol.* 70, 1288–1291.

Tu, W., Mao, H., Zheng, J., Liu, Y., Chiu, S. S., Qin, G., et al. (2010). Cytotoxic T lymphocytes established by seasonal human influenza cross-react against 2009 pandemic H1N1 influenza virus. *J. Virol.* 84, 6527–6535. doi: 10.1128/JVI.00519-10

Tu, W., Zheng, J., Liu, Y., Sia, S. F., Liu, M., Qin, G., et al. (2011). The aminobisphosphonate pamidronate controls influenza pathogenesis by expanding a gammadelta T cell population in humanized mice. *J. Exp. Med.* 208, 1511–1522. doi: 10.1084/jem.20110226

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

Ulmer, J. B., Fu, T. M., Deck, R. R., Friedman, A., Guan, L., Dewitt, C., et al. (1998). Protective CD4+ and CD8+ T cells against influenza virus induced by vaccination with nucleoprotein DNA. *J. Virol.* 72, 5648–5653.

Valkenburg, S. A., Quinones-Parra, S., Gras, S., Komadina, N., McVernon, J., Wang, Z., et al. (2013). Acute emergence and reversion of influenza A virus quasispecies within CD8(+) T cell antigenic peptides. *Nat. Commun.* 4:2663. doi: 10.1038/ncomms3663

Valkenburg, S. A., Venturi, V., Dang, T. H., Bird, N. L., Doherty, P. C., Turner, S. J., et al. (2012). Early priming minimizes the age-related immune compromise of CD8 T cell diversity and function. *PLoS Pathog.* 8:e1002544. doi: 10.1371/annotation/e142f9de-7f30-4759-bda1-a651e86d5ba6

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

Vantourout, P., and Hayday, A. (2013). Six-of-the-best: unique contributions of gammadelta T cells to immunology. *Nat. Rev. Immunol.* 13, 88–100. doi: 10.1038/nri3384

Virelizier, J. L., Allison, A. C., Oxford, J. S., and Schild, G. C. (1977). Early presence of ribonucleoprotein antigen on surface of influenza virus-infected cells. *Nature* 266, 52–54. doi: 10.1038/266052a0

Voeten, J. T., Bestebroer, T. M., Nieuwkoop, N. J., Fouchier, R. A., Osterhaus, A. D., and Rimmelzwaan, G. F. (2000). Antigenic drift in the influenza A virus (H3N2) nucleoprotein and escape from recognition by cytotoxic T lymphocytes. *J. Virol.* 74, 6800–6807. doi: 10.1128/JVI.74.15.6800-6807.2000

Wahl, A., Schafer, F., Bardet, W., Buchli, R., Air, G. M., and Hildebrand, W. H. (2009). HLA class I molecules consistently present internal influenza epitopes. *Proc. Natl. Acad. Sci. U.S.A.* 106, 540–545. doi: 10.1073/pnas.0811271106

Wang, T. T., Tan, G. S., Hai, R., Pica, N., Ngai, L., Ekiert, D. C., et al. (2010). Vaccination with a synthetic peptide from the influenza virus hemagglutinin provides protection against distinct viral subtypes. *Proc. Natl. Acad. Sci. U.S.A.* 107, 18979–18984. doi: 10.1073/pnas.1013387107

Wei, C. J., Boyington, J. C., McTamney, P. M., Kong, W. P., Pearce, M. B., Xu, L., et al. (2010). Induction of broadly neutralizing H1N1 influenza antibodies by vaccination. *Science* 329, 1060–1064. doi: 10.1126/science.1192517

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

Wilkinson, T. M., Li, C. K., Chui, C. S., Huang, A. K., Perkins, M., Liebner, J. C., et al. (2012). Preexisting influenza-specific CD4+ T cells correlate with disease protection against influenza challenge in humans. *Nat. Med.* 18, 274–280. doi: 10.1038/nm.2612

Wu, C., Zanker, D., Valkenburg, S., Tan, B., Kedzierska, K., Zou, Q. M., et al. (2011). Systematic identification of immunodominant CD8+ T-cell responses to influenza A virus in HLA-A2 individuals. *Proc. Natl. Acad. Sci. U.S.A.* 108, 9178–9183. doi: 10.1073/pnas.1105624108

Wu, J. T., Ma, E. S., Lee, C. K., Chu, D. K., Ho, P. L., Shen, A. L., et al. (2010). The infection attack rate and severity of 2009 pandemic H1N1 influenza in Hong Kong. *Clin. Infect. Dis.* 51, 1184–1191. doi: 10.1086/656740

Yap, K. L., Ada, G. L., and McKenzie, I. F. (1978). Transfer of specific cytotoxic T lymphocytes protects mice inoculated with influenza virus. *Nature* 273, 238–239. doi: 10.1038/273238a0

Yewdell, J. W., Bennink, J. R., Smith, G. L., and Moss, B. (1985). Influenza A virus nucleoprotein is a major target antigen for cross-reactive anti-influenza A virus cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. U.S.A.* 82, 1785–1789. doi: 10.1073/pnas.82.6.1785

Yewdell, J. W., Frank, E., and Gerhard, W. (1981). Expression of influenza A virus internal antigens on the surface of infected P815 cells. *J. Immunol.* 126, 1814–1819.

Yoshida, R., Igarashi, M., Ozaki, H., Kishida, N., Tomabechi, D., Kida, H., et al. (2009). Cross-protective potential of a novel monoclonal antibody directed against antigenic site B of the hemagglutinin of influenza A viruses. *PLoS Pathog.* 5:e1000350. doi: 10.1371/journal.ppat.1000350

Zhao, Y., Zhang, Y. H., Denney, L., Young, D., Powell, T. J., Peng, Y. C., et al. (2012). High levels of virus-specific CD4+ T cells predict severe pandemic influenza A virus infection. *Am. J. Respir. Crit. Care Med.* 186, 1292–1297. doi: 10.1164/rccm.201207-1245OC

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.

Received: 31 March 2014; accepted: 23 May 2014; published online: 12 June 2014.
*Citation: Quiñones-Parra S, Loh L, Brown LE, Kedzierska K and Valkenburg SA (2014) Universal immunity to influenza must outwit immune evasion. Front. Microbiol. **5**:285. doi: 10.3389/fmicb.2014.00285*
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Microbiology.
Copyright © 2014 Quiñones-Parra, Loh, Brown, Kedzierska and Valkenburg. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 04 November 2014
doi: 10.3389/fmicb.2014.00572

How can we design better vaccines to prevent HIV infection in women?

Hannah Rafferty, Sengeziwe Sibeko and Sarah Rowland-Jones*

Nuffield Department of Medicine, University of Oxford, Oxford, UK

Edited by:
Magdalena Plebanski, Monash University, Australia

Reviewed by:
Dirk Dittmer, University of North Carolina at Chapel Hill, USA
Heinrich Korner, Menzies Research Institute Tasmania, Australia

***Correspondence:**
Sarah Rowland-Jones, University of Oxford, NDM Research Building, Old Road Campus, Roosevelt Drive, Oxford, OX3 7FZ, UK
e-mail: sarah.rowland-jones@ndm.ox.ac.uk

The human immunodeficiency virus (HIV) burden in women continues to increase, and heterosexual contact is now the most common route of infection worldwide. Effective protection of women against HIV-1 infection may require a vaccine specifically targeting mucosal immune responses in the female genital tract (FGT). To achieve this goal, a much better understanding of the immunology of the FGT is needed. Here we review the architecture of the immune system of the FGT, recent studies of potential methods to achieve the goal of mucosal protection in women, including systemic-prime, mucosal-boost, FGT-tropic vectors and immune response altering adjuvants. Advances in other fields that enhance our understanding of female genital immune correlates and the interplay between hormonal and immunological systems may also help to achieve protection of women from HIV infection.

Keywords: vaccines, HIV, women, genital tract, mucosal

INTRODUCTION

The burden of human immunodeficiency virus (HIV) infection in the twenty-first century falls disproportionately on women, particularly in the developing world. Women in Sub-Saharan Africa have, on average, a 60% increased risk of HIV infection compared with their male counterparts (Magadi, 2011) and now account for 58% of HIV-infected adults in the region (UNAIDS, 2012a). Despite these statistics little attention has been paid to developing vaccine candidates that specifically protect the FGT. The holy grail of HIV prophylactics is a vaccine preventing acquisition, achieving "sterilizing immunity." Unfortunately such vaccines have proven difficult to develop, due to HIV's numerous immune evasion strategies and the speed and strength of immune response required to prevent virus dissemination. Most previous vaccine strategies have focused on achieving systemic immunity with conventional intramuscular immunization. However, HIV is arguably a mucosal disease, with acquisition most common *via* mucosal routes. The ability of HIV to overcome the epithelial barrier and innate immune responses, together with the delayed development of adaptive immune responses, means that there is a very narrow time-window for protection against acquisition at the mucosa. Furthermore, systemic vaccines do not elicit sufficient local immune responses, including secretory Immunoglobulin A (sIgA) (Baral et al., 2012), to prevent infection, so it seems likely that a mucosal strategy such as a vaccine or microbicide, eliciting both systemic and mucosal immune responses, will be most effective in preventing acquisition. Given the biological differences between the immunology of the FGT and other mucosal compartments, a mucosal vaccine specifically targeting the FGT would seem to be the best way to protect women against the spread of HIV.

EPIDEMIOLOGY OF HIV-1 INFECTION IN WOMEN

Vaginal heterosexual sex is the most common route of transmission worldwide (Kalichman et al., 2011; UNAIDS, 2012b), and women are believed to have double the risk of infection via this route compared to men (Boily et al., 2009). Young women (aged 15–24) are particularly susceptible, accounting for 22% of all new infections (Rodriguez-Garcia et al., 2013). Various factors, both biological and social, may contribute to the high rates of HIV infection in young women. At a social level gender biases are common, particularly in developing countries. The frequency of violence against women combined with their lower socioeconomic status leads to power imbalances. These relationship dynamics give women little ability to negotiate safer sexual practices or the use of contraceptives; hence women are less able to protect themselves actively against infection (Stein, 1990). This is compounded by unequal access to education, with studies suggesting women have consistently poorer knowledge of the benefits of condoms in HIV prevention (UNAIDS, 2012a). In addition, other female populations are pivotal in disease spread. Female sex workers contribute heavily to HIV-1 transmission due to their high HIV prevalence, estimated at 12% worldwide (Baral et al., 2012), along with increased sexual activity. These factors led to direct implication of the sex trade in 10% of Ugandan HIV diagnoses in 2010 (Government of Uganda, 2008). Pregnant women can transmit HIV during pregnancy, labor or breastfeeding, and may also be more likely to acquire HIV than their non-pregnant counterparts (Drake et al., 2014). Effective protection of women is therefore likely to have a large impact on HIV transmission to men and children, especially in high prevalence regions.

IMMUNITY IN THE FGT

ANATOMY AND IMMUNOLOGICAL STRUCTURE OF THE FGT

The FGT can be divided into two distinct regions: the lower consisting of vulva and vagina, and the upper of ovaries, fallopian tubes and uterus, including the ectocervix and endocervix. The vagina was previously thought to be the site of HIV-1 acquisition; however it is now thought that the cervix, particularly the endocervix and the area between the endocervix and ectocervix known as the transformation zone, are particularly susceptible to infection (Nuovo et al., 1993). This is probably due to an abundance of potential HIV target cells, $CD4^+$ T-cells, macrophages and dendritic cells, in this region (Pudney et al., 2005), which separates the richly colonized lower reproductive tract and the relatively sterile upper tract. In adolescence, the columnar epithelium of the endocervix extends down into the ectocervix, a phenomenon known as cervical ectopy. This exposes a greater area of more susceptible tissue to potential infection and may contribute to the high risk of HIV infection in adolescent girls.

Cervico-vaginal fluid (CVF) is secreted throughout the FGT mucosa and constitutes the first line of mucosal defense: CVF contains an array of soluble factors including chemokines, cytokines and anti-microbial peptides, many of which have potent anti-HIV activity. Intriguingly, the CVF of younger women, particularly those with cervical ectopy, shows increased levels of pro-inflammatory cytokines (Hwang et al., 2011). This may further increase their susceptibility to HIV infection, as inflammation in the genital tract has been associated with increased HIV infection risk in several studies (Levinson et al., 2009; Naranbhai et al., 2012).

The FGT is unique among mucosal surfaces in that it largely lacks organized lymphoid elements, possessing instead small numbers of mononuclear cells scattered throughout the sub-epithelial stroma (Yeaman et al., 1997). This is in marked contrast to the resident immune system of the intestinal mucosa, which consists of clearly-defined lymphoid patches, sub-mucosal lymphocytes, and a large population of intraepithelial lymphocytes poised between crypt epithelial cells (Perry et al., 1998). The absence of a follicular structure means that it is difficult to identify an FGT immune inductive site, responsible for initiating an immune response. Therefore, induction of immunity to genital pathogens is assumed to occur outside the genital tract, followed by recruitment of re-circulating cells into infected sites through the common mucosal immune system (CMIS) (Kantele et al., 1998). There is some evidence that suggests FGT induction sites may be associated with nasal-associated lymphoid tissue (NALT), gut-associated lymphoid tissue (GALT), or bronchial-associated lymphoid tissue (BALT), but none of these preferentially induce local FGT B cells (Mestecky and Russell, 2000) (see **Figure 1** for details of general inductive and effector sites). An understanding of the pathways that direct lymphocyte trafficking to the FGT is essential for the development of mucosal vaccines (Perry et al., 1998).

MUCOSAL HOMING

In the mouse, T lymphocyte recruitment to the genital mucosa is directed by the same set of interactions that direct T cells to systemic sites of inflammation, which are distinct from those that dictate traffic to the intestinal mucosa. The homing pathways defined for the intestinal mucosa are assumed to be relevant to all mucosal sites, but are not well represented in the genital tract. This presents yet another area for further investigation if a successful vaccine is to be developed.

IMMUNOLOGICAL ENDOCRINE INTERPLAY

HORMONAL EFFECTS ON IMMUNE RESPONSES IN THE FGT

An important difference between the FGT and other mucosal sites is the influence of female hormones. These not only produce the menstrual cycle, but also affect the immunity of the FGT. Unfortunately this topic has not been extensively researched and hence knowledge relating to vaccine design is limited. It is known that some of the anti-viral proteins in CVF are regulated by hormone status: for example levels of HBD2 and SLP1, two anti-HIV peptides, are lower in CVF during ovulation (Keller et al., 2007). In contrast, oestradiol secretion enhances the secretion of anti-microbial peptides, whilst simultaneously suppressing the secretion of pro-inflammatory cytokines and chemokines (Fahey et al., 2008): these observations would predict lower HIV susceptibility in the first half of the cycle. Macaque studies suggest that females are more susceptible to simian immunodeficiency virus (SIV) vaginal challenge during the luteal (progesterone dominant) phase of the menstrual cycle (Vishwanathan et al., 2011). The luteal phase can be thought of as a time of relative immune suppression in the FGT in order to optimize conditions for fertilization and implantation. The secretion of mucus, as well as anti-microbial peptides, by the endocervix also varies during the menstrual cycle, which may influence susceptibility to infection (Radtke et al., 2012). These factors suggest that there are distinct patterns of immune response and differing susceptibility to infection during the three phases of the menstrual cycle. Although data from human studies are lacking, it has been proposed that women have a distinct "window of vulnerability" to HIV infection in the 7–10 days following ovulation (Wira and Fahey, 2008). Thus future vaccine and microbicide trials need to take account of the menstrual cycle of female participants in order adequately to assess protection from and susceptibility to HIV infection.

MUCOSAL TRANSMISSION

INITIAL EVENTS IN HIV-1 INFECTION IN THE FGT

Most of our current understanding of the process of HIV mucosal transmission comes from animal models and *in vitro* studies, for example using cervical explants. There is a welcome trend to make SIV models more physiological, with lower doses of SIV used repeatedly in a mucosal challenge. In many previous trials macaques were given high doses of SIV, often intravenously, which is unlikely to represent the early events of HIV-1 infection in women (Haase, 2011). It is still not entirely clear where in the human FGT HIV is most likely to establish primary infection. Transmission studies by Miller and colleagues showed that both vagina and cervix could be sites of primary SIV infection in the SIV/Rhesus macaque model (Miller, 1998). More recent evidence suggests that primary infection takes place predominantly in the cervix following vaginal SIV exposure in macaques, particularly the endocervix and transformation zone (junction between endocervix and ectocervix) (Li et al., 2009a), where the target-cell

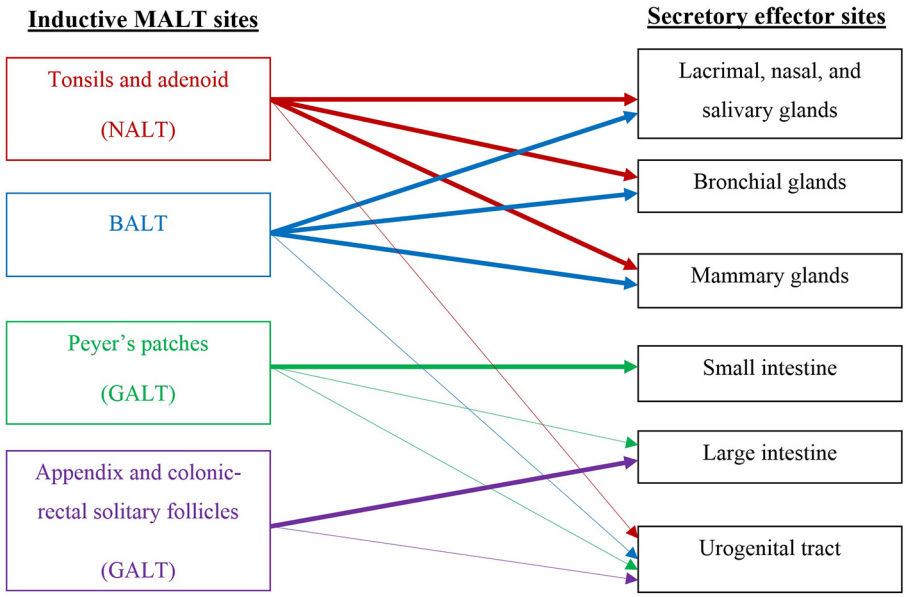

FIGURE 1 | Schematic of the common mucosal immune system (CMIS) relevant to the stimulation of vaccine-induced responses in the female genital tract.

density [T cells and antigen presenting cells (APCs)] and turnover is greatest (Li et al., 2009b), and where breaks in the mucosa often occur (Norvell et al., 1984).

It was initially thought that HIV first infected vaginal APCs such as macrophages and Langerhans cells, with subsequent rounds of replication occurring in the draining lymph nodes. This was thought to be followed by spread to more proximal lymphoid nodes and finally to the bloodstream and distant lymphoid tissue (Miller, 1998). Subsequently Zhang et al showed that the first cell to be infected is the endocervical intraepithelial resting $CD4^+$ T cell (Zhang et al., 1999). Human cervical explant culture models confirmed that memory $CD4^+$ T cells were the first infected during HIV transmission across the cervical mucosa (Gupta et al., 2002) (see **Figure 2**).

Most of our understanding of the very early events following HIV transmission has come from the macaque model of acute SIV infection. Even when large amounts of viral RNA are used in the inoculum, only small foci of tissue-associated viral RNA are found in the first 3–4 days after infection, consistent with a limited founder population of infected cells (Haase, 2011). These clusters of 40–50 infected cells are most consistently found in the endocervix and transformation zone, and expand locally by recruitment of susceptible cells. These observations suggest that there is a critical "window of opportunity" in the first few days after infection, when a targeted immune response involving virus-specific antibodies and/or cytotoxic T lymphocytes (CTL), could control and clear the initial infection before local expansion and subsequent dissemination into the lymphatics. In macaque studies, an influx of SIV-specific CTL was identified that the authors described as generally "too little," i.e., at too low an effector-to-target ratio to control the infection, and "too late" (Li et al., 2009b).

CORRELATES OF HIV IMMUNITY IN THE FGT

It is still not clear what responses a vaccine should elicit for protection of the FGT against HIV-1, but some valuable insights have come from studying highly-exposed seronegative subjects (HESNs). SIgA, the major immunoglobulin class involved in mucosal immunity, specific for HIV has been found in the genital fluids of HESN women in several studies (Mazzoli et al., 1997; Devito et al., 2000a,b, 2002; Belec et al., 2001; Broliden et al., 2001; Freeman et al., 2006; Tudor et al., 2009) suggesting it may be important in the protective immune response (Kaul et al., 2001). Further investigations of the HIV IgA response showed these antibodies were directed toward gp41 and were able to inhibit HIV-1 transcytosis and neutralize virions (Devito et al., 2000a,b; Belec et al., 2001; Tudor et al., 2009). HIV-1-specific-immunoglobulin G (IgG) has also been found in the FGT of HESNs (Belec et al., 2001; Buchacz et al., 2001). However, another group found no detectable HIV-1 specific vaginal IgG or IgA in a population of HESNs in the Gambia (Dorrell et al., 2000).

Cellular responses may also contribute to protection against HIV infection. HIV-specific $CD8^+$ cytotoxic T-lymphocytes (CTLs) have been detected in the cervical mucosa of HESN sex workers (Kaul et al., 2000, 2003), where they were enriched relative to responses detected in blood (Kaul et al., 2000; Iqbal et al., 2005).

However, others suggest it is not the immune response against HIV-1 that provides protection, but rather the overall immune quiescence of the FGT (Card et al., 2012; Lajoie et al., 2012). This group reported lower levels of pro-inflammatory cytokines in female HESNs compared to HIV-negative controls (Card et al., 2013), as well as a lower level of expression of genes crucial for HIV replication (McLaren et al., 2010; Songok et al., 2012). In contrast, FGT inflammation is associated with an increased risk

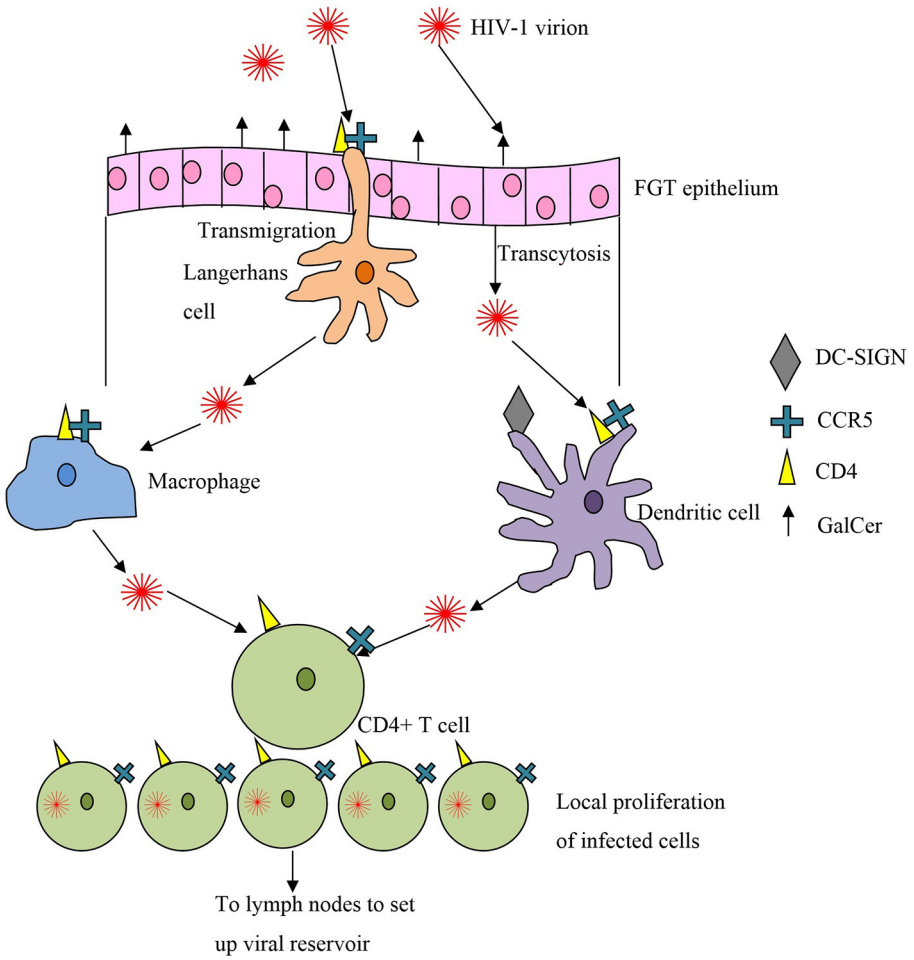

FIGURE 2 | Crossing the first line of defense, the epithelial mucosa, and targets of HIV infection.

of HIV infection, presumably due to the recruitment of activated $CD4^+$ T-cells (Cohen, 2004; Freeman et al., 2006).

Given that the main function of the FGT is its role in reproduction, it is not surprising that immune tolerance is an important feature of the FGT. Tolerance facilitates fetal implantation in the uterus and allows commensal organisms to colonize the lower tract. Whilst this may contribute to protection against genital infection, tolerance mechanisms, including regulatory T-cells and TGF-β secretion, must therefore be overcome by an induced vaccine response, requiring a highly immunogenic preparation.

DESIRABLE ATTRIBUTES OF A MUCOSAL VACCINE

THE NEED FOR A MUCOSAL VACCINE

Timing is of the essence to achieve a protective immune response against HIV. The response must be sufficiently rapid to stop the infection before the virus disseminates, by which time it is beyond control (Haase, 2010). Systemic memory responses are too slow to prevent HIV infection at the mucosa; instead a large pool of effector cells at the FGT mucosal surface, ready for immediate mobilization, is more likely to confer protection. Direct comparison of mucosal and systemic vaccination routes has shown the mucosal route alone can induce mucosal memory populations of CTLs (Gallichan and Rosenthal, 1996) and high-avidity CTLs (Ranasinghe and Ramshaw, 2009), matching the CTL profiles of HIV controllers (Mothe et al., 2012). Systemic vaccines can elicit FGT IgG, but in comparison to mucosal vaccines induce little or no SIgA, which is produced locally by plasma cells in the FGT stroma (Nardelli-Haefliger et al., 1999; Pattani et al., 2012). Despite the dominance of the IgG subtype in the lower FGT, SIgA has a decisive role in protection, acting as the first defense against the virus by preventing attachment and hence acquisition (Neutra and Kozlowski, 2006; Brandtzaeg, 2007). Unlike IgG, IgA does not activate the complement system and so can be thought of as anti-inflammatory, important for HIV protection. The impact of a mucosal response to HIV was practically demonstrated by the success of the 1% tenofovir microbicide (Abdool Karim et al., 2010). Mucosal vaccines can also stimulate lymphatic and systemic immune responses (Belyakov et al., 1998), acting as a catch-all for virions that pass into the circulation. These factors suggest a mucosal vaccine specific for the FGT would provide the immune response most likely to prevent HIV acquisition (see **Figure 3** and **Box 1**).

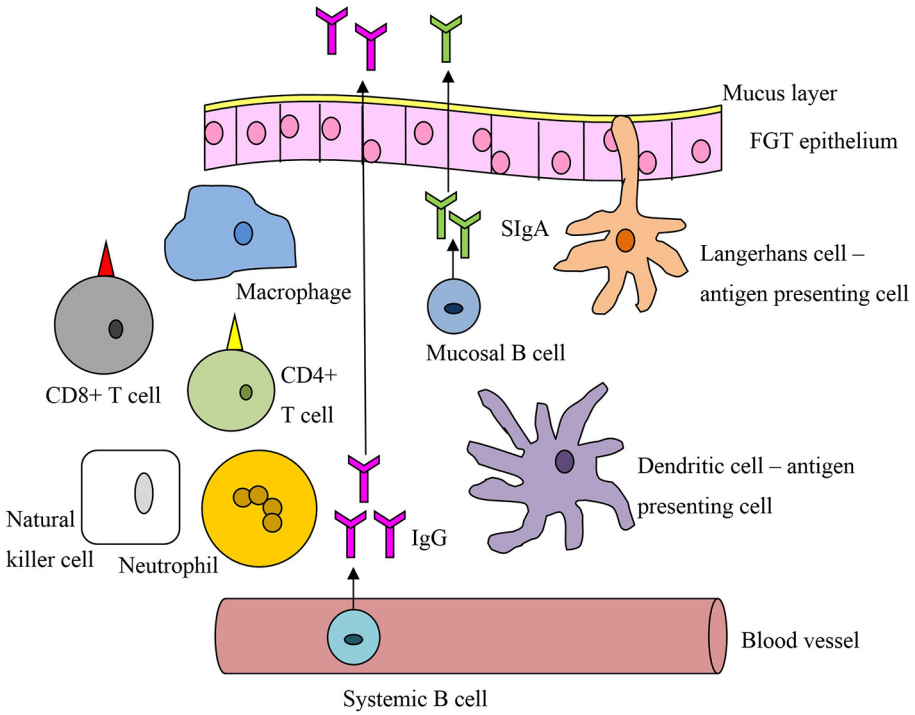

FIGURE 3 | A general schematic of vaccine-related immune cells active at the mucosal interface of the female genital tract.

Box 1 | Challenges in developing mucosal vaccines against HIV infection.

- The gross architecture of the FGT immune system is not typical of other mucosal surfaces—it lacks lymphoid aggregates such as Peyer's patches in the gastrointestinal tract, which comprise B and T cell zones and are responsible for immune induction.
- Correlates of protection against HIV at this site are not well understood for various reasons, including difficulty in establishing appropriate models for studying the HIV-immune system interaction at the mucosal interface.
- Immune tolerance is a feature of the FGT, in view of widespread microbial colonization in its lower tract and the principle function of the upper tract in reproduction—this impedes the mounting of desirable immune responses to locally administered vaccines.
- Systemic immunization, the commonest route of administration of vaccines, is ineffective in generating protective immunity at the mucosa - systemic vaccines do not elicit sufficient local immune responses including SIgA.
- Protein antigens are poor immunogens when given mucosally. Instead of response induction, they lead to immunological tolerance or unresponsiveness known as mucosally-induced tolerance. Consequently, adjuvants are needed to ensure an adequate mucosal immune response is mounted.

ROUTE OF ADMINISTRATION AND THE DOSING STRATEGY

As described above, no specific induction site has been characterized in the FGT (see **Figure 1**), although immune responses can be generated by APCs in the sub-mucosa (Wira and Fahey, 2008). Several possible routes have been investigated including oral, rectal, vaginal, and intranasal. Intranasal administration seems to be a promising strategy in terms of immune response and application. Rhesus macaques showed SIV-specific IgA, IgG and CTLs in cervico-vaginal washes post intranasal immunization with SIV-p55gag with cholera toxin adjuvant (Imaoka et al., 1998). A study in human volunteers also showed an increase in vaginal cholera toxin B (CTB)-specific IgA and IgG with a strong systemic response following intranasal-immunization with CTB subunit (Bergquist et al., 1997). However, a Phase 1 trial of an intranasal HIV-1 vaccine using recombinant HIV-1-gp160 yielded no antibodies in serum or secretory fluids (Pialoux et al., 2008).

It may be better to use a combined prime-boost strategy to elicit FGT immunity. This can, however, be complex as the vaccine response is affected by both the route and the timing of immunization, particularly the interval between the prime and the boost. Rhesus Macaques were immunized with simian-HIV-SF162P3 P1 and recombinant gp41 subunit antigens grafted onto virosomes delivered first intramuscularly (IM) then boosted with intranasal (IN) application (Bomsel et al., 2011). Four of five macaques in the IM/IN group were fully protected against 13 vaginal SHIV challenges delivered over 9 weeks. The other macaques only showed transient infection while

none seroconverted to p27gag-SIV. In contrast, only one of six macaques given just the intramuscular formulation was fully protected. All placebo immunized animals seroconverted. Protected animals showed cervicovaginal antigen-specific IgA which inhibited HIV-1 transcytosis and IgG with neutralizing or antibody-dependent cytotoxicity effects. CTLs were not assessed. Some cross-clade transcytosis-inhibition was found in the IM/IN group, suggesting the generation of more broadly neutralizing antibodies. The animals lacked neutralizing antibodies in serum, further emphasizing the importance of a mucosal response for HIV protection.

A recent phase I placebo-controlled trial tested a virosome harboring surface HIV-1 gp41-derived P1 lipidated peptides delivered as an intramuscular prime, then by intranasal boost (Leroux-Roels et al., 2013). The vaccine was safe and well tolerated. P1-specific serum IgG and IgA were induced in all participants receiving the high dose of vaccine. Analysis did not reveal a statistically significant increase in mucosal P1-specifc IgA, despite being detected in 63 and 43% of the low and high dose participants respectively. However, there was an unexpectedly high pre-immune vaginal reactivity toward the P1 antigen, which may have skewed the results. IgA expression is influenced by hormonal changes so sampling during different phases of the menstrual cycle may explain these findings. Vaginal and rectal IgG did increase significantly over the weeks of vaccination for both the high and the low dose groups. These vaginal antibodies were further investigated and were shown to possess the ability to inhibit HIV-1 transcytosis. This result shows promise, demonstrating both the safety and immunogenicity of mucosal vaccines.

A study looking at HSV-2 infection used a novel prime-boost strategy known as "prime-pull," which could be extrapolated to an HIV vaccine (Shin and Iwasaki, 2012). The "prime-pull" technique involves the mucosal application of chemokines after immunization to recruit primed cells to the mucosa. Mice were immunized systemically, then chemokines CXCL9 and CXCL10 were applied directly to the vaginal mucosa. CTLs were recruited to the FGT and established a long-term population of memory $CD8^+$ T cells. $CD4^+$ T cells were initially recruited to the FGT but not retained there long-term. The initial $CD4^+$ T-cell influx could potentially increase HIV risk, but after the effector phase these cells withdrew, so overall the risk is deemed negligible. In addition, no markers of inflammation were found, and the strategy led to complete protection from vaginal HSV-2 infection.

Following this promising result the "prime-pull" technique was recently adapted to HIV. Mice were immunized intranasally with HIV-1-gp140 and then either the cytokine CCL28 or the toll-like receptor 4 ligand (TLR4) MPLA was administered to the vaginal mucosa (Tregoning et al., 2013). The application of CCL28 post-intranasal vaccine did not increase vaginal B cells or antibodies; however MPLA application caused significant increases in HIV-1 specific vaginal IgA and serum IgG and IgA. The authors concede that, as homing mechanisms in the urogenital tract are not well understood, CCL28 may not have been the best cytokine to use, and suggest that other chemokines involved in B-cell recruitment should be tested. It may be that a single cytokine is insufficient and several acting in concert would provide a better "pull" toward the urogenital tract. On the other hand the success of MPLA adds another dimension to this "prime-pull" strategy; different TLR agonists should also now be investigated.

ADJUVANTS

As seen with the "prime-pull" strategy described above, most vaccines require an adjuvant to boost the immunogenicity of the construct. Adjuvants are substances that possess the biological capacity to enhance, prolong or accelerate the quality of specific immune responses to vaccine antigens. With regards to mucosal vaccines, adjuvants can be broadly classified into those that play an immunostimulatory role and those that facilitate vaccine delivery for the induction of protective immunity via the CMIS (Yuki and Kiyono, 2003).

Few adjuvant studies have focused on boosting mucosal HIV-specific immunity. Chemokines and cytokines are widely thought to be the most effective adjuvants for HIV-1 vaccines. When CCL28 was used to adjuvant an $HIV\text{-}1_{IIIB}$ virus like particle (VLP) construct, enhanced neutralizing capabilities against HIV-1 clade B laboratory isolates and an HIV-1 clade C primary isolate were found in vaginal secretions and sera of mice (Rainone et al., 2011). Increased env-specific interferon gamma (IFN-γ) and interleukin (IL)-45 were also seen, with increased serum IgA, both non-specific and specific for HIV-1. More recently, mice were immunized either IM or IN with HIV-1 gp140 co-delivered with plasmid CCL19 or CCL28 (Hu et al., 2013). Both IM and IN protocols enhanced serum IgG responses, and both cytokines enhanced vaginal IgG and IgA responses, but only when given via the IN route. The vaginal antibodies showed neutralizing activity against both homologous and heterologous HIV-1.

A novel approach to using cytokines as adjuvants employed soluble IL-13 receptors to antagonize the IL-13 response (Ranasinghe et al., 2013). Recombinant poxviruses that co-expressed HIV-1-gag/pol with IL-13Rα2 soluble receptors were given via intranasal-prime, intramuscular-boost to female mice. The transient blockade of IL-13 resulted in the generation of high-avidity CTLs in the iliac and genito-rectal nodes (which drain the FGT), which were not found in the control protocol without soluble IL-13Rα2: high-avidity CTLs were more protective, shown by greater protection following an intranasal challenge with gag-expressing influenza in the IL-13Rα2 group. The stimulation of high-avidity CTLs matches the CTL profiles of HIV controllers (Mothe et al., 2012). This study presents an interesting alternative to the conventional addition of cytokines. Potentially a mixture of addition and blockade of cytokine pathways will generate a suitable immune response in the FGT.

FGT TROPIC VECTORS

Given the mucosal site of HIV acquisition, effective vectors should exhibit mucosal tropism, ideally specific for the FGT. Adenovirus, a commonly used vector, targets the mucosa but is not FGT-specific: this may contribute to its lack of success in clinical trials to prevent HIV infection. There is controversy surrounding the use of adenovirus as a vector after the STEP trial (HVTN502/Merck023), the first clinical trial to examine an HIV prophylactic vaccine using adenovirus as a vector, suggested

that adenovirus priming may actually increase the risk of HIV infection (Buchbinder et al., 2008).

Human papillomavirus (HPV) is a FGT tropic virus that infects cervicovaginal keratinocytes, lying senescent for long periods of time. A recent study in Cynomolgus and Rhesus Macaques used HPV pseudovirosomes to deliver SIV-Gag-DNA (Gordon et al., 2012). Gag-specific IgA, IgG and CD4/8+ T cell responses were found in the serum and vaginal tract, which rapidly expanded following intravaginal SIV exposure, suggesting the formation of memory populations. However limitations of this study were that only low levels of vaginal IgG and IgA were induced, and the vaccine led to a substantial $CD4^+$ T cell response that could increase HIV susceptibility: there was no protection against vaginal SIV challenge. Furthermore, unattenuated HPV has been shown to increase CXCL8 levels, potentially increasing susceptibility to HIV-1 in cervical tissues and upregulating HIV-1 proliferation (Narimatsu et al., 2005).

These are early days in mucosal targeting of vaccines: with subsequent testing and refining, better results may be achieved. Other sexually transmitted disease (STD) vectors such as HSV-2 could also be tested as vectors. HSV-1 has been used as a vector in mice (Parker et al., 2007), using an HSV-1 vector expressing the HIV-1 gag gene for intraperitoneal immunization. Strong gag-specific CD8+ responses were elicited (Parker et al., 2007) which persisted 9 months post-immunization. HSV-2 is closely related to HSV-1, but is acquired through the genital mucosa suggesting it may be a more appropriate HIV vaccine vector. However, further research is needed to improve immune responses and reduce potentially harmful mucosal inflammation.

FUTURE DIRECTIONS

Despite the possibilities highlighted in these studies, several factors must be addressed to improve the development of an effective HIV vaccine. We strongly recommend that immune responses in the FGT should be measured as an integral part of every HIV vaccine trial. Even though precise correlates of immunity are not yet known, it seems reasonable to assume that local immune responses to HIV in the FGT will play an important part in protection against sexual acquisition. Ensuring that FGT responses are always measured provides a timely reminder of the site where most HIV infections in the world are acquired, and should inform future trials and vaccine design. Correlates of FGT inflammation should also be investigated to ensure a vaccine does not increase HIV susceptibility. A focus on FGT responses requires a distinct agenda from the outset so that a laboratory science program is incorporated and prioritized within the parent clinical trial protocol. It is also important to study participants with breakthrough infections, and collect and store relevant, appropriate, and appropriately timed biological specimens, collected as close as possible to the estimated time of infection, (Sibeko and Makvandi-Nejad, 2013).

Our lack of knowledge in key aspects of FGT immunology remains a major problem for mucosal vaccine development. Characterization of FGT homing pathways would greatly improve mucosal vaccine design, as would clear evidence of which compartment in the human FGT is most susceptible to HIV infection. HIV predominantly affects women in developing countries, and therefore for maximal public health benefit new vaccines should be cheap, store well, and preferably not require administration by medical practitioners.

Despite having a significant impact on the immunology of the FGT, the changing levels of estrogen and progesterone throughout the menstrual cycle have rarely been considered in natural history or vaccine studies (Hickey et al., 2011). A better understanding of the immunological milieu in different menstrual phases may suggest a specific point of the cycle when vaccination would be most beneficial. Previous studies suggest that intravaginal vaccination is most effective in women during the follicular phase of the cycle (Kozlowski et al., 2002). The effects of hormones on other mucosal routes have not yet been characterized. The cycle stage should therefore be an important consideration in vaccine trials, and vaccines should be tested during different hormonal stages to assess the most effective timing of administration. Adolescence is an important period when major hormonal fluctuations occur. The HIV-1 incidence in the 15–24 year age group is twice as high in women compared to men (UNAIDS, 2012a), highlighting the importance and challenges of vaccine efficacy in this vulnerable group.

Differential immune responses for adults and adolescents against HSV-2 and *E. Coli* suggest that correlates of mucosal immunity may differ substantially in different age-groups (Madan et al., 2012). It may therefore be necessary to look systematically at different populations and to include adolescents in future vaccine trials. Furthermore, substantial hormonal fluctuations also occur during pregnancy and with the use of hormonal contraceptives. In the future, vaccine assessment should extend to these groups in clinical trials to ensure optimal protection of women at different stages of life.

CONCLUSIONS

The dynamics of HIV infection are changing, with more women infected than in previous years and at younger ages than their male counterparts. This trend probably reflects a combination of socioeconomic and biological factors. Preventing infection in women will have a major impact on HIV incidence in their partners and children. A vaccine specifically targeting the FGT may be needed to induce an immune response that is able to contain HIV prior to dissemination. As mucosal vaccines induce SIgA and mucosal CTL memory responses more successfully than their systemic counterparts, a mucosal vaccine specifically targeting the FGT appears to be the best option for preventing HIV acquisition. Several strategies look promising, with systemic-prime, mucosal-boost, FGT tropic vectors, and adjuvants tailoring the immune response to the FGT all yielding encouraging results in animal models. However, much more must be done: future vaccine trials must put more emphasis on the immune responses of the FGT and consider hormonal effects on mucosal immunity from the outset. Further research on mucosal vaccines specifically targeting these issues may finally yield the protective vaccine needed to protect women and the wider population from the spread of HIV.

ACKNOWLEDGMENT

Dr. Sibeko is in receipt of an Oxford Nuffield Medical Fellowship.

REFERENCES

Abdool Karim, Q. A. K. S., Frohlich, J. A., Grobler, A. C., Baxter, C., Mansoor, L. E., Kharsany, A. B., et al. (2010). Effectiveness and safety of tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women. *Science* 329, 1168–1174. doi: 10.1126/science.1193748

Baral, S., Beyrer, C., Muessig, K., Poteat, T., Wirtz, A. L., Decker, M. R., et al. (2012). Burden of HIV among female sex workers in low-income and middle-income countries: a systematic review and meta-analysis. *Lancet Infect. Dis.* 12, 538–549. doi: 10.1016/S1473-3099(12)70066-X

Belec, L., Ghys, P. D., Hocini, H., Nkengasong, J. N., Tranchot-Diallo, J., Diallo, M. O., et al. (2001). Cervicovaginal secretory antibodies to human immunodeficiency virus type 1 (HIV-1) that block viral transcytosis through tight epithelial barriers in highly exposed HIV-1-seronegative African women. *J. Infect. Dis.* 184, 1412–1422. doi: 10.1086/324375

Belyakov, I. M., Derby, M. A., Ahlers, J. D., Kelsall, B. L., Earl, P., Moss, B., et al. (1998). Mucosal immunization with HIV-1 peptide vaccine induces mucosal and systemic cytotoxic T lymphocytes and protective immunity in mice against intrarectal recombinant HIV-vaccinia challenge. *Proc. Natl. Acad. Sci. U.S.A.* 95, 1709–1714. doi: 10.1073/pnas.95.4.1709

Bergquist, C., Johansson, E. L., Lagergard, T., Holmgren, J., and Rudin, A. (1997). Intranasal vaccination of humans with recombinant cholera toxin B subunit induces systemic and local antibody responses in the upper respiratory tract and the vagina. *Infect. Immun.* 65, 2676–2684.

Boily, M. C., Baggaley, R. F., and Masse, B. (2009). The role of heterosexual anal intercourse for HIV transmission in developing countries: are we ready to draw conclusions? *Sex. Transm. Infect.* 85, 408–410. doi: 10.1136/sti.2009.037499

Bomsel, M., Tudor, D., Drillet, A. S., Alfsen, A., Ganor, Y., Roger, M. G., et al. (2011). Immunization with HIV-1 gp41 subunit virosomes induces mucosal antibodies protecting nonhuman primates against vaginal SHIV challenges. *Immunity* 34, 269–280. doi: 10.1016/j.immuni.2011.01.015

Brandtzaeg, P. (2007). Induction of secretory immunity and memory at mucosal surfaces. *Vaccine* 25, 5467–5484. doi: 10.1016/j.vaccine.2006.12.001

Broliden, K., Hinkula, J., Devito, C., Kiama, P., Kimani, J., Trabbatoni, D., et al. (2001). Functional HIV-1 specific IgA antibodies in HIV-1 exposed, persistently IgG seronegative female sex workers. *Immunol. Lett.* 79, 29–36. doi: 10.1016/S0165-2478(01)00263-2

Buchacz, K., Parekh, B. S., Padian, N. S., van der Straten, A., Phillips, S., Jonte, J., et al. (2001). HIV-specific IgG in cervicovaginal secretions of exposed HIV-uninfected female sexual partners of HIV-infected men. *AIDS Res. Hum. Retroviruses* 17, 1689–1693. doi: 10.1089/08892220152741388

Buchbinder, S. P. M. D., Duerr, A., Fitzgerald, D. W., Mogg, R., Li, D., Gilbert, P. B., et al. (2008). Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. *Lancet* 372, 1881–1893. doi: 10.1016/S0140-6736(08)61591-3

Card, C. M., Ball, T. B., and Fowke, K. R. (2013). Immune quiescence: a model of protection against HIV infection. *Retrovirology* 10:141. doi: 10.1186/1742-4690-10-141

Card, C. M., Rutherford, W. J., Ramdahin, S., Yao, X., Kimani, M., Wachihi, C., et al. (2012). Reduced cellular susceptibility to *in vitro* HIV infection is associated with CD4+ T cell quiescence. *PLoS ONE* 7:e45911. doi: 10.1371/journal.pone.0045911

Cohen, M. S. (2004). HIV and sexually transmitted diseases: lethal synergy. *Top. HIV Med.* 12, 104–117.

Devito, C., Broliden, K., Kaul, R., Svensson, L., Johansen, K., Kiama, P., et al. (2000a). Mucosal and plasma IgA from HIV-1-exposed uninfected individuals inhibit HIV-1 transcytosis across human epithelial cells. *J. Immunol.* 165, 5170–5176. doi: 10.4049/jimmunol.165.9.5170

Devito, C., Hinkula, J., Kaul, R., Kimani, J., Kiama, P., Lopalco, L., et al. (2002). Cross-clade HIV-1-specific neutralizing IgA in mucosal and systemic compartments of HIV-1-exposed, persistently seronegative subjects. *J. Acquir. Immune Defic. Syndr.* 30, 413–420. doi: 10.1097/00042560-200208010-00007

Devito, C., Hinkula, J., Kaul, R., Lopalco, L., Bwayo, J. J., Plummer, F., et al. (2000b). Mucosal and plasma IgA from HIV-exposed seronegative individuals neutralize a primary HIV-1 isolate. *AIDS* 14, 1917–1920. doi: 10.1097/00002030-200009080-00006

Dorrell, L., Hessell, A. J., Wang, M., Whittle, H., Sabally, S., Rowland-Jones, S., et al. (2000). Absence of specific mucosal antibody responses in HIV-exposed uninfected sex workers from the Gambia. *AIDS* 14, 1117–1122. doi: 10.1097/00002030-200006160-00008

Drake, A. L., Wagner, A., Richardson, B., and John-Stewart, G. (2014). Incident HIV during pregnancy and postpartum and risk of mother-to-child HIV transmission: a systematic review and meta-analysis. *PLoS Med.* 11:e1001608. doi: 10.1371/journal.pmed.1001608

Fahey, J. V., Wright, J. A., Shen, L., Smith, J. M., Ghosh, M., Rossoll, R. M., et al. (2008). Estradiol selectively regulates innate immune function by polarized human uterine epithelial cells in culture. *Mucosal Immunol.* 1, 317–325. doi: 10.1038/mi.2008.20

Freeman, E. E., Weiss, H. A., Glynn, J. R., Cross, P. L., Whitworth, J. A., and Hayes, R. J. (2006). Herpes simplex virus 2 infection increases HIV acquisition in men and women: systematic review and meta-analysis of longitudinal studies. *AIDS* 20, 73–83. doi: 10.1097/01.aids.0000198081.09337.a7

Gallichan, W. S., and Rosenthal, K. L. (1996). Long-lived cytotoxic T lymphocyte memory in mucosal tissues after mucosal but not systemic immunization. *J. Exp. Med.* 184, 1879–1890. doi: 10.1084/jem.184.5.1879

Gordon, S. N., Kines, R. C., Kutsyna, G., Ma, Z.-M., Hryniewicz, A., Roberts, J. N., et al. (2012). Targeting the vaginal mucosa with human papillomavirus pseudovirion vaccines delivering simian immunodeficiency virus DNA. *J. Immunol.* 188, 714–723. doi: 10.4049/jimmunol.1101404

Government of Uganda. (2008). *Uganda: UNGASS Country Progress Report*. Kampala.

Gupta, P., Collins, K. B., Ratner, D., Watkins, S., Naus, G. J., Landers, D. V., et al. (2002). Memory CD4(+) T cells are the earliest detectable human immunodeficiency virus type 1 (HIV-1)-infected cells in the female genital mucosal tissue during HIV-1 transmission in an organ culture system. *J. Virol.* 76, 9868–9876. doi: 10.1128/JVI.76.19.9868-9876.2002

Haase, A. T. (2010). Targeting early infection to prevent HIV-1 mucosal transmission. *Nature* 464, 217–223. doi: 10.1038/nature08757

Haase, A. T. (2011). Early events in sexual transmission of HIV and SIV and opportunities for interventions. *Annu. Rev. Med.* 62, 127–139. doi: 10.1146/annurev-med-080709-124959

Hickey, D. K., Patel, M. V., Fahey, J. V., and Wira, C. R. (2011). Innate and adaptive immunity at mucosal surfaces of the female reproductive tract: stratification and integration of immune protection against the transmission of sexually transmitted infections. *J. Reprod. Immunol.* 88, 185–194. doi: 10.1016/j.jri.2011.01.005

Hu, K., Luo, S., Tong, L., Huang, X., Jin, W., Huang, W., et al. (2013). CCL19 and CCL28 augment mucosal and systemic immune responses to HIV-1 gp140 by mobilizing responsive immunocytes into secondary lymph nodes and mucosal tissue. *J. Immunol.* 191, 1935–1947. doi: 10.4049/jimmunol.1300120

Hwang, L. Y., Scott, M. E., Ma, Y., and Moscicki, A. B. (2011). Higher levels of cervicovaginal inflammatory and regulatory cytokines and chemokines in healthy young women with immature cervical epithelium. *J. Reprod. Immunol.* 88, 66–71. doi: 10.1016/j.jri.2010.07.008

Imaoka, K., Miller, C. J., Kubota, M., McChesney, M. B., Lohman, B., Yamamoto, M., et al. (1998). Nasal immunization of nonhuman primates with simian immunodeficiency virus p55gag and cholera toxin adjuvant induces Th1/Th2 help for virus-specific immune responses in reproductive tissues. *J. Immunol.* 161, 5952–5958.

Iqbal, S. M., Ball, T. B., Kimani, J., Kiama, P., Thottingal, P., Embree, J. E., et al. (2005). Elevated T cell counts and RANTES expression in the genital mucosa of HIV-1-resistant Kenyan commercial sex workers. *J. Infect. Dis.* 192, 728–738. doi: 10.1086/432482

Kalichman, S. C., Pinkerton, S. D., Carey, M. P., Cain, D., Mehlomakulu, V., Carey, K. B., et al. (2011). Heterosexual anal intercourse and HIV infection risks in the context of alcohol serving venues, Cape Town, South Africa. *BMC Public Health* 11:807. doi: 10.1186/1471-2458-11-807

Kantele, A., Häkkinen, M., Moldoveanu, Z., Lu, A., Savilahti, E., Alvarez, R. D., et al. (1998). Differences in immune responses induced by oral and rectal immunizations with Salmonella typhi Ty21a: evidence for compartmentalization within the common mucosal immune system in humans. *Infect. Immun.* 66, 5630–5635.

Kaul, R., Plummer, F., Clerici, M., Bomsel, M., Lopalco, L., and Broliden, K. (2001). Mucosal IgA in exposed, uninfected subjects: evidence for a role in protection against HIV infection. *AIDS* 15, 431–442. doi: 10.1097/00002030-200102160-00026

Kaul, R., Plummer, F. A., Kimani, J., Dong, T., Kiama, P., Rostron, T., et al. (2000). HIV-1-Specific mucosal CD8+ lymphocyte responses in the cervix

of HIV-1- resistant prostitutes in Nairobi. *J. Immunol.* 164, 1602–1611. doi: 10.4049/jimmunol.164.3.1602

Kaul, R., Thottingal, P., Kimani, J., Kiama, P., Waigwa, C. W., Bwayo, J. J., et al. (2003). Quantitative *ex vivo* analysis of functional virus-specific CD8 T lymphocytes in the blood and genital tract of HIV-infected women. *AIDS* 17, 1139–1144. doi: 10.1097/00002030-200305230-00004

Keller, M. J., Guzman, E., Hazrati, E., Kasowitz, A., Cheshenko, N., Wallenstein, S., et al. (2007). PRO 2000 elicits a decline in genital tract immune mediators without compromising intrinsic antimicrobial activity. *AIDS* 21, 467–476. doi: 10.1097/QAD.0b013e328013d9b5

Kozlowski, P. A., Williams, S. B., Lynch, R. M., Flanigan, T. P., Patterson, R. R., Cu-Uvin, S., et al. (2002). Differential induction of mucosal and systemic antibody responses in women after nasal, rectal, or vaginal immunization: influence of the menstrual cycle. *J. Immunol.* 169, 566–574. doi: 10.4049/jimmunol.169.1.566

Lajoie, J., Juno, J., Burgener, A., Rahman, S., Mogk, K., Wachihi, C., et al. (2012). A distinct cytokine and chemokine profile at the genital mucosa is associated with HIV-1 protection among HIV-exposed seronegative commercial sex workers. *Mucosal Immunol.* 5, 277–287. doi: 10.1038/mi.2012.7

Leroux-Roels, G., Maes, C., Clement, F., van Engelenburg, F., van den Dobbelsteen, M., Adler, M., et al. (2013). Randomized phase I: safety, immunogenicity and Mucosal antiviral activity in young healthy women vaccinated with HIV-1 Gp41 P1 peptide on virosomes. *PLoS ONE* 8:e55438. doi: 10.1371/journal.pone.0055438

Levinson, P., Kaul, R., Kimani, J., Ngugi, E., Moses, S., MacDonald, K. S., et al. (2009). Levels of innate immune factors in genital fluids: association of alpha defensins and LL-37 with genital infections and increased HIV acquisition. *AIDS* 23, 309–317. doi: 10.1097/QAD.0b013e328321809c

Li, Q., Estes, J. D., Schlievert, P. M., Duan, L., Brosnahan, A. J., Southern, P. J., et al. (2009b). Glycerol monolaurate prevents mucosal SIV transmission. *Nature* 458, 1034–1038. doi: 10.1038/nature07831

Li, Q., Skinner, P. J., Ha, S. J., Duan, L., Mattila, T. L., Hage, A., et al. (2009a). Visualizing antigen-specific and infected cells *in situ* predicts outcomes in early viral infection. *Science* 323, 1726–1729. doi: 10.1126/science.1168676

Madan, R. P., Carpenter, C., Fiedler, T., Kalyoussef, S., McAndrew, T. C., Viswanathan, S., et al. (2012). Altered biomarkers of mucosal immunity and reduced vaginal lactobacillus concentrations in sexually active female adolescents. *PLoS ONE* 7:e40415. doi: 10.1371/journal.pone.0040415

Magadi, M. A. (2011). Understanding the gender disparity in HIV infection across countries in sub-Saharan Africa: evidence from the Demographic and Health Surveys. *Sociol. Health Illn.* 33, 522–539. doi: 10.1111/j.1467-9566.2010.01304.x

Mazzoli, S., Trabbatoni, D., Lo Caputo, S., Piconi, S., Ble, C., Meacci, F., et al. (1997). HIV-specific mucosal and cellular immunity in HIV-seronegative partners of HIV-seropositive individuals. *Nat. Med.* 3, 1250–1257. doi: 10.1038/nm1197-1250

McLaren, P. J., Ball, T. B., Wachihi, C., Jaoko, W., Kelvin, D. J., Danesh, A., et al. (2010). HIV-exposed seronegative commercial sex workers show a quiescent phenotype in the CD4+ T cell compartment and reduced expression of HIV-dependent host factors. *J. Infect. Dis.* 202(Suppl. 3), S339–S344. doi: 10.1086/655968

Mestecky, J., and Russell, M. W. (2000). Induction of mucosal immune responses in the human genital tract. *FEMS Immunol. Med. Microbiol.* 27, 351–355. doi: 10.1111/j.1574-695X.2000.tb01449.x

Miller, C. J. (1998). Localization of Simian immunodeficiency virus-infected cells in the genital tract of male and female Rhesus macaques. *J. Reprod. Immunol.* 41, 331–339. doi: 10.1016/S0165-0378(98)00069-2

Mothe, B., Llano, A., Ibarrondo, J., Zamarreno, J., Schiaulini, M., Miranda, C., et al. (2012). CTL responses of high functional avidity and broad variant cross-reactivity are associated with HIV control. *PLoS ONE* 7:e29717. doi: 10.1371/journal.pone.0029717

Naranbhai, V., Abdool Karim, S. S., Altfeld, M., Samsunder, N., Durgiah, R., Sibeko, S., et al. (2012). Innate immune activation enhances hiv acquisition in women, diminishing the effectiveness of tenofovir microbicide gel. *J. Infect. Dis.* 206, 993–1001. doi: 10.1093/infdis/jis465

Nardelli-Haefliger, D., Roden, R., Balmelli, C., Potts, A., Schiller, J., and De Grandi, P. (1999). Mucosal but not parenteral immunization with purified human papillomavirus type 16 virus-like particles induces neutralizing titers of antibodies throughout the estrous cycle of mice. *J. Virol.* 73, 9609–9613.

Narimatsu, R., Wolday, D., and Patterson, B. K. (2005). IL-8 increases transmission of HIV type 1 in cervical explant tissue. *AIDS Res. Hum. Retroviruses* 21, 228–233. doi: 10.1089/aid.2005.21.228

Neutra, M. R., and Kozlowski, P. A. (2006). Mucosal vaccines: the promise and the challenge. *Nat. Rev. Immunol.* 6, 148–158. doi: 10.1038/nri1777

Norvell, M. K., Benrubi, G. I., and Thompson, R. J. (1984). Investigation of microtrauma after sexual intercourse. *J. Reprod. Med.* 29, 269–271.

Nuovo, G. J., Forde, A., MacConnell, P., and Fahrenwald, R. (1993). *In situ* detection of PCR-amplified HIV-1 nucleic acids and tumor necrosis factor cDNA in cervical tissues. *Am. J. Pathol.* 143, 40–48.

Parker, S. D., Rottinghaus, S. T., Zajac, A. J., Yue, L., Hunter, E., Whitley, R. J., et al. (2007). HIV-$1_{89.6}$ Gag expressed from a replication competent HSV-1 vector elicits persistent cellular immune responses in mice. *Vaccine* 25, 6764–6773. doi: 10.1016/j.vaccine.2007.06.064

Pattani, A., McKay, P. F., Garland, M. J., Curran, R. M., Migalska, K., Cassidy, C. M., et al. (2012). Microneedle mediated intradermal delivery of adjuvanted recombinant HIV-1 CN54gp140 effectively primes mucosal boost inoculations. *J. Control. Release* 162, 529–537. doi: 10.1016/j.jconrel.2012.07.039

Perry, L. L., Feilzer, K., Portis, J. L., and Caldwell, H. D. (1998). Distinct homing pathways direct T lymphocytes to the genital and intestinal mucosae in Chlamydia-infected mice. *J. Immunol.* 160, 2905–2914.

Pialoux, G., Hocini, H., Perusat, S., Silberman, B., Salmon-Ceron, D., Slama, L., et al. (2008). Phase I study of a candidate vaccine based on recombinant HIV-1 gp160 (MN/LAI) administered by the mucosal route to HIV-seronegative volunteers: the ANRS VAC14 study. *Vaccine* 26, 2657–2666. doi: 10.1016/j.vaccine.2007.11.002

Pudney, J., Quayle, A. J., and Anderson, D. J. (2005). Immunological microenvironments in the human vagina and cervix: mediators of cellular immunity are concentrated in the cervical transformation zone. *Biol. Reprod.* 73, 1253–1263. doi: 10.1095/biolreprod.105.043133

Radtke, A. L., Quayle, A. J., and Herbst-Kralovetz, M. M. (2012). Microbial products alter the expression of membrane-associated mucin and antimicrobial peptides in a three-dimensional human endocervical epithelial cell model 1. *Biol. Reprod.* 87:132. doi: 10.1095/biolreprod.112.103366

Rainone, V., Dubois, G., Temchura, V., Überla, K., Clivio, A., Nebuloni, M., et al. (2011). CCL28 induces mucosal homing of HIV-1-specific IgA-secreting plasma cells in mice immunized with HIV-1 virus-like particles. *PLoS ONE* 6:e26979. doi: 10.1371/journal.pone.0026979

Ranasinghe, C., and Ramshaw, I. A. (2009). Immunisation route-dependent expression of IL-4/IL-13 can modulate HIV-specific CD8(+) CTL avidity. *Eur. J. Immunol.* 39, 1819–1830. doi: 10.1002/eji.200838995

Ranasinghe, C., Trivedi, S., Stambas, J., and Jackson, R. (2013). Unique IL-13Rα2-based HIV-1 vaccine strategy to enhance mucosal immunity, CD8+ T-cell avidity and protective immunity. *Mucosal Immunol.* 6, 1068–1080. doi: 10.1038/mi.2013.1

Rodriguez-Garcia, M., Patel, M. V., and Wira, C. R. (2013). Innate and adaptive anti-HIV immune responses in the female reproductive tract. *J. Reprod. Immunol.* 97, 74–84. doi: 10.1016/j.jri.2012.10.010

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

Sibeko, S., and Makvandi-Nejad, S. (2013). From the laboratory to clinical trials and back again: lessons learned from HIV prevention trials. *Am. J. Reprod. Immunol.* 69(Suppl. 1), 106–115. doi: 10.1111/aji.12045

Songok, E. M., Luo, M., Liang, B., McLaren, P., Kaefer, N., Apidi, W., et al. (2012). Microarray analysis of HIV resistant female sex workers reveal a gene expression signature pattern reminiscent of a lowered immune activation state. *PLoS ONE* 7:e30048. doi: 10.1371/journal.pone.0030048

Stein, Z. A. (1990). HIV prevention: the need for methods women can use. *Am. J. Public Health* 80, 460–462. doi: 10.2105/AJPH.80.4.460

Tregoning, J. S., Buffa, V., Oszmiana, A., Klein, K., Walters, A. A., and Shattock, R. J. (2013). A "prime-pull" vaccine strategy has a modest effect on local and systemic antibody responses to HIV gp140 in mice. *PLoS ONE* 8:e80559. doi: 10.1371/journal.pone.0080559

Tudor, D., Derrien, M., Diomede, L., Drillet, A. S., Houimel, M., Moog, C., et al. (2009). HIV-1 gp41-specific monoclonal mucosal IgAs derived from highly

exposed but IgG-seronegative individuals block HIV-1 epithelial transcytosis and neutralize CD4(+) cell infection: an IgA gene and functional analysis. *Mucosal Immunol.* 2, 412–426. doi: 10.1038/mi.2009.89

UNAIDS. (2012a). *UNAIDS Report on the Global AIDS Epidemic in Global Report. 2012.* Geneva.

UNAIDS. (2012b). *Women Outloud: How Women Living with HIV will Help the World End AIDS.* Geneva.

Vishwanathan, S. A., Guenthner, P. C., Lin, C. Y., Dobard, C., Sharma, S., Adams, D. R., et al. (2011). High susceptibility to repeated, low-dose, vaginal SHIV exposure late in the luteal phase of the menstrual cycle of pigtail macaques. *J. Acquir. Immune Defic. Syndr.* 57, 261–264. doi: 10.1097/QAI.0b013e318220ebd3

Wira, C. R., and Fahey, J. V. (2008). A new strategy to understand how HIV infects women: identification of a window of vulnerability during the menstrual cycle. *AIDS* 22, 1909–1917. doi: 10.1097/QAD.0b013e3283060ea4

Yeaman, G. R., Guyre, P. M., Fanger, M. W., Collins, J. E., White, H. D., Rathbun, W., et al. (1997). Unique CD8+ T cell-rich lymphoid aggregates in human uterine endometrium. *J. Leukoc. Biol.* 61, 427–435.

Yuki, Y., and Kiyono, H. (2003). New generation of mucosal adjuvants for the induction of protective immunity. *Rev. Med. Virol.* 13, 293–310. doi: 10.1002/rmv.398

Zhang, Z., Schuler, T., Zupancic, M., Wietgrefe, S., Staskus, K. A., Reimann, K. A., et al. (1999). Sexual transmission and propagation of SIV and HIV in resting and activated CD4+ T cells. *Science* 286, 1353–1357. doi: 10.1126/science.286.5443.1353

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.

Received: 27 June 2014; accepted: 09 October 2014; published online: 04 November 2014.

*Citation: Rafferty H, Sibeko S and Rowland-Jones S (2014) How can we design better vaccines to prevent HIV infection in women? Front. Microbiol. **5**:572. doi: 10.3389/fmicb.2014.00572*

This article was submitted to Microbial Immunology, a section of the journal Frontiers in Microbiology.

Copyright © 2014 Rafferty, Sibeko and Rowland-Jones. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 08 May 2014
doi: 10.3389/fimmu.2014.00202

Induction of potent and long-lived antibody and cellular immune responses in the genitorectal mucosa could be the critical determinant of HIV vaccine efficacy

Nadia Chanzu*[1] *and Beatrice Ondondo*[2]

[1] *Institute of Tropical and Infectious Diseases, College of Health Sciences, University of Nairobi, Nairobi, Kenya*
[2] *The Jenner Institute, Nuffield Department of Medicine, University of Oxford, Oxford, UK*

Edited by:
Magdalena Plebanski, Monash University, Australia

Reviewed by:
Mirko Trilling, University Duisburg-Essen, Germany; Essen University Hospital, Germany
Emilio Luis Malchiodi, University of Buenos Aires, Argentina
John Stambas, Deakin University, Australia

***Correspondence:**
Beatrice Ondondo, The Jenner Institute, University of Oxford, ORCRB, Roosevelt Drive, Oxford OX3 7DQ, UK
e-mail: beatrice.ondondo@ndm.ox.ac.uk

The field of HIV prevention has indeed progressed in leaps and bounds, but with major limitations of the current prevention and treatment options, the world remains desperate for an HIV vaccine. Sadly, this continues to be elusive, because more than 30 years since its discovery there is no licensed HIV vaccine. Research aiming to define immunological biomarkers to accurately predict vaccine efficacy have focused mainly on systemic immune responses, and as such, studies defining correlates of protection in the genitorectal mucosa, the primary target site for HIV entry and seeding are sparse. Clearly, difficulties in sampling and analysis of mucosal specimens, as well as their limited size have been a major deterrent in characterizing the type (mucosal antibodies, cytokines, chemokines, or CTL), threshold (magnitude, depth, and breadth) and viral inhibitory capacity of HIV-1-specific immune responses in the genitorectal mucosa, where they are needed to immediately block HIV acquisition and arrest subsequent virus dissemination. Nevertheless, a few studies document the existence of HIV-specific immune responses in the genitorectal mucosa of HIV-infected aviremic and viremic controllers, as well as in highly exposed persistently seronegative (HEPS) individuals with natural resistance to HIV-1. Some of these responses strongly correlate with protection from HIV acquisition and/or disease progression, thus providing significant clues of the ideal components of an efficacious HIV vaccine. In this study, we provide an overview of the key features of protective immune responses found in HEPS, elite and viremic controllers, and discuss how these can be achieved through mucosal immunization. Inevitably, HIV vaccine development research will have to consider strategies that elicit potent antibody and cellular immune responses within the genitorectal mucosa or induction of systemic immune cells with an inherent potential to home and persist at mucosal sites of HIV entry.

Keywords: HIV-1, HIV vaccines, elite controllers, long-term non-progressors, highly exposed persistently seronegative, mucosal immunity

INTRODUCTION

The past three decades have witnessed the transformation of HIV/AIDS from a fatality to a chronic manageable disease but the situation remains far from ideal as the epidemic continues to spread. According to the UNAIDS report 2013, an estimated 35 million people are living with HIV, 2.3 million new infections, and 1.6 million AIDS-related deaths were documented at the end of 2012. The field of HIV prevention and treatment has progressed significantly over the years and has had a huge impact on the HIV pandemic. Anti-retroviral therapy (ART), more especially highly active ART (HAART) has been quite instrumental in the fight against HIV/AIDS and represents the most significant achievement that has transformed an epidemic that threatened to wipe out humanity (1). HAART has not only increased the lifespan of infected people (2), but has had other significant health benefits including reduced risk of opportunistic infections such as tuberculosis and AIDS-defining cancers, namely Kaposi sarcoma and cervical cancer (1, 3). Moving a notch higher, pre-exposure prophylaxis (PrEP) and post-exposure (PEP) trials demonstrate outstanding success as evidenced by significant reductions in the risk of HIV-1 infection via heterosexual and homosexual transmission, as well as injecting drug usage (4–8). In breakthrough infections, however, early treatment not only provides an immune advantage such as functional cure demonstrated in a few studies including the VISCONTI study, the Mississippi baby, and the recently reported Long Beach baby (9–12), but also dramatically reduces the risk of secondary transmission as demonstrated in studies with sero-discordant couples, where the infected partner initiates early treatment (13) and studies the prevention of mother-to-child transmission (MTCT) (14, 15).

The recent introduction of universal testing and treatment (UTT) followed by immediate initiation of ART to all those testing HIV positive irrespective of clinical stage or CD4 count (16–19) will have a huge impact on the epidemic, although the reality is that wider scale implementation will inevitably be logistically and financially overwhelming (20). Microbicides (8) and male

circumcision (21–23) have also contributed significantly to the reduced risk of infection and/or transmission, and have great potential if deployed on a larger scale. Despite the tremendous progress in the field, challenges remain and a vaccine is still of top most priority. An HIV vaccine would not only ease the cost of burden of therapy globally, but will also have a huge public health impact. Experts agree that a comprehensive package combining an effective, safe, well-tolerated, accessible, and affordable HIV vaccine with HAART, and the current HIV preventive technologies would ultimately bring the epidemic to an end. Thus, a combination of prophylactic and therapeutic vaccines that prevent infection and afford superior control of viremia in breakthrough infections will significantly reduce the spread of infection and disease (3). The successful HIV vaccine candidates are expected to induce potent broadly neutralizing antibodies (bNAbs) and high titers of non-neutralizing antibodies, in addition to robust cellular immune responses with virus inhibitory effector functions in order to offer long-lasting sterilizing immunity, or in the case of breakthrough infections, to at least increase the threshold of HIV titers required for infection, reduce virus load setting point, and reduce secondary transmission rates (24–28). Most importantly, since a significantly large proportion of HIV infections are transmitted sexually, both heterosexual (29, 30) and homosexual, especially in men who have sex with men (31), prophylactic vaccine candidates should be effective at all possible portals of HIV-1 entry, with key focus on the genital and rectal mucosa (32).

HIV-1 VACCINE CANDIDATES: PROGRESS SO FAR

Several factors that have rendered HIV vaccine design an unprecedented challenge have been extensively discussed in several independent reviews. These factors include the enormous HIV virion diversity (up to 35% in gp120) culminating in many HIV-1 subtypes/clades and circulating recombinant forms (33, 34); the well-documented immune evasion strategies (35) and immune escape (36); persistence of the virus in latent reservoirs that cannot be effectively cleared with HAART (37, 38); the immunoregulatory facet of HIV comprising memory CD4+ T-cell depletion from mucosal sites including the gut-associated lymphoid tissue (GALT) (39, 40) and MHC class 1 down-regulation by Nef (41) among others; and the overall lack of definitive correlates of immune protection coupled with imperfect correlations or discrepancies between systemic and mucosal immune responses (42–45). Despite these challenges, the field of HIV vaccine trials has evolved over the years, and currently, more than 200 vaccine trials (IAVI Clinical Trials Database 2014: http://iavireport.org/Trials-Database) have been conducted since the launch of the first ever HIV vaccine trial in the mid 1980s (46). Of these, a few vaccine candidates that showed modest immunogenicity in the initial stages of evaluation were implemented at Phase IIb and III to test their efficacy in HIV control (summarized in **Table 1**). These vaccines were designed to induce T cells alone (HVTN502 and HVTN503), T cells in combination with antibodies (HVTN505 and RV144), or antibodies alone (VAX003 and VAX004).

The major goal of T cell vaccines is to induce and maintain a high level of potent and fully functional effector T cells that will rapidly become home to mucosal sites, the first portal of entry, and abort early HIV infection (54). Evaluation of MRKAd5 vaccine (*gag/pol/nef*) in the STEP (HVTN502) and Phambili (HVTN503) Phase IIb trials demonstrated induction of relatively strong and durable T cell responses (47, 55); however, in both studies, the vaccine failed to prevent infection or control early viremia in breakthrough infections. Further analysis of breakthrough infections uncovered the existence of vaccine-induced selective pressure (56), suggesting a strong vaccine-induced immune response, and also highlighting the possibility that limited breadth of vaccine-stimulated responses might have impacted on the potential to contain virus replication during acute infection. A carefully designed follow-on Phase IIb clinical trial (HVTN505) that tested a multiclade immunogen expressing *gag/pol/nef/env* was also halted for futility (49). This vaccine induced both T cell and antibody responses (strong IgG binding antibodies to gp140), as well as some neutralizing activity, but clearly these did not correlate with protection, and were instead skewed toward increased risk of HIV acquisition. Although the failure of these vaccines to protect against infection and the unexpected association with increased risk of HIV acquisition are a huge setback in the development of T cell vaccines, there is still cause for optimism, as follow-up analysis of the HIV-infected STEP study participants revealed a correlation of vaccine-induced Gag-specific T cells with reduced plasma viremia, independent of HLA influence (57). Furthermore, we have recently demonstrated induction of broad and very high magnitude, polyfunctional CD8+ and CD4+ T cell responses in a Phase I clinical trial of a T cell vaccine candidate (HIVconsv), expressing *gag/pol/vif/env* sequences that were assembled from the most conserved regions of HIV-1 (58, 59). Of key importance is the observation that HIVconsv vaccine-induced CD8+ effector T cells could recognize HIV-infected autologous CD4+ T cells and achieved up to 5.79 $\log_{10}$ inhibition of virus replication, suggesting that such vaccine-induced cytotoxic T cells may have great potential to impact post-infection virus replication. Indeed, these findings were corroborated in a challenge study where rhesus macaques immunized with SIVconsv (an equivalent of HIVconsv) showed robust and polyfunctional T cell responses that protected them from the pathogenic SIVmac251 (60). Independently, a T cell-based vaccine expressing SIV Gag was shown to elicit high magnitude, broad, and polyfunctional cellular immune responses that were associated with reduced SIVmac251 virus load set point, as well as decreased AIDS mortality (61). However, the efficacy of HIVconsv in preventing HIV-1 acquisition or lowering virus set points remains to be tested in efficacy trials, and if achieved, will be a significant milestone for T cell vaccines.

As it is speculated that sterilizing immunity against HIV-1 will largely depend on induction of potent bNAbs (in combination with strong antiviral T cell responses), antibody-based vaccines remain attractive in HIV vaccine development strategies although their potential benefit in terms of preventing HIV acquisition or controlling replication in humans is yet to be sufficiently demonstrated (51–53). These Phase III clinical trials (VAX003 and VAX004) tested the monovalent subtype B and bivalent subtype B/E rgp120 vaccines and showed induction of complex and robust immune responses comprising binding and neutralizing antibody responses to gp120 (**Table 1**), but no reduction in the incidence of HIV-1 was observed among the vaccinees. Although the high-risk nature of VAX003 and VAX004 trial participants might have

Table 1 | Phase IIb and III HIV vaccine efficacy trials.

Vaccine trial	Vaccine candidate and immunogens	Specimens collected		Most significant immune response elicited	Reference
		Systemic	Mucosal		
HVTN 502/Merck 023 STEP Study (Phase IIb/prophylactic)	MRKAd5 HIV-1 *gag/pol/nef*	Serum, plasma, PBMCs	Not collected	T cell response	Buchbinder et al. (47)
HVTN 503 Phambili Study (Phase IIb/prophylactic)	MRKAd5 HIV-1 *gag/pol/nef*	Serum, plasma, PBMCs	Not collected	T cell response	Gray et al. (48)
HVTN 505 (Phase IIb/prophylactic)	VRC-HIVDNA016-00-VP/ VRC-HIVADV014-00-VP	Serum, plasma, PBMCs	Not collected	T cell and antibody responses (gp140 binding IgG)	Hammer et al. (49)
RV144 (Phase III/prophylactic)	ALVAC-HIV vCP1521/ AIDSVAX-gp120 B/E	Serum, plasma, PBMCs	Collected but inadequate	T cell and antibody responses (non-neutralizing antibodies to the V1/V2 loop)	Rerks-Ngarm et al. (50)
VAX 003 (Phase III/prophylactic)	AIDSVAX B/E (gp120)	Serum, plasma, PBMCs	Not collected	Antibody response (binding and neutralizing antibodies to gp120)	Pitisuttithum et al. (51)
VAX 004 (Phase III/prophylactic)	AIDSVAX B/B (gp120)	Serum, plasma, PBMCs	Not collected	Antibody response (binding and neutralizing antibodies to gp120)	Flynn et al. (52), Gilbert et al. (53)

PBMCs, peripheral blood mononuclear cells.

had an influence on vaccine efficacy, the failure of these trials still highlighted legitimate limitations of antibody-based vaccines, in terms of preventing HIV acquisition or post-infection virus replication. Nonetheless, studies in non-human primates (NHPs) have provided solid evidence that bNAbs can be very effective in the control and elimination of experimental SIV or SHIV infections (62–64). This has paved way for the identification and isolation of a number of potent and broadly neutralizing monoclonal antibodies (65–71), as discussed in later sections.

Although the focus is largely on bNAbs, non-neutralizing antibodies may potentially play a significant role in HIV-1 acquisition and progression by acting via Fc-receptor-mediated binding of infected cells to trigger recruitment of effector cells with cellular cytotoxic activities such as antibody-dependent cell-mediated cytotoxicity (ADCC) or secretion of antiviral cytokines that inhibit virus replication, i.e. antibody-dependent cellular virus inhibition (ADCVI) (72, 73). Vaccine challenge studies in NHPs revealed a correlation of such antibody-dependent cytotoxicity and viral inhibition with reduced viral loads (74–77), thus demonstrating their potential involvement in protection. Furthermore, high levels of ADCC-mediating antibodies correlate with HIV suppression in elite controllers (78). The protective effect of non-neutralizing antibodies was indeed confirmed in the Thai Phase III RV144 trial, where potent non-neutralizing antibodies to the V1/V2 loop correlated significantly with protection from HIV acquisition (50, 79, 80). Additionally, correlation of ADCC with reduced risk of infection was also confirmed (79). The RV144 study tested the safety and efficacy of a prime-boost regimen comprising an ALVAC-HIV (a canary pox vector expressing HIV-1 *env/gag/pro*) prime and AIDSVAX-gp120 B/E (recombinant gp120) boost in heterosexual individuals at various levels of risk of HIV infection. These vaccines were designed to induce both antibody and cell-mediated immune responses and to complement each other in order to maximize protection. Hence, with 74 HIV infections among the placebo recipients compared to only 51 in the vaccinees, RV144 achieved 31.2% efficacy and although there was no effect on viral load following infection, it so far remains the most encouraging vaccine study to date. Thus, although highly elusive, the unsatisfactory outcomes of three large clinical trials (47–49, 55, 81) mean that now more than ever, the world is more desperate for a safe and an effective vaccine to prevent HIV infection and/or control progression to AIDS.

NOVEL VACCINE DESIGN AND DELIVERY STRATEGIES TO IMPROVE IMMUNOGENICITY AND EFFICACY

As much as poor immunogenicity might be blamed for the apparent lack of efficacy of several HIV vaccine candidates tested to date, the observation that vaccine-induced immune responses consistently waned over time suggests that even with the most immunogenic of vaccines, protection may be limited to only those individuals who get exposed to HIV-1 within the first few months following immunization. Although this can be effectively overcome by multiple booster immunizations, the costs and compliance issues will certainly be prohibitive. Therefore, novel strategies that circumvent these pre-existing hurdles are urgently required in order to design vaccines with improved immunogenicity and long-term efficacy. For instance, it is anticipated that strategies targeting the B cell maturation pathway to induce preferential maturation of naïve B cell precursors of potent and bNAbs could achieve long-term protection against HIV acquisition and dissemination (82, 83). Additionally, adjuvants that activate enzymes regulating somatic mutation, such as activation-induced cytidine deaminase (AID), could be utilized to potentially boost the chances of producing bNAbs (84). Durability of antibody responses may

be enhanced by use of vectored immunoprophylaxis, an approach where immunoglobulin genes are inserted in a viral vector, which then provides life-long expression of high titers of the respective monoclonal bNAbs following a single intramuscular injection (85). The success of this strategy has been very recently demonstrated in a study using adeno-associated virus (AAV) encoding bNAbs against HIV, which was shown to induce prolonged antibody expression and long-lasting protection of humanized mice from high-dose intravenous and vaginal challenges with diverse HIV strains (85, 86).

Cytokines that can directly enhance B cell maturation into long-lived antibody-secreting cells (ASC), such as IL-4, IL-5, and IL-6, may be used as genetic adjuvants to increase NAb titers. The efficacy of this approach is documented by a Friend virus challenge study where co-delivery of adenovirus vectors encoding IL-5, IL-6, or IL-23 together with adenoviral vector expressing the Friend virus surface envelope protein gp70 (Ad.pIXgp70) significantly controlled virus replication and enhanced protection (87). In particular, mice co-immunized with IL-5 and IL-23-encoding adenoviruses produced higher titers of NAbs (87). Genetic adjuvants encoding type 1 interferons (IFNs) (88) and certain chemokines such as CCL3, CCL19, and CCL28 (89, 90) were also found to improve adenovirus vector-mediated immunity to Friend virus. Chemokines that attract specialized antigen-presenting cells can thus enhance vaccine uptake and increase the magnitude of the immune response. When adenovirus vector encoding CCL3 (a DC chemo-attractant) was co-delivered with adenovirus vectors expressing *gag/env* antigens of Friend virus, improved protection that correlated with enhanced virus-specific CD4+ T cells and higher NAb titers was observed (89). Similarly, CCL3 co-delivery of HIV antigens (*gag/pol/env*) induced higher titers of HIV-specific binding and neutralizing antibodies compared to delivery of antigens alone (89). Co-delivery of CCL19 and CCL28 significantly augmented mucosal and systemic antibody responses and also enhanced their neutralizing activity against homologous and heterologous HIV-1 strains (90). Thus, the adjuvant effect of these cytokines and chemokines could be synchronized with antigen delivery to enhance HIV vaccine efficacy.

Following the recent demonstration that contrary to the non-replicating adenovirus vector, a replicating cytomegalovirus-vectored vaccine offered superior protection of rhesus macaques from repeated mucosal challenges with the highly pathogenic SIVmac239 (91), the focus is shifting toward live vectors that induce stable effector memory rather than central memory CD8+ T cell responses (92). Seemingly, persistent but low-level replication of vaccine delivery vectors not only correlates with stimulation of potent effector memory T cells with enhanced antiviral capacity, but also provides stable immune-surveillance capable of clearing latent viral reserves (93). Such like vectors hold great promise for successful HIV vaccines, although they will need to be carefully selected to avoid induction of active disease in vaccine recipients, or possible antagonism of vaccine-elicited immune responses.

Immunological pressure exerted on HIV by vaccine-stimulated CD8+ T cells is thought to have caused early viral escape and contributed to the lack of protection among the STEP study vaccinees. Thus, vaccine approaches utilizing immunogens that are derived from conserved regions of HIV-1 (58, 59) or conserved consensus mosaics (94, 95) may possibly limit escape and offer better protection. Mosaic vaccines are designed to maximize coverage of global antigen epitopes and to therefore overcome HIV diversity by eliciting broad multi-clade immune responses (95, 96). Such increased diversity of immunogens (multiple epitopes and their variants) greatly increases the chances of matching vaccine-induced immune responses to the antigenic phenotype of the infecting founder virus or circulating HIV strains. Mosaic vaccines have shown great promise in NHP studies where comparatively superior breadth and depth of T cell responses was demonstrated (94, 97, 98). Furthermore, polyvalent mosaics were shown to induce both neutralizing antibodies and cellular responses (99), which effectively protected animals from high-dose challenge infections and were also effective at controlling breakthrough virus replication. Thus, to overcome the problem of enormous HIV diversity, while at the same time improving longevity of vaccine-stimulated immunity, a combination of replicating vectors (with an excellent safety profile) with both T cell- and antibody-inducing mosaic vaccines might be a much better strategy.

HIV infection activates immune regulatory pathways by increasing the frequency of regulatory T cells (Tregs) and generation of functionally impaired, exhausted CD8+ and CD4+ T cells which are inadequate to control virus replication and prevent disease progression (100). Thus, strategies that could enhance immunogenicity and overall vaccine efficacy, with particular emphasis on the control of breakthrough infections, may include concurrent depletion of Tregs and blockade of inhibitory pathways such as the programed death-1/ligand (PD-1/PD-L1) and the T cell immunoglobulin and mucin protein 3 (Tim-3) pathways during immunization. These strategies function to overcome negative regulation and restore immune function in exhausted T cells, and may be particularly attractive in therapeutic vaccines for HIV-1 where they could reduce virus loads and help to maintain an aviremic state (101, 102).

CORRELATES OF PROTECTION AGAINST HIV/AIDS

Despite intensive research, immunological biomarkers that could accurately predict reduced HIV-1 acquisition and transmission are not yet defined, thus making it a huge challenge to gage the potential efficacy of HIV vaccine candidates prior to the costly large-scale efficacy trials. This is further complicated by the fact that there is not a single documented case of immune-mediated HIV-1 clearance in infected individuals. Although the recent RV144 trial has provided significant clues of possible correlates of risk versus protection (79), the limited number of infected vaccinees studied makes it hard to draw definitive conclusions. Thus, attempts to define immune correlates of protection are still based on data largely arising from studies of long-term non-progressors (LTNPs) and HIV controllers, a rare group of individuals comprising both elite and viremic controllers with spontaneous HIV-1 control in the absence of treatment (103) and also from individuals who are highly exposed to the virus but remain persistently uninfected highly exposed persistently seronegative (HEPS) such as commercial sex workers, the uninfected partners of sero-discordant couples, or exposed healthcare providers. These studies have unraveled a number of protective factors which can be broadly divided

into two categories, namely host/viral-related or immune-related factors, and are discussed in detail below.

HOST GENETIC FACTORS THAT CORRELATE WITH PROTECTION

Data from large numbers of non-progressor/slow progressor cohorts indicate that protective HLA alleles such as HLA-B27, HLA-B51, HLA-B57 and HLA-B58 which are associated with better virus control are overrepresented in these groups, thus implicating a very strong role for host genetics in the course of HIV infection and disease (104–106). Incidentally, genome-wide association and HLA class 1 analysis of HIV-specific T cell responses in the MRKAd5 vaccinees revealed that high-magnitude responses were associated with HLA-B27, -B51 and -B57, while HLA-B08 and -B45 were associated with lower level responses (107). Other host genetic factors such as the CCR5Δ32 and CCR2-64I mutations in chemokine receptor genes (108–110) as well as the killer-cell immunoglobulin-like (KIR) receptor polymorphisms (111–115) have also been associated with slow progression or resistance to infection (116). In fact individuals who are homozygous for the 32 base pair deletion in CCR5 are completely protected from HIV infection, and the protective function of this mutation has been recently demonstrated in the only patient to achieve sterilizing cure of HIV-1 following stem cell transplant from a CCR5Δ32 homozygous donor (117–120). Additionally, there are intrinsic host-resistant factors such as the restriction factors TRIM-5α, APOBEC3G, SAMHD1, and tetherin (121–123), which control HIV replication through various mechanisms, and any genetic alterations in their expression levels or patterns can alter the rate of HIV acquisition and progression (124, 125). However, it is imperative to note that the presence of these protective factors, chemokine receptor mutations, or HLA haplotypes *per se*, is not sufficient to confer a slow progression phenotype, as several studies indicate rapid progression of some infected individuals bearing these protective HLA and KIR genotypes. Additionally, it is known that some individuals without these protective genetic characteristics control HIV replication or persistently evade infection, thus strongly implicating alternative explanations for the attenuated disease course, such as immune-mediated protection.

IMMUNE-MEDIATED CORRELATES OF PROTECTION

Studies of HIV-infected controllers indicate that robust, broadly directed, highly proliferative, polyfunctional cytotoxic CD8+ and CD4+ T cell responses target conserved regions of HIV-1 such as Gag correlate with reduced virus replication (126–134). In particular, CD8+ T cells from HIV-1 controllers display enhanced capacity to inhibit HIV-1 replication (129, 135–138) either via direct killing of infected cells or by secretion of antiviral factors known to suppress HIV replication (139). These cells also produce higher levels of IL-2 (140), have superior capacity for clonal expansion, and contain more granzyme B and perforin (138, 141). In some cases, these cells can exhibit the exceptional capacity to supress viral replication *in vitro* without prior antigen re-stimulation (127, 129). However, it is worth noting that although control of HIV-1 in various independent (but small) slow progressor cohorts correlates significantly with enhanced magnitude and breadth of T cell responses, a study looking at a larger sample size ($n = 124$) of well-defined elite controllers showed that elite control of HIV-1 was in fact associated with the lowest magnitude and breadth of IFN-γ responses, as well as the lowest titers of broadly cross-reactive neutralizing antibodies (142). This confirms that the quality of the response rather than the quantity remains important in virus control. Thus, in agreement with other studies, preferential targeting of Gag and co-secretion of both IFN-γ and IL-2 were correlated with virus control among the elite controllers than the chronic progressors and viremic controllers in this study (142).

In addition to controlling virus replication, it has recently been reported that HIV-specific CD8+ CTLs are an absolute requirement for the elimination of latent viral reservoirs following reactivation (143). Since elite controllers harbor significantly lower latent viral reservoirs (144–146), this suggests a strong link between the presence of HIV-specific CD8+ T cells with potent cytotoxic activity and controlled latency. Indeed, this was confirmed in a very recent study which demonstrated the exceptional ability of primary CD8+ T cells from elite suppressors to effectively eliminate precursors of latently infected cells (147). Moreover, elite controllers are known to develop potent effector memory (T_{EM}) rather than central memory (T_{CM}) T cells (148) which are not only more effective at suppressing viral control but are also known to resist apoptosis, thus capable of protecting against disease progression and establishment of latent reservoirs for extended periods in the absence of HAART (103, 149–151). Besides T cells, natural killer (NK) cells, known to inhibit HIV replication through a variety of mechanisms (152), are also numerically elevated in blood of HEPS, where they are thought to protect from HIV-1 acquisition by secretion of antiviral factors (153–155). Indeed, increased production of pro-inflammatory cytokines such as TNF-α and IFN-γ by KIR3DL1/HLA-Bw4 NK cells is associated with lower viral loads and slower progression of infected individuals (113, 156, 157). Moreover, preserved NK cell function (which is lost in progressive HIV-1 infection) has been linked with asymptomatic HIV-2 infection (158). Also, increased levels of the beta-chemokines RANTES, MIP-1α, and MIP-1β, all of which are known to bind the HIV-1 co-receptor, CCR5, is linked with protection from HIV acquisition in cohorts of high-risk women (159, 160). These chemokines have been directly associated with resistance to HIV-1 infection, reduced viral replication, and subsequently delayed disease progression (161–163).

The studies described in this section mainly relate to systemic immune responses. However, it is vital that immune-mediated correlates of protection in the genitorectal mucosa are characterized since they are indispensable in defining the overall efficacy of HIV vaccines. The next section briefly highlights the immune responses found in the genitorectal mucosa that have been correlated with protection.

IMMUNE-MEDIATED CORRELATES OF PROTECTION IN THE GENITORECTAL MUCOSA

The observation that multiple exposures to high virus load is required for successful heterosexual HIV-1 transmission suggests the existence of robust mucosal innate immunity that must be actively involved in the control of HIV-1 infection and virus dissemination. Thus, immune responses at the genital mucosa especially those of the innate immune system provide the first line of defense and are critical in preventing HIV-1 infection (164)

or destruction of HIV-1 target cells at the mucosa, before the development of adaptive immune responses. Plausibly, vaccine-mediated stimulation of components of the innate immune system such as dendritic cells, NK, and NKT cells as well as macrophages in the genitorectal mucosa could have a significant reduction in HIV-1 acquisition by production of antiviral cytokines such as type 1 IFNs or even by acting through ADCC to destroy infected cells. Alternatively, the type 1 IFNs together with other cytokines including IL-15 and IL-18 can serve to augment both innate and adaptive immune responses. However, this review will only discuss the adaptive humoral and cell-mediated immune responses to HIV-1 in the genitorectal mucosa as they form a significant part of the critical determinants of vaccine-induced immunity and might therefore hold the key to accelerating HIV-1 vaccine development. Several HIV-1 vaccine candidates tested for immunogenicity and efficacy up to advanced stages (**Table 1**) did not yield any mucosal data (165), especially the RV144 trial, the only one to show vaccine efficacy. As such, the definitive correlates of vaccine efficacy in the genital mucosa still remain unknown. This section discusses the various mucosal immune responses which have been linked with natural resistance to HIV-1 in HEPS or attenuated disease course in HIV controllers.

PROTECTIVE CELLULAR IMMUNE RESPONSES IN THE GENITORECTAL MUCOSA

A large body of evidence documents the existence of HIV-specific cellular immune responses in blood of HEPS (166–170), although there have been lots of skepticism concerning the occurrence of adaptive immune responses without active HIV infection or replication. It has been speculated that these responses arise from abortive (failed) infections that are effectively cleared before the virus successfully establishes its reservoirs. A study designed specifically to address the question as to whether HEPS truly make HIV-specific T cell responses recruited sero-discordant couples in Malawi (HPTN 052 cohort) and in the UK (CHAVI 002, St. Mary's cohort) and used a very sensitive (cultured Elispot) assay able to detect very low frequency T cell responses (171). This study confirmed the existence of HIV-specific CD8+ and CD4+ T cell responses that were mapped to T cell epitopes frequently targeted in HIV-infected individuals. Interestingly, the responses were maintained across multiple visits in the absence of HIV infection, as no virus could be detected even with the highly sensitive transcription-mediated amplification assay (TMA). A recent study of sero-discordant couples in Uganda reported similar findings (172), thus confirming that exposure to HIV can prime adaptive immune responses that can be boosted by repeated exposures.

There is also strong evidence that mucosal HIV-specific CD8+ and CD4+ T cells modulate HIV-1 disease course, as they can control post-infection virus replication and persistence by directly killing infected target cells or secreting a number of antiviral cytokines. IFN-γ-producing HIV-specific CD8+ T cells found in the genital mucosa of HEPS were thought to be responsible for their natural HIV-1 resistance (173). This study also revealed an enrichment of IFN-γ-producing HIV-specific CD8+ T cells in the cervical mucosa as opposed to the systemic compartment, thus strongly suggesting a role in protection against HIV-1 acquisition. However, although multiple exposures to replication-competent HIV-1 is well-documented in these individuals, in the absence of confirmed productive HIV-1 infection, it is difficult to judge whether the existence of HIV-specific T cells is indeed cause of HIV-1 resistance or merely a marker of exposure. Nevertheless, human studies assessing immune responses in mucosal compartments of HIV-1 infected individuals revealed an inverse correlation of magnitude and poly-functionality of rectal HIV-1 Gag-specific CD8+ and CD4+ T cell responses and viral load (45, 174, 175), highly suggesting that mucosal T cells do exert anti-HIV activity in the rectal mucosa. Elite control of HIV was significantly associated with strong and polyfunctional T cells, secreting CD107a and MIP-1-β among other cytokines. Intriguingly, the mucosal immune responses in HIV-1 controllers were significantly higher and more polyfunctional than those in progressors, while the systemic immune responses remained indistinguishable (45). These observations highlight the potential discrepancies between blood and mucosal immune responses and further demonstrate the importance of generating protective immune responses within the local sites of virus entry.

PROTECTIVE HUMORAL IMMUNE RESPONSES IN THE GENITORECTAL MUCOSA

HIV-specific mucosal IgG and IgA antibodies, especially those with demonstrated HIV-1-neutralizing activity form the pillar that protects against HIV-1 acquisition, at least in cohorts of highly exposed seronegative individuals (176–180). These antibodies can act by inhibiting various mucosal HIV-1 entry pathways such as epithelial transcytosis (181, 182) and by binding to the HIV-1 virus or by neutralizing the virus to prevent infection of CD4+ T cells by primary HIV-1 isolates (183). Alternatively, they could also serve to aggregate HIV virions and inhibit movement through cervical mucus. Contrary to individuals with progressive HIV-1 infection, the IgA antibodies found in HEPS mainly recognize the conformationally conserved regions of the gp41 subunit of HIV-1 envelope, thus indicating potential for cross-clade neutralization (178, 184, 185). HIV-specific IgA with potential neutralizing activity (179, 186) has been detected in the cervico-vaginal secretions of women (176, 187, 188) and urethral swabs of heterosexual men (189) with natural HIV-1 resistance, indicating a possible role in blocking HIV-1 acquisition. In a recent study, HIV-1-neutralizing IgA was found in the cervico-vaginal secretions of highly exposed seronegative women in a prospective discordant couple cohort study (190), thus providing definitive evidence of antiviral activity in mucosal secretions after HIV-1 exposure. One major shortcoming is that maintenance of adequate levels of these antibodies requires continuous exposure to HIV-1 (186), suggesting perhaps a lack of effective formation of memory B cells. Despite the encouraging neutralization activity of HIV-1-specific IgA antibodies and the fact that secretory IgAs are a major component of the mucosal antibody responses, not many IgA monoclonal antibodies have been isolated. However, one study indicates that human monoclonal Fab IgAs directed at gp41, which were constructed by genetic engineering of mucosal cervical B lymphocytes from HEPS, exhibited good neutralization potential and were more potent at blocking transcytosis (183). The protective effect of such conserved and naturally induced antibodies implicates them as desirable components of mucosal HIV-1 vaccines, where they can potentially

abort HIV-1 infection by targeting cell-free virus to prevent entry into mucosal tissues. This concept was demonstrated in an animal study where a gp41 subunit HIV-1 vaccine-induced vaginal IgAs capable of blocking transcytosis, as well as vaginal IgGs with neutralizing or ADCC activities (43). These antibodies provided sterilizing immunity in *Macaca mulatta* monkeys challenged with SHIV-SF162P3, in the absence of systemic neutralizing antibodies, again reinforcing the importance of generating immune responses in the genitorectal mucosa.

The studies described here convincingly demonstrate that these immuno-functional parameters are predictive of slow progression in a majority of HIV controllers or sterile protection in those individuals who resist HIV-1 infection despite multiple exposures to high doses of intact replication-competent HIV-1 viruses (171). Therefore, it is plausible that vaccine strategies that can induce such immune responses would have a significantly greater chance of either preventing infection or achieving functional cure in breakthrough infections. This was to some extent demonstrated in the STEP trial where vaccine-induced T cells correlated with virus control in a few of the infected vaccinees (57). Thus, collectively, these observations indicate that a myriad of factors encompassing host genetics, immunological parameters, and viral determinants act together to bring about the attenuated disease course (103, 191) or resistance to HIV. However, although it is conceivable that vaccines can be engineered to mimic protective immune responses, little can be done to influence the host genetic factors. Therefore, vaccine development efforts need to focus on the induction of mucosal (and systemic) immune responses which confer protection independent of host genetic factors.

CORRELATES OF IMMUNE PROTECTION IN THE CONTEXT OF VACCINE EFFICACY: ARE THEY RELIABLE?

To put all these into the context of vaccine efficacy, it is worth a reminder that despite the documentation of immune responses that seemingly correlate with virus control in infected or highly exposed uninfected individuals, the ultimate proof of correlates of protection can only come from efficacy trials in humans. Can we say that so far the predicted correlates of protection have translated into vaccine efficacy? As an example, protection in the RV144 trial was not related to bNAbs or strong CD8+ CTL responses as both of these were not detected. Instead only CD4+ T cells, ADCC and neutralizing antibodies to the easy to neutralize tier-1 viruses were observed. Furthermore, the RV144 trial revealed that although high levels of plasma IgG antibodies targeted to the variable regions V1/V2 loop of the envelope gp120 were associated with protection against HIV-1 acquisition, envelope-specific IgA antibodies actually mitigated the effects of protective antibodies and were associated with increased risk of infection (79).

Incidentally, high levels of such IgA-binding antibodies to gp140 were also detected in the HVTN505 study vaccinees, where they are speculated to have influenced the risk of HIV acquisition (49). These observations immediately prompt the need to further investigate the role of IgA in HIV-1 acquisition since it has been significantly associated with mucosal immunity to HIV-1 in several studies of HEPS (188, 190, 192). Moreover, these observations indicate that protection from infection is not necessarily mediated by neutralizing antibodies or robust CTLs as inferred from studies in HEPS, elite controllers, and LTNPs, thus emphasizing the need to re-define correlates of protection, and perhaps keenly study the role of non-neutralizing antibodies in protection versus risk. It is possible that vaccines inducing high levels of IgG and lower levels of IgA, concurrently with broadly directed high-magnitude cellular immune responses at mucosal sites will more likely protect against infection and post-infection virus replication. It remains possible that the efficacy of RV144 may have been due in part to the presence of vaccine-induced antibody and cellular immunity in the genital mucosa.

More intriguing, however, is the fact that all the HIV-1 vaccine candidates tested for efficacy showed modest immune responses in preclinical and early stages of clinical evaluation but have shown no efficacy in the longer term. Take for instance, the MRKAd5 vaccine that induced robust responses in majority of vaccinees but failed to prevent HIV infection or post-infection viremia (47, 55). Although this vaccine was not expected to prevent infection, the fact that it did not attenuate disease course despite the observed immune responses begs the question as to whether the current measurements of evaluating vaccine immunogenicity during the preclinical and Phase I clinical trials (193) are robust enough to predict vaccine failure. Apart from the inevitable discrepancies between animal models and humans, this might also perhaps suggest qualitative and quantitative differences between the vaccine-stimulated responses and those required to prevent HIV-1 acquisition at the first point of encounter (genitorectal mucosa). Indeed, in addition to paucity of CD4+ T cell responses, the CD8+ T cell responses in the MRKAd5 vaccinees were found to be of low magnitude, narrowly directed, less polyfunctional, and targeted mostly Pol and Nef (55), contrary to what has been correlated with spontaneous HIV-1 control. Furthermore, as most vaccine-stimulated responses are tested by IFN-γ ELISPOT in peripheral blood samples, it could be though that potent responses are elicited in the systemic compartment, their migration to mucosal portals of entry where they are critical may be a limitation. Indeed, some studies have revealed a lack of correlation in the magnitude, quality, breadth, and functional capacity of T cell responses between blood (systemic) and mucosal samples (43–45). Thus, in addition to induction of robust systemic antibody and cell-mediated immunity, vaccine strategies that focus on generating local immune responses within the genitorectal mucosa, such as the "prime-pull" (194) approach may be more successful at reducing the replication and dissemination of transmitted founder viruses. Moreover, it remains possible that other parameters of the mucosal immune response such as proliferation, *in vitro* viral inhibition, and ADCC could be important (74, 111, 195, 196). Thus, accurate correlates of protection should include active sampling of mucosal compartments and measure several immunological and phenotypic parameters, including expression of mucosal homing receptors and ligands.

Recent studies document the antiviral potential of vaccine-stimulated T cells against various HIV-1 isolates (58) or SIV (SIVmac239 and SIVsmE660) (197, 198) using *in vitro* virus suppression assays (VIA). The general expectation is that VIA (199, 200), which demonstrate active inhibition of the replication of intact virus *in vitro* (or *ex vivo*) would correlate with *in vivo* inhibition of HIV replication (198, 201), and would therefore be a

more accurate prediction of vaccine efficacy. In fact the *in vitro* antiviral inhibitory capacity of CD8+ T cells measured by VIA was shown to accurately predict CD4+ T cell loss during early HIV-1 infection (201). However, the *in vitro* VIA did not predict vaccine efficacy or *in vivo* inhibition of virus replication in a challenge SIV study in NHPs (197), suggesting that more robust markers to predict vaccine efficacy are needed. Alternatively, this discrepancy could be attributed to lower frequencies of vaccine-stimulated T cells within the genital mucosa, and it might be that VIA performed with T cells isolated from the genital mucosa will give a better correlation with *in vivo* HIV or SIV control. Thus, studies that assess mucosal B or T cell antiviral capacity, whether vaccine stimulated (human and NHP) or HIV induced, for example in LTNPs, elite controllers and HEPS will be important in informing research aiming to identify correlates of vaccine efficacy.

On a completely separate platform, increased susceptibility to HIV-1 acquisition in vaccinees in the STEP and Phambili studies might to a certain extent reflect the dark side of vaccine-mediated immune activation that may create readily available HIV-1 targets (55, 202, 203). This hypothesis was proven in a challenge study where immunized rhesus monkeys exhibiting higher frequencies of IFN-γ secreting T cells were more susceptible to SIV infection (203). This strongly suggests that induction of sub-optimal vaccine-specific T cells (without robust antiviral effector functions) could increase the risk of HIV-1 acquisition in vaccinated individuals. Other risk factors such as herpes simplex virus type 2 (HSV-2) infection may significantly alter the immune milieu in the genitorectal mucosa (204–206) and affect HIV-1 vaccine efficacy. Indeed, HSV2 infection correlated with a fivefold increased risk of HIV-1 acquisition in heterosexual men in the HVTN505/Phambili study (48) and in homosexual men in the STEP study (47). HSV2 infection subverts cellular immune responses directed to HIV-1 (205), disrupts mucosal integrity, and induces massive recruitment of HIV-1 targets (CCR5+CD4+ T cells and immature DCs expressing DC-SIGN) to the genitorectal mucosa (207). These observations highlight the additional challenge to clearly define benchmark features that distinguish protective versus detrimental vaccine-induced immune cells that accelerate HIV-1 acquisition and disease progression. This is more especially due to the seemingly unavoidable paradox, where imprinting a mucosal homing phenotype on vaccine-induced immune cells is critical to prevent HIV-1 acquisition, but also poses a significant risk of increased susceptibility.

BROADLY NEUTRALIZING ANTIBODIES ARE CRITICAL DETERMINANTS FOR STERILIZING IMMUNITY

The occurrence of systemic and mucosal HIV-specific neutralizing IgG antibodies is also documented in LTNPs (135, 208–210), where they were initially linked with slow disease progression. However, recent studies indicate that their role in controlling established infection is quite limited and that high titers and breadth are in fact a result of higher virus loads (211). Indeed, such bNAbs are frequently detected in a very small proportion of HIV-infected individuals known as elite neutralizers (212), but they do not prevent disease progression (213), presumably due to rapid virus escape. Thus, a major limitation of antibodies is the high escape rate, meaning that even with the most excellent bNAbs, protection may only be transient as mutational escape occurs within a relatively short time. Moreover, several studies indicate that bNAbs are found at much lower levels among the Elite and viremic controllers (142, 214, 215), and at comparatively higher levels in chronic progressors (142), not only suggesting a lack of influence on virus replication and disease progression (211, 213), but most importantly also reinforcing the fact that neutralization breadth increases with prolonged exposure to HIV. These observations, together with the fact that bNAbs take several years to develop, incited skepticism and questioning of the potential relevance of bNAbs in preventing HIV acquisition and disease progression. Nonetheless, the possibility that bNAbs could achieve virus neutralization and block virus entry if present within the genital mucosa before infection, either by passive administration or if induced by immunization has been a cause worth fighting for.

A large panel of bNAbs (both first and second generation) have thus been extensively characterized (71), and some such as PG9 and PG16 which are found within the conserved domain of the V1/V2 loop were shown to induce potent neutralizing activity on 70–80% of circulating HIV-1 isolates (66, 216). The CD4 binding site monoclonal antibody VRC01, in particular, displays very broad neutralizing activity (>90%) against primary isolates of envelope pseudoviruses (70). These monoclonal bNAbs have been quite successful in animal studies, for instance, vaginal administration of B12 or intravenous delivery of 2F5 and 4E10 mAbs protected macaques from intravaginal or intrarectal SHIV challenges (217, 218), raising the possibility that bNAbs can attenuate HIV-1 acquisition and disease progression. Indeed, this has been demonstrated in recent passive transfer studies in NHPs showing that if present at sufficient levels and well before virus challenge, then bNAbs can in fact abort infection to achieve sterilizing immunity (77, 218–221). The main caveats making it difficult to extrapolate the relevance of NHP studies to humans are (1) unlike natural HIV-1 infection, where individuals are exposed to diverse HIV-1 strains with differing neutralization sensitivities, NHP studies often use a homogeneous challenge virus that is usually highly susceptible to neutralization. (2) Some NHP studies have used high doses of antibodies that are unlikely to be attained by immunization. (3) Some NHP studies use high doses of the challenge viruses, which can significantly mask the vaccine-mediated protection. Nonetheless, several monoclonal bNAbs with potent neutralizing activity such as the PGT, PG, and VRC series, among others (66, 70, 216) afforded sterile protection at very low serum concentrations, even with high-dose challenges (85, 219, 220, 222, 223). Furthermore, bispecific bNAbs combining the inhibitory activity of an antibody directed to domain 2 of human CD4 (ibalizumab) with either PG9 (PG9-iMab) or PG16 (PG16-iMab) exhibited high potency and neutralized 118 viruses at very low (picomolar) concentrations (224). Hopefully, such remarkable neutralization breadth and potency can be achieved in a clinical setting, via immunization or passive administration.

These observations are very encouraging as they identify the particular antibody target epitopes as excellent templates for vaccine design, and also shed some light on the threshold level of antibodies required to achieve sterilizing immunity in humans. This also raises hopes that antibody vaccines based on such potent and bNAbs would achieve significant control of virus replication

in infected individuals and possibly offer sterilizing immunity in healthy vaccinated subjects. However, no vaccine has induced bNAbs so far, and the only evidence that bNAbs could offer some protection in humans comes from earlier clinical studies where passive administration of the bNAbs, 2G12, 2F5, and 4E10 was shown to transiently delay viral rebound after HAART cessation (225, 226), but the protection was very limited. In a recent Phase I clinical trial of the modified trimeric V2-deleted envelope vaccine, potent neutralizing antibodies were induced in human volunteers, but these were of very limited breadth (227), despite enhanced neutralization breadth in animal studies (228, 229). Thus, the greatest challenge for antibody vaccines is to induce bNAbs that are potent enough to recapitulate the neutralization spectra observed in elite neutralizers and to neutralize many virus isolates including the most resistant, heterologous tier-2 and tier-3 viruses. The remarkable efficacy of monoclonal bNAbs in the vast majority of animal studies discussed here may partly be due to intravenous delivery, which ensures broad anatomic dissemination including the genitorectal mucosa. However, it is still uncertain whether bNAbs induced by parenteral immunization will traverse to the genitorectal mucosa, thus strongly arguing for immunization strategies to induce potent bNAbs localized within or in close proximity to the genitorectal mucosa.

EXPERIMENTAL HIV-1 VACCINES TARGETING MUCOSAL SITES

HIV-1 mucosal vaccinology is still in its infancy and remains a challenge despite the intense interest within the HIV/AIDS field. A mucosal vaccine that interferes with HIV-1 attachment and blocks subsequent steps including crossing of the epithelial barriers within mucosal surfaces to infect target cells, while at the same time inducing potent systemic antibody and cellular immunity would have a greater potential for enhanced efficacy. Although several HIV-1 vaccine candidates administered by intramuscular injection stimulate robust systemic immune responses in peripheral blood, whether or not these vaccine-elicited T cells or antibody-secreting plasma cells can migrate to the genitorectal mucosa is not well documented. This is a fundamental requirement for a successful HIV-1 vaccine, therefore immunization modalities that either generate immune responses *in situ*, i.e., within the genital mucosa, or strategies that drive recruitment of systemically induced vaccine-specific immune cells to the genital areas are highly desirable. The quality of mucosally induced vaccine-specific immune responses and the degree to which they can disseminate to other anatomic compartments largely depends on the route of vaccine administration (230–233), besides the properties of the immunogen. Thus, it is important that vaccine delivery routes are carefully selected or optimized in order to maximize immune control in multiple sites. Perhaps the most important delivery routes are those that demonstrate potential to stimulate both antibody and cellular responses in the genitorectal mucosa, as well as other mucosal sites and within various systemic compartments. Some of these characteristics have been traditionally attributed to mucosal immunization including intranasal, intravaginal, intrarectal, and oral or sublingual delivery routes (230, 234, 235). Possibly, delivery of a vaccine at one mucosal site may induce immunity at peripheral mucosal sites via the common mucosal immune system (236, 237), although this hypothesis is disputed by studies showing compartmentalization of the mucosal immune network (238).

Despite the near consensus for mucosal delivery of immunogens being the best way to trigger mucosal immune responses (230, 239), there are still controversies as to whether mucosal antigen delivery alone can effectively induce systemic immune responses. This is largely due to the tissue-specific imprinting of chemokine receptors and adhesion molecules on immune cells following activation within mucosal inductive sites. Perhaps, the sublingual vaccination route which has been shown to induce broadly disseminated mucosal and systemic immune responses (230) may be considered as a more suitable alternative for delivery of HIV vaccines. Intrarectal administration of a DNA/MVA vaccine encoding HIV immunogens was also shown to elicit both systemic and mucosal virus-specific immune responses that were associated with delayed progression to AIDS following SHIV89.6P challenge (240). Another possible alternative is to combine both intramuscular and intranasal delivery, a strategy that has been quite successful in enhancing vaccine-induced HIV-specific immune responses in both the systemic and vaginal compartments in NHP studies (241–243). These observations demonstrate that mucosally delivered vaccines undoubtedly elicit local immune responses that are capable of disseminating to other systemic compartments. In the following sections, we highlight some of the studies which have successfully employed mucosal vaccine delivery with or without mucosal adjuvants to elicit potent immune responses in the genitorectal mucosa.

MUCOSAL IMMUNIZATION WITHOUT ADJUVANTATION

Active mucosal immunization has been shown to induce potent cell-mediated and antibody responses at the genital mucosa in animal studies (244–246). In particular, intranasal vaccine delivery induces robust antibody and T cell immune responses in the genital mucosa, possibly due to targeting of dendritic cells in multiple organs such as the respiratory system, the gut mucosa, and the spleen (247, 248). Intranasal delivery of a number of HIV vaccine approaches such as DNA, peptide, live bacterial, and viral vectors induced strong CD8+ T cell responses and/or antibody responses (comprising IgG and IgA and sometimes neutralizing antibodies in vaginal washes) in mice and macaques (233, 249–251). Very recently, studies in NHPs have demonstrated that intranasal and oral vaccine administration routes were consistently and significantly better than intramuscular administration, and elicited mucosal and systemic immune responses that protected rhesus macaques from disease progression following intrarectal or vaginal challenges (252–256). These mucosal immunization routes induced high-magnitude polyfunctional CD8+ and CD4+ T cells in the rectum and vagina, which correlated with the extent of viral control.

Although mucosal (intranasal or intrarectal) delivery of DNA vaccines enhances vaccine-specific mucosal responses, it has been suggested that the quality, longevity and peripheral distribution of memory T cell responses in the genital mucosa could be improved by systemic (or intravaginal) administration of live vaccines (231, 257, 258). Live recombinant vaccine delivery vectors introduced via intramuscular, intrarectal, oral or intravaginal routes in a prime-boost strategy induced robust HIV-specific T cell responses

in the vaginal mucosa as well as in the spleen, possibly due to active systemic infection that stimulates potent immune responses (257). In this study, intravaginal delivery of attenuated recombinant Listeria monocytogenes expressing Gag (rLm-gag) as a prime in combination with replication-defective adenovirus serotype 5 (Ad5) expressing Gag (rAd5-gag) as a boost induced robust Gag-specific CTL responses in the vaginal mucosa and these persisted for at least 5 months. The persisting CTLs were of effector memory phenotype and possessed strong cytotoxic activity which protected against a vaccinia-Gag challenge.

Influenza virus targets the respiratory system and is thus well-adapted for stimulating mucosal immunity. Therefore, recombinant influenza virus vectors expressing foreign genes efficiently stimulate potent long-lasting antibody and T cell immune responses in mucosal and systemic compartments (259–261). Chimeric influenza virus expressing HIV-1 gp120 V3 loop peptide (IHIGPGRAFTYTT) induced robust NAb and CTL responses following mucosal immunization in mice (262). In a separate study, intranasal delivery of recombinant influenza expressing the gp41 ELDKWA epitope also stimulated persistent NAb and IgA responses in nasal, vaginal, and intestinal secretions (263–265). Moreover, H1N1 and H3N2 influenza viruses expressing SIV CD8+ T cell epitopes induced T cells with the mucosal homing ($\alpha_4\beta_7$) integrin following intranasal or intratracheal vaccine delivery in pigtail macaques (266). Of particular relevance to induction of long-lived vaccine-specific immunity by repeated immunization, influenza virus vectors when combined with other vectors in mucosal (intraperitoneal and intranasal) prime-boost immunization protocols, have proven effective at priming HIV-specific mucosal immune responses that could be augmented by recombinant MVA in BALB/c mice (267). This demonstrates the potential utility of influenza virus vectors in effective prime-boost immunization regimens to generate mucosal immune responses in the genitorectal draining lymph nodes to combat HIV infection. The possible limitation of influenza virus vectors is the insert capacity which may limit the size of antigens that could be delivered.

Several studies indicate that poxvirus vectors can also induce mucosal immune responses to foreign antigens. In particular, some studies have reported induction of immune responses in the genitorectal mucosa [as well as in the Peyer's patches (PP) and lamina propria], that controlled SHIV replication in mice and NHPs following mucosal immunization with recombinant vaccinia virus (268–270). Mucosal (intranasal and intrarectal) delivery of non-replicating rMVA vaccines in a DNA-prime and MVA-boost strategy also induced robust antibody and cellular immune responses in the systemic compartment as well as in the genitorectal mucosa, which controlled SHIV replication and disease progression (240, 271). Mucosal vaccination with other poxvirus vectors including NYVAC and ALVAC also induced antigen-specific responses in mucosal compartments (272).

A number of studies also demonstrate induction of long-lived mucosal immunity following systemic immunization with live virus vectors such as Ad5 and NYVAC, owing to acquisition of mucosal homing properties by vaccine-induced CD8+ and CD4+ T cells (272–275). In some cases, the immune responses elicited following intramuscular delivery were superior or equivalent to those elicited by mucosal immunization. Intramuscular delivery of the SIV antigens; gag/pol or gag/pol/env by Ad35-prime followed with Ad26-boost in rhesus macaques induced potent NAb and cellular immune responses in the periphery and within the colorectal mucosa (276). Both peripheral and mucosal immune responses, especially Env-specific IgG correlated with reduced risk of SIV_{MAC} acquisition during intrarectal challenges. This is indeed very encouraging and may obviate the need for the more invasive genital mucosal immunization methods (intravaginal and intrarectal), although factors such as activation status and the inflammatory state of the host could affect mucosal recruitment and retention, as well as memory reactivation. Additionally, any impairment in the migratory capacity of vaccine-induced immune cells, possibly because differential up-regulation or down-regulation of mucosal homing integrins would significantly affect the biological relevance of the vaccine in the genitorectal mucosa.

MUCOSAL IMMUNIZATION WITH ADJUVANTATION

Mucosal adjuvants such as the non-toxic B subunit of cholera toxin (CTB) or heat labile toxin B subunit (LT-B) are known to boost protective antibody and cellular immune responses following mucosal immunization, and could therefore impact significantly on HIV-1 vaccine efficacy (244, 277). These mucosal adjuvants have been very successful in a number of experimental animal studies. For instance, intrarectal immunization with a synthetic peptide vaccine incorporating the mutant form of heat labile toxin, LT(R192G), as an adjuvant induced mucosal and systemic SIV-specific CTL responses that correlated with viral clearance in challenge experiments (278). Furthermore, intranasal co-administration of HIV-1 envelope antigens in a DNA/MVA or MVA/MVA immunization together with cholera toxin (CT) significantly enhanced antibody and cellular immune responses in the mucosa as well as systemic compartments (279). Other adjuvants known to enhance mucosal immune responses include immunostimulatory CpG motifs and pro-inflammatory cytokines such as IL-1α, IL-12, and IL-18 (44, 244, 280, 281). CpG adjuvantation in particular was shown to significantly enhance vaccine-induced antibody and cellular immune responses following mucosal delivery and to provide protection from mucosal virus challenge (282–284). The glycolipid α-GalCer also shows promise as a mucosal adjuvant which could be used with DNA vaccines (285). The use of non-replicating virosome vectors, known for their intrinsic adjuvant properties and efficient targeting of antigen presenting cells (286) may be another useful delivery platform to enhance mucosal immune responses. Intramuscular and intranasal delivery of a gp41 subunit antigen grafted on virosomes was shown to protect monkeys against SHIV challenge following induction of vaginal IgA and IgG with potent transcytosis blockade activities as well as neutralizing and ADCC activities (43). Intriguingly, protection of vaccinated animals was mediated by the mucosal antibody activities and not the serum circulating HIV-1 antibodies or bNAbs, suggesting that mucosal responses can prevent HIV acquisition in the absence of other systemic responses including bNAbs. Feasibility of the virosome delivery method for induction of mucosal antibodies in humans has been recently demonstrated in a Phase I proof-of-principle study using HIV-1 gp41-derived peptides (287). In this study, both serum IgG and IgA, as well

as vaginal and rectal IgG were induced, but neutralization activity was not detected. However, vaginal secretions were shown to inhibit HIV-1 transcytosis, demonstrating potential to reduce HIV acquisition.

Ultimately, the goal for mucosal HIV-1 vaccine delivery is to generate local antibody and mucosal T cells with antiviral activities, but also with intrinsic ability to disseminate systemically in order to combat HIV-1 infection and spread. Alternatively, the use of tissue-specific adhesion molecules or chemokine-mediated site-specific directed migration of vaccine-stimulated immune cells from mucosal immune inductive sites to peripheral mucosal and systemic effector sites (230, 288) may be useful delivery strategies for HIV vaccines.

MUCOSAL HOMING MARKERS ON IMMUNE EFFECTOR CELLS

The migration of effector/memory T cells and ASC such as those that secrete IgA (IgA-ASC) to various extra-lymphoid tissues including the gut and genitorectal mucosa is facilitated by specific homing receptors on immune cells, together with their cognate ligands, which are expressed in the destination tissues. Migration to the gut for instance requires up-regulation of the chemokine receptors CCR9 and CCR10, as well as the mucosal integrin $\alpha_4\beta_7$ (289–292). The $\alpha_4\beta_7$ integrin, also known as lymphocyte Peyer's patch adhesion molecule-1 (LPAM-1) is a mucosal homing receptor that binds MAdCAM-1, a mucosal vascular addressin selectively expressed on intestinal mucosal endothelium. CCL25, the ligand for CCR9, is expressed mainly by small intestine endothelial and epithelial cells (293, 294), while CCL27 and CCL28 (the ligands for CCR10) are expressed in several mucosal tissues. Thus, binding of these receptors to their respective ligands mediates selective lymphocyte homing to and retention within the intestinal lamina propria and the PP (295–298). Co-expression of CCR9 or CCR10 with $\alpha_4\beta_7$ is therefore a characteristic phenotype of gut homing immune cells. This guided migration is important for tissue-targeted immune activities such as that demonstrated in murine rotavirus infection, where memory/effector CD8+ T cells expressing high levels of $\alpha_4\beta_7$ (i.e., $\alpha_4\beta_7^{hi}CD44^{hi}$) homed preferentially to intestinal tissues and were more effective at pathogen clearance compared to cells with $\alpha_4\beta_7^{lo}CD44^{hi}$ phenotype (299). Moreover, expression of gut homing receptors on CD4+ T cells was shown to be important for mucosal immune reconstitution following HAART, as failed reconstitution was linked to defective homing (300).

While migration of vaccine-induced T and B cells to the gut is crucial to prevent establishment of HIV-1 reservoirs and CD4+ T cell destruction (301), migration to the genitorectal mucosa is critical for preventing HIV-1 acquisition. Although migration to these distinct mucosal sites may be governed by distinct signals, homing to the genitorectal mucosa also requires a B7 integrin, $\alpha_E\beta_7$ (CD103), which is known to mediate lymphocyte recruitment to various mucosal tissues (including the genital mucosa) by binding to epithelial cadherin (E-cadherin) (302–305). A recent study reported isolation of a functional subset of HIV-specific CD8+CD103+IFN-γ+ T cells in samples from the cervical mucosa of HIV-infected individuals (306). CXCR3 expression is up-regulated following lymphocyte activation, and allows migration of CXCR3+ cells to inflamed sites where the cognate ligands, CXCL9 and CXCL10, are up-regulated in response to inflammatory stimuli. Thus, expression of CD103 and CXCR3 by activated B and T cells is likely to direct their migration to the genitorectal mucosa, especially if some degree of inflammation is induced (194).

Naïve lymphocytes express CD62L and CCR7, the major lymph node homing markers, which allow them to circulate through various lymphoid organs, under homeostatic conditions. Upon antigen encounter, they differentiate into activated cells expressing unique adhesion receptors that are imprinted based on the site of antigen exposure (307). For instance, systemic antigen exposure can confer multiple homing signatures, whereas oral exposure preferentially induces higher levels of gut homing receptors (289). This could in part be due to increased expression of retinoic acid receptors on dendritic cells and macrophages in gut-associated lymphoid tissues [PP, mesenteric lymph nodes (MLN) and intestinal lamina propria] which facilitate imprinting of gut homing properties on activated T and B cells by generating retinoic acid to up-regulate CCR9, CCR10, and $\alpha_4\beta_7$ (308–311). Thus, targeting delivery of HIV-1 antigens for activation within the PP and MLN via mucosal immunization may lead to induction of $\alpha_E\beta_7^{hi}/CD44^{hi}$, $\alpha_4\beta_7^{hi}$/CCR9+, or $\alpha_4\beta_7^{hi}$/CCR10+ immune cells with the ability to access multiple mucosal compartments, including the genital and rectal mucosa.

FACTORS THAT LIMIT ASSESSMENT OF IMMUNE RESPONSES IN THE GENITORECTAL MUCOSA

Some of the difficulties arising from studying mucosal sites include the heterogeneity in frequencies and distribution of various immune cell phenotypes, especially in the female genital tract. For instance, the frequency of CD4+ and CD8+ T cells, B cells and NK cells, as well as other antigen presenting cells varies significantly between the lower vaginal mucosa, the ectocervix, and the transformation zone (312). Furthermore, these vary significantly between individuals, owing to factors such as the menstrual cycle and hormonal regulation of the immune system, including levels of IgG and IgA antibodies (209, 312, 313). Such inconsistencies, especially in the integrity of the protective mucus barrier (314–316) and the frequency of activated HIV target cells have great influence on HIV acquisition and control (317–319). Other obstacles relate to the invasiveness of mucosal sampling procedures to obtain cervi-covaginal lavage, swabs, or rectal biopsies and the accompanying time-consuming procedures for isolation of cells from the biopsies (320). Furthermore, the cell yields are characteristically very low and inadequate for comprehensive functional analysis studies. Despite these challenges, procedures to collect, process, and analyze mucosal samples in clinical trials are actively being developed by groups such as the HIV Vaccine Trials Network (HVTN) and the Mucosal Immunology Group (MIG). With such collaborative efforts, several mucosal samples including semen, saliva, rectal and cervical secretions, as well as rectal and foreskin biopsies can now be collected and tested. Sample collection methods (including cups, adsorbent wicks, or sponges for vaginal and rectal secretions) as well as cryopreservation techniques and ultra-sensitive analytical assays that utilize minimal sample volumes and cell numbers are being developed and optimized.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

A successful HIV-1 vaccine will have to stimulate both antibody and cell-mediated immune responses within the mucosal sites of transmission and in blood, while concurrently avoiding recruitment of activated HIV-1 target cells to the genital mucosa. Owing to the enormous hurdles relating to mucosal sampling methodologies and limited sample volumes, immune correlates of protection against HIV-1 in the genital mucosa have not been routinely tested during clinical trials that evaluate immunogenicity and vaccine efficacy (165). However, the field is progressing steadily and it is anticipated that routine assessment of mucosal immune responses induced by immunization will be incorporated in clinical trials. Given the detection of antibody and cellular immune responses that correlate with protection from HIV-1 acquisition as observed in HEPS and from disease progression as seen in aviremic and viremic controllers, together with vaccine-induced responses in the STEP and RV144 trials (and the ongoing RV152 follow-up study), there indeed are several clues of the sort of immune responses that would correlate with HIV-1 vaccine efficacy. All evidence assessed to date indicates that the most successful strategy will induce high titers of both bNAbs and non-neutralizing antibodies to block mucosal transmission of multiple HIV-1 isolates, together with a strong polyfunctional T cell immune response with high antiviral capacity to rapidly target and kill any HIV-1 infected cells at the genitorectal mucosa and prevent systemic spread or establishment of latent reservoirs, before virus diversification. Above all, to attain desirable efficacy levels, vaccine-stimulated responses will indeed have to be present within the genitorectal mucosa prior to HIV-1 exposure. And to maintain sustained HIV immune surveillance, vaccines will need to induce stable, long-lasting B and T cell memory within the genitorectal mucosa, perhaps by employing vectored immuno-prophylaxis (85) or sustained antigen release (321) strategies.

As far as vaccine delivery modalities are concerned, several proof-of-principle studies highlight the feasibility of inducing potent immune responses in the systemic and mucosal compartments by delivering vaccines through intranasal, intravaginal, intrarectal, and oral/sublingual routes in various combinations of heterologous prime-boost immunization strategies. Although quite few, such strategies have shown improved vaccine immunogenicity in human studies and the efficacy of these mucosally redirected immune responses needs to be evaluated in larger clinical trials. Possible concerns about induction of tolerance following mucosal immunization (288) will need to be addressed, although this could be overcome by initial systemic priming followed with mucosal boosting, or perhaps by use of carefully designed vaccine delivery and dosage regimens. Moreover, experimental studies in animals demonstrate robust responses following mucosal priming and mucosal boosting (241, 243), suggesting that the benefits of mucosal vaccine delivery may by far outweigh the risk of tolerance induction.

It remains possible that live viral vectors will be the most effective delivery method to induce potent mucosal immune responses following the most preferred, non-invasive parenteral delivery. However, until this is tested extensively and found comparable to or better than mucosal vaccines, the search for suitable delivery platforms to induce protective T cell and antibody responses and to provide a local immune barrier at the genital mucosa must continue. Perhaps the most practical way to ensure that sufficient numbers of protective CTLs home to the genital mucosa following parenteral vaccine delivery is the innovative "prime and pull" approach that was proposed by Shin and Iwasaki (194). The potential of this strategy was proven in mice studies where priming with a model HSV-2 vaccine elicit systemic T cell responses, followed by the guided migration of these T cells by applying CXCL9 and CXCL10 chemokines in the vaginal mucosa, thus leading to increased recruitment of CD8+ and CD4+ T cells (194). This immunization strategy generated a local memory T cell pool which was stable and persisted for a long time. However, when tested in the context of HIV vaccines, the prime-pull strategy achieved only a modest effect on local and systemic antibody responses (322). Despite this, the enormous potential of this strategy to significantly enhance the magnitude and longevity of HIV vaccine-induced T and B cells in the genital mucosa warrants further testing.

In conclusion, the data available in the field thus far point to the imminent possibility of a vaccine that can stimulate the greatly desired protective mucosal and systemic immune responses. It might be that a carefully selected combination of immunogens, adjuvants, delivery vectors, and immunization routes may possibly yield an HIV-1 vaccine that induces optimal activation of the innate immune system and elicit protective antibody and T cell responses in both the mucosal and systemic compartments. So far, mucosal immunization seems to hold promise as the ultimate modality to ensure sustained levels of potent antibody and cellular immune responses at the genital mucosa, where they are required to arrest initial breakthrough infections. Moreover, since systemic responses do not accurately represent local immunity at the genitorectal mucosa, comprehensive immuno-functional and phenotypic characterization of the mucosal anti-HIV-1 immune response that correlates with *in vivo* virus inhibition, together with the mechanisms involved, will be crucial to the design of an efficacious vaccine for HIV-1. These will include accurate quantification of threshold titers of the mucosal antibody and T cell responses that would be sufficient to prevent infection.

ACKNOWLEDGMENTS

Beatrice Ondondo is an immunologist employed on a research grant supported by MRC, UK. Nadia Chanzu is a Ph.D. student funded by the GHRI grants program and CIHR, IID and GHTP.

REFERENCES

1. Volberding PA, Deeks SG. Antiretroviral therapy and management of HIV infection. *Lancet* (2010) **376**(9734):49–62. doi:10.1016/S0140-6736(10)60676-9
2. Palella FJ Jr, Delaney KM, Moorman AC, Loveless MO, Fuhrer J, Satten GA, et al. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators. *N Engl J Med* (1998) **338**(13):853–60. doi:10.1056/NEJM199803263381301
3. Gamble LJ, Matthews QL. Current progress in the development of a prophylactic vaccine for HIV-1. *Drug Des Devel Ther* (2010) **5**:9–26. doi:10.2147/DDDT.S6959
4. Choopanya K, Martin M, Suntharasamai P, Sangkum U, Mock PA, Leethochawalit M, et al. Antiretroviral prophylaxis for HIV infection in injecting drug users in Bangkok, Thailand (the Bangkok Tenofovir Study): a randomised, double-blind, placebo-controlled phase 3 trial. *Lancet* (2013) **381**(9883):2083–90. doi:10.1016/S0140-6736(13)61127-7

5. Thigpen MC, Kebaabetswe PM, Paxton LA, Smith DK, Rose CE, Segolodi TM, et al. Antiretroviral preexposure prophylaxis for heterosexual HIV transmission in Botswana. *N Engl J Med* (2012) **367**(5):423–34. doi:10.1056/NEJMoa1110711
6. Grant RM, Lama JR, Anderson PL, McMahan V, Liu AY, Vargas L, et al. Preexposure chemoprophylaxis for HIV prevention in men who have sex with men. *N Engl J Med* (2010) **363**(27):2587–99. doi:10.1056/NEJMoa1011205
7. Baeten JM, Donnell D, Ndase P, Mugo NR, Campbell JD, Wangisi J, et al. Antiretroviral prophylaxis for HIV prevention in heterosexual men and women. *N Engl J Med* (2012) **367**(5):399–410. doi:10.1056/NEJMoa1108524
8. Abdool Karim Q, Abdool Karim SS, Frohlich JA, Grobler AC, Baxter C, Mansoor LE, et al. Effectiveness and safety of tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women. *Science* (2010) **329**(5996):1168–74. doi:10.1126/science.1193748
9. Saez-Cirion A, Bacchus C, Hocqueloux L, Avettand-Fenoel V, Girault I, Lecuroux C, et al. Post-treatment HIV-1 controllers with a long-term virological remission after the interruption of early initiated antiretroviral therapy ANRS VISCONTI Study. *PLoS Pathog* (2013) **9**(3):e1003211. doi:10.1371/journal.ppat.1003211
10. Dolgin E. New, intensive trials planned on heels of Mississippi HIV "cure". *Nat Med* (2013) **19**(4):380–1. doi:10.1038/nm0413-380
11. Hocqueloux L, Prazuck T, Avettand-Fenoel V, Lafeuillade A, Cardon B, Viard JP, et al. Long-term immunovirologic control following antiretroviral therapy interruption in patients treated at the time of primary HIV-1 infection. *AIDS* (2010) **24**(10):1598–601. doi:10.1097/QAD.0b013e32833b61ba
12. Persaud D, Gay H, Ziemniak C, Chen YH, Piatak M Jr, Chun TW, et al. Absence of detectable HIV-1 viremia after treatment cessation in an infant. *N Engl J Med* (2013) **369**(19):1828–35. doi:10.1056/NEJMoa1302976
13. Cohen MS, Chen YQ, McCauley M, Gamble T, Hosseinipour MC, Kumarasamy N, et al. Prevention of HIV-1 infection with early antiretroviral therapy. *N Engl J Med* (2011) **365**(6):493–505. doi:10.1056/NEJMoa1105243
14. Palombi L, Marazzi MC, Voetberg A, Magid NA. Treatment acceleration program and the experience of the DREAM program in prevention of mother-to-child transmission of HIV. *AIDS* (2007) **21**(Suppl 4):S65–71. doi:10.1097/01.aids.0000279708.09180.f5
15. Kilewo C, Karlsson K, Ngarina M, Massawe A, Lyamuya E, Swai A, et al. Prevention of mother-to-child transmission of HIV-1 through breastfeeding by treating mothers with triple antiretroviral therapy in Dar es Salaam, Tanzania: the Mitra Plus study. *J Acquir Immune Defic Syndr* (2009) **52**(3):406–16. doi:10.1097/QAI.0b013e3181b323ff
16. Granich RM, Gilks CF, Dye C, De Cock KM, Williams BG. Universal voluntary HIV testing with immediate antiretroviral therapy as a strategy for elimination of HIV transmission: a mathematical model. *Lancet* (2009) **373**(9657):48–57. doi:10.1016/S0140-6736(08)61697-9
17. Dodd PJ, Garnett GP, Hallett TB. Examining the promise of HIV elimination by "test and treat" in hyperendemic settings. *AIDS* (2010) **24**(5):729–35. doi:10.1097/QAD.0b013e32833433fe
18. Dieffenbach CW, Fauci AS. Universal voluntary testing and treatment for prevention of HIV transmission. *JAMA* (2009) **301**(22):2380–2. doi:10.1001/jama.2009.828
19. Garnett GP, Baggaley RF. Treating our way out of the HIV pandemic: could we, would we, should we? *Lancet* (2009) **373**(9657):9–11. doi:10.1016/S0140-6736(08)61698-0
20. Hayes R, Sabapathy K, Fidler S. Universal testing and treatment as an HIV prevention strategy: research questions and methods. *Curr HIV Res* (2011) **9**(6):429–45. doi:10.2174/157016211798038515
21. Bailey RC, Moses S, Parker CB, Agot K, Maclean I, Krieger JN, et al. Male circumcision for HIV prevention in young men in Kisumu, Kenya: a randomised controlled trial. *Lancet* (2007) **369**(9562):643–56. doi:10.1016/S0140-6736(07)60312-2
22. Gray RH, Kigozi G, Serwadda D, Makumbi F, Watya S, Nalugoda F, et al. Male circumcision for HIV prevention in men in Rakai, Uganda: a randomised trial. *Lancet* (2007) **369**(9562):657–66. doi:10.1016/S0140-6736(07)60313-4
23. Auvert B, Taljaard D, Lagarde E, Sobngwi-Tambekou J, Sitta R, Puren A. Randomized, controlled intervention trial of male circumcision for reduction of HIV infection risk: the ANRS 1265 trial. *PLoS Med* (2005) **2**(11):e298. doi:10.1371/journal.pmed.0020298
24. McMichael AJ. HIV vaccines. *Annu Rev Immunol* (2006) **24**:227–55. doi:10.1146/annurev.immunol.24.021605.090605
25. Emini EA, Koff WC. AIDS/HIV. Developing an AIDS vaccine: need, uncertainty, hope. *Science* (2004) **304**(5679):1913–4. doi:10.1126/science.1100368
26. Thorner AR, Barouch DH. HIV-1 vaccine development: progress and prospects. *Curr Infect Dis Rep* (2007) **9**(1):71–5. doi:10.1007/s11908-007-0025-0
27. Robinson HL, Amara RR. T cell vaccines for microbial infections. *Nat Med* (2005) **11**(4 Suppl):S25–32. doi:10.1038/nm1212
28. Sekaly RP. The failed HIV Merck vaccine study: a step back or a launching point for future vaccine development? *J Exp Med* (2008) **205**(1):7–12. doi:10.1084/jem.20072681
29. Wang L, Wang N, Wang L, Li D, Jia M, Gao X, et al. The 2007 estimates for people at risk for and living with HIV in China: progress and challenges. *J Acquir Immune Defic Syndr* (2009) **50**(4):414–8. doi:10.1097/QAI.0b013e3181958530
30. Des Jarlais DC, Arasteh K, Semaan S, Wood E. HIV among injecting drug users: current epidemiology, biologic markers, respondent-driven sampling, and supervised-injection facilities. *Curr Opin HIV AIDS* (2009) **4**(4):308–13. doi:10.1097/COH.0b013e32832bbc6f
31. Sullivan PS, Hamouda O, Delpech V, Geduld JE, Prejean J, Semaille C, et al. Reemergence of the HIV epidemic among men who have sex with men in North America, Western Europe, and Australia, 1996-2005. *Ann Epidemiol* (2009) **19**(6):423–31. doi:10.1016/j.annepidem.2009.03.004
32. Shacklett BL. Mucosal immunity to HIV: a review of recent literature. *Curr Opin HIV AIDS* (2008) **3**(5):541–7. doi:10.1097/COH.0b013e32830ab9ee
33. Louwagie J, McCutchan FE, Peeters M, Brennan TP, Sanders-Buell E, Eddy GA, et al. Phylogenetic analysis of gag genes from 70 international HIV-1 isolates provides evidence for multiple genotypes. *AIDS* (1993) **7**(6):769–80. doi:10.1097/00002030-199306000-00003
34. Hemelaar J, Gouws E, Ghys PD, Osmanov S; WHO-UNAIDS Network for HIV Isolation and Characterisation. Global trends in molecular epidemiology of HIV-1 during 2000-2007. *AIDS* (2011) **25**(5):679–89. doi:10.1097/QAD.0b013e328342ff93
35. Collins DR, Collins KL. HIV-1 accessory proteins adapt cellular adaptors to facilitate immune evasion. *PLoS Pathog* (2014) **10**(1):e1003851. doi:10.1371/journal.ppat.1003851
36. Goonetilleke N, Liu MK, Salazar-Gonzalez JF, Ferrari G, Giorgi E, Ganusov VV, et al. The first T cell response to transmitted/founder virus contributes to the control of acute viremia in HIV-1 infection. *J Exp Med* (2009) **206**(6):1253–72. doi:10.1084/jem.20090365
37. Chun TW, Engel D, Berrey MM, Shea T, Corey L, Fauci AS. Early establishment of a pool of latently infected, resting CD4(+) T cells during primary HIV-1 infection. *Proc Natl Acad Sci U S A* (1998) **95**(15):8869–73. doi:10.1073/pnas.95.15.8869
38. Finzi D, Blankson J, Siliciano JD, Margolick JB, Chadwick K, Pierson T, et al. Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. *Nat Med* (1999) **5**(5):512–7. doi:10.1038/8394
39. Mehandru S, Poles MA, Tenner-Racz K, Horowitz A, Hurley A, Hogan C, et al. Primary HIV-1 infection is associated with preferential depletion of CD4+ T lymphocytes from effector sites in the gastrointestinal tract. *J Exp Med* (2004) **200**(6):761–70. doi:10.1084/jem.20041196
40. Guadalupe M, Reay E, Sankaran S, Prindiville T, Flamm J, McNeil A, et al. Severe CD4+ T-cell depletion in gut lymphoid tissue during primary human immunodeficiency virus type 1 infection and substantial delay in restoration following highly active antiretroviral therapy. *J Virol* (2003) **77**(21):11708–17. doi:10.1128/JVI.77.21.11708-11717.2003
41. Schwartz O, Marechal V, Le Gall S, Lemonnier F, Heard JM. Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein. *Nat Med* (1996) **2**(3):338–42. doi:10.1038/nm0396-338
42. Yu M, Vajdy M. Mucosal HIV transmission and vaccination strategies through oral compared with vaginal and rectal routes. *Expert Opin Biol Ther* (2010) **10**(8):1181–95. doi:10.1517/14712598.2010.496776
43. Bomsel M, Tudor D, Drillet AS, Alfsen A, Ganor Y, Roger MG, et al. Immunization with HIV-1 gp41 subunit virosomes induces mucosal antibodies protecting nonhuman primates against vaginal SHIV challenges. *Immunity* (2011) **34**(2):269–80. doi:10.1016/j.immuni.2011.01.015

44. Belyakov IM, Ahlers JD, Brandwein BY, Earl P, Kelsall BL, Moss B, et al. The importance of local mucosal HIV-specific CD8(+) cytotoxic T lymphocytes for resistance to mucosal viral transmission in mice and enhancement of resistance by local administration of IL-12. *J Clin Invest* (1998) **102**(12):2072–81. doi:10.1172/JCI5102
45. Ferre AL, Hunt PW, Critchfield JW, Young DH, Morris MM, Garcia JC, et al. Mucosal immune responses to HIV-1 in elite controllers: a potential correlate of immune control. *Blood* (2009) **113**(17):3978–89. doi:10.1182/blood-2008-10-182709
46. Esparza J. A brief history of the global effort to develop a preventive HIV vaccine. *Vaccine* (2013) **31**(35):3502–18. doi:10.1016/j.vaccine.2013.05.018
47. Buchbinder SP, Mehrotra DV, Duerr A, Fitzgerald DW, Mogg R, Li D, et al. Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. *Lancet* (2008) **372**(9653):1881–93. doi:10.1016/S0140-6736(08)61591-3
48. Gray GE, Allen M, Moodie Z, Churchyard G, Bekker LG, Nchabeleng M, et al. Safety and efficacy of the HVTN 503/Phambili study of a clade-B-based HIV-1 vaccine in South Africa: a double-blind, randomised, placebo-controlled test-of-concept phase 2b study. *Lancet Infect Dis* (2011) **11**(7):507–15. doi:10.1016/S1473-3099(11)70098-6
49. Hammer SM, Sobieszczyk ME, Janes H, Karuna ST, Mulligan MJ, Grove D, et al. Efficacy trial of a DNA/rAd5 HIV-1 preventive vaccine. *N Engl J Med* (2013) **369**(22):2083–92. doi:10.1056/NEJMoa1310566
50. Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S, Kaewkungwal J, Chiu J, Paris R, et al. Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. *N Engl J Med* (2009) **361**(23):2209–20. doi:10.1056/NEJMoa0908492
51. Pitisuttithum P, Gilbert P, Gurwith M, Heyward W, Martin M, van Griensven F, et al. Randomized, double-blind, placebo-controlled efficacy trial of a bivalent recombinant glycoprotein 120 HIV-1 vaccine among injection drug users in Bangkok, Thailand. *J Infect Dis* (2006) **194**(12):1661–71. doi:10.1086/508748
52. Flynn NM, Forthal DN, Harro CD, Judson FN, Mayer KH, Para MF, et al. Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection. *J Infect Dis* (2005) **191**(5):654–65. doi:10.1086/428404
53. Gilbert PB, Peterson ML, Follmann D, Hudgens MG, Francis DP, Gurwith M, et al. Correlation between immunologic responses to a recombinant glycoprotein 120 vaccine and incidence of HIV-1 infection in a phase 3 HIV-1 preventive vaccine trial. *J Infect Dis* (2005) **191**(5):666–77. doi:10.1086/428405
54. Schiffner T, Sattentau QJ, Dorrell L. Development of prophylactic vaccines against HIV-1. *Retrovirology* (2013) **10**:72. doi:10.1186/1742-4690-10-72
55. McElrath MJ, De Rosa SC, Moodie Z, Dubey S, Kierstead L, Janes H, et al. HIV-1 vaccine-induced immunity in the test-of-concept Step Study: a case-cohort analysis. *Lancet* (2008) **372**(9653):1894–905. doi:10.1016/S0140-6736(08)61592-5
56. Rolland M, Tovanabutra S, deCamp AC, Frahm N, Gilbert PB, Sanders-Buell E, et al. Genetic impact of vaccination on breakthrough HIV-1 sequences from the STEP trial. *Nat Med* (2011) **17**(3):366–71. doi:10.1038/nm.2316
57. Janes H, Friedrich DP, Krambrink A, Smith RJ, Kallas EG, Horton H, et al. Vaccine-induced gag-specific T cells are associated with reduced viremia after HIV-1 infection. *J Infect Dis* (2013) **208**(8):1231–9. doi:10.1093/infdis/jit322
58. Borthwick N, Ahmed T, Ondondo B, Hayes P, Rose A, Ebrahimsa U, et al. Vaccine-elicited human T cells recognizing conserved protein regions inhibit HIV-1. *Mol Ther* (2013) **22**(2):464–75. doi:10.1038/mt.2013.248
59. Letourneau S, Im EJ, Mashishi T, Brereton C, Bridgeman A, Yang H, et al. Design and pre-clinical evaluation of a universal HIV-1 vaccine. *PLoS One* (2007) **2**(10):e984. doi:10.1371/journal.pone.0000984
60. Koopman G, Beenhakker N, Nieuwenhuis I, Doxiadis G, Mooij P, Drijfhout JW, et al. DNA/long peptide vaccination against conserved regions of SIV induces partial protection against SIVmac251 challenge. *AIDS* (2013) **27**:2841–51. doi:10.1097/QAD.0000000000000047
61. Liu J, O'Brien KL, Lynch DM, Simmons NL, La Porte A, Riggs AM, et al. Immune control of an SIV challenge by a T-cell-based vaccine in rhesus monkeys. *Nature* (2009) **457**(7225):87–91. doi:10.1038/nature07469
62. Mascola JR, Montefiori DC. The role of antibodies in HIV vaccines. *Annu Rev Immunol* (2010) **28**:413–44. doi:10.1146/annurev-immunol-030409-101256
63. Mascola JR, Stiegler G, VanCott TC, Katinger H, Carpenter CB, Hanson CE, et al. Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. *Nat Med* (2000) **6**(2):207–10. doi:10.1038/72318
64. Baba TW, Liska V, Hofmann-Lehmann R, Vlasak J, Xu W, Ayehunie S, et al. Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection. *Nat Med* (2000) **6**(2):200–6. doi:10.1038/72309
65. Clapham PR, Lu S. Vaccinology: precisely tuned antibodies nab HIV. *Nature* (2011) **477**(7365):416–7. doi:10.1038/477416a
66. Walker LM, Phogat SK, Chan-Hui PY, Wagner D, Phung P, Goss JL, et al. Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target. *Science* (2009) **326**(5950):285–9. doi:10.1126/science.1178746
67. Scheid JF, Mouquet H, Ueberheide B, Diskin R, Klein F, Oliveira TY, et al. Sequence and structural convergence of broad and potent HIV antibodies that mimic CD4 binding. *Science* (2011) **333**(6049):1633–7. doi:10.1126/science.1207227
68. Bonsignori M, Hwang KK, Chen X, Tsao CY, Morris L, Gray E, et al. Analysis of a clonal lineage of HIV-1 envelope V2/V3 conformational epitope-specific broadly neutralizing antibodies and their inferred unmutated common ancestors. *J Virol* (2011) **85**(19):9998–10009. doi:10.1128/JVI.05045-11
69. Corti D, Langedijk JP, Hinz A, Seaman MS, Vanzetta F, Fernandez-Rodriguez BM, et al. Analysis of memory B cell responses and isolation of novel monoclonal antibodies with neutralizing breadth from HIV-1-infected individuals. *PLoS One* (2010) **5**(1):e8805. doi:10.1371/journal.pone.0008805
70. Wu X, Yang ZY, Li Y, Hogerkorp CM, Schief WR, Seaman MS, et al. Rational design of envelope identifies broadly neutralizing human monoclonal antibodies to HIV-1. *Science* (2010) **329**(5993):856–61. doi:10.1126/science.1187659
71. Klein F, Mouquet H, Dosenovic P, Scheid JF, Scharf L, Nussenzweig MC. Antibodies in HIV-1 vaccine development and therapy. *Science* (2013) **341**(6151):1199–204. doi:10.1126/science.1241144
72. Florese RH, Demberg T, Xiao P, Kuller L, Larsen K, Summers LE, et al. Contribution of nonneutralizing vaccine-elicited antibody activities to improved protective efficacy in rhesus macaques immunized with Tat/Env compared with multigenic vaccines. *J Immunol* (2009) **182**(6):3718–27. doi:10.4049/jimmunol.0803115
73. Forthal DN, Landucci G, Phan TB, Becerra J. Interactions between natural killer cells and antibody Fc result in enhanced antibody neutralization of human immunodeficiency virus type 1. *J Virol* (2005) **79**(4):2042–9. doi:10.1128/JVI.79.4.2042-2049.2005
74. Hidajat R, Xiao P, Zhou Q, Venzon D, Summers LE, Kalyanaraman VS, et al. Correlation of vaccine-elicited systemic and mucosal nonneutralizing antibody activities with reduced acute viremia following intrarectal simian immunodeficiency virus SIVmac251 challenge of rhesus macaques. *J Virol* (2009) **83**(2):791–801. doi:10.1128/JVI.01672-08
75. Xiao P, Zhao J, Patterson LJ, Brocca-Cofano E, Venzon D, Kozlowski PA, et al. Multiple vaccine-elicited nonneutralizing antienvelope antibody activities contribute to protective efficacy by reducing both acute and chronic viremia following simian/human immunodeficiency virus SHIV89.6P challenge in rhesus macaques. *J Virol* (2010) **84**(14):7161–73. doi:10.1128/JVI.00410-10
76. Gomez-Roman VR, Patterson LJ, Venzon D, Liewehr D, Aldrich K, Florese R, et al. Vaccine-elicited antibodies mediate antibody-dependent cellular cytotoxicity correlated with significantly reduced acute viremia in rhesus macaques challenged with SIVmac251. *J Immunol* (2005) **174**(4):2185–9. doi:10.4049/jimmunol.174.4.2185
77. Moog C, Dereuddre-Bosquet N, Teillaud JL, Biedma ME, Holl V, Van Ham G, et al. Protective effect of vaginal application of neutralizing and nonneutralizing inhibitory antibodies against vaginal SHIV challenge in macaques. *Mucosal Immunol* (2014) **7**(1):46–56. doi:10.1038/mi.2013.23
78. Lambotte O, Ferrari G, Moog C, Yates NL, Liao HX, Parks RJ, et al. Heterogeneous neutralizing antibody and antibody-dependent cell cytotoxicity responses in HIV-1 elite controllers. *AIDS* (2009) **23**(8):897–906. doi:10.1097/QAD.0b013e328329f97d
79. Haynes BF, Gilbert PB, McElrath MJ, Zolla-Pazner S, Tomaras GD, Alam SM, et al. Immune-correlates analysis of an HIV-1 vaccine efficacy trial. *N Engl J Med* (2012) **366**(14):1275–86. doi:10.1056/NEJMoa1113425
80. Rolland M, Edlefsen PT, Larsen BB, Tovanabutra S, Sanders-Buell E, Hertz T, et al. Increased HIV-1 vaccine efficacy against viruses with genetic signatures in Env V2. *Nature* (2012) **490**(7420):417–20. doi:10.1038/nature11519
81. Gray G, Buchbinder S, Duerr A. Overview of STEP and Phambili trial results: two phase IIb test-of-concept studies investigating the efficacy of MRK adenovirus type 5 gag/pol/nef subtype B HIV vaccine. *Curr Opin HIV AIDS* (2010) **5**(5):357–61. doi:10.1097/COH.0b013e32833d2d2b

82. Haynes BF, Kelsoe G, Harrison SC, Kepler TB. B-cell-lineage immunogen design in vaccine development with HIV-1 as a case study. *Nat Biotechnol* (2012) **30**(5):423–33. doi:10.1038/nbt.2197
83. Liao HX, Lynch R, Zhou T, Gao F, Alam SM, Boyd SD, et al. Co-evolution of a broadly neutralizing HIV-1 antibody and founder virus. *Nature* (2013) **496**(7446):469–76. doi:10.1038/nature12053
84. Alt FW, Zhang Y, Meng FL, Guo C, Schwer B. Mechanisms of programmed DNA lesions and genomic instability in the immune system. *Cell* (2013) **152**(3):417–29. doi:10.1016/j.cell.2013.01.007
85. Balazs AB, Chen J, Hong CM, Rao DS, Yang L, Baltimore D. Antibody-based protection against HIV infection by vectored immunoprophylaxis. *Nature* (2012) **481**(7379):81–4. doi:10.1038/nature10660
86. Balazs AB, Ouyang Y, Hong CM, Chen J, Nguyen SM, Rao DS, et al. Vectored immunoprophylaxis protects humanized mice from mucosal HIV transmission. *Nat Med* (2014) **20**(3):296–300. doi:10.1038/nm.3471
87. Ohs I, Windmann S, Wildner O, Dittmer U, Bayer W. Interleukin-encoding adenoviral vectors as genetic adjuvant for vaccination against retroviral infection. *PLoS One* (2013) **8**(12):e82528. doi:10.1371/journal.pone.0082528
88. Bayer W, Lietz R, Ontikatze T, Johrden L, Tenbusch M, Nabi G, et al. Improved vaccine protection against retrovirus infection after co-administration of adenoviral vectors encoding viral antigens and type I interferon subtypes. *Retrovirology* (2011) **8**:75. doi:10.1186/1742-4690-8-75
89. Lietz R, Bayer W, Ontikatze T, Johrden L, Tenbusch M, Storcksdieck Genannt Bonsmann M, et al. Codelivery of the chemokine CCL3 by an adenovirus-based vaccine improves protection from retrovirus infection. *J Virol* (2012) **86**(3):1706–16. doi:10.1128/JVI.06244-11
90. Hu K, Luo S, Tong L, Huang X, Jin W, Huang W, et al. CCL19 and CCL28 augment mucosal and systemic immune responses to HIV-1 gp140 by mobilizing responsive immunocytes into secondary lymph nodes and mucosal tissue. *J Immunol* (2013) **191**(4):1935–47. doi:10.4049/jimmunol.1300120
91. Hansen SG, Ford JC, Lewis MS, Ventura AB, Hughes CM, Coyne-Johnson L, et al. Profound early control of highly pathogenic SIV by an effector memory T-cell vaccine. *Nature* (2011) **473**(7348):523–7. doi:10.1038/nature10003
92. Hansen SG, Vieville C, Whizin N, Coyne-Johnson L, Siess DC, Drummond DD, et al. Effector memory T cell responses are associated with protection of rhesus monkeys from mucosal simian immunodeficiency virus challenge. *Nat Med* (2009) **15**(3):293–9. doi:10.1038/nm.1935
93. Hansen SG, Piatak M Jr, Ventura AB, Hughes CM, Gilbride RM, Ford JC, et al. Immune clearance of highly pathogenic SIV infection. *Nature* (2013) **502**(7469):100–4. doi:10.1038/nature12519
94. Barouch DH, Stephenson KE, Borducchi EN, Smith K, Stanley K, McNally AG, et al. Protective efficacy of a global HIV-1 mosaic vaccine against heterologous SHIV challenges in rhesus monkeys. *Cell* (2013) **155**(3):531–9. doi:10.1016/j.cell.2013.09.061
95. Fischer W, Perkins S, Theiler J, Bhattacharya T, Yusim K, Funkhouser R, et al. Polyvalent vaccines for optimal coverage of potential T-cell epitopes in global HIV-1 variants. *Nat Med* (2007) **13**(1):100–6. doi:10.1038/nm1461
96. Palesch D, Kirchhoff F. First steps toward a globally effective HIV/AIDS vaccine. *Cell* (2013) **155**(3):495–7. doi:10.1016/j.cell.2013.10.012
97. Barouch DH, O'Brien KL, Simmons NL, King SL, Abbink P, Maxfield LF, et al. Mosaic HIV-1 vaccines expand the breadth and depth of cellular immune responses in rhesus monkeys. *Nat Med* (2010) **16**(3):319–23. doi:10.1038/nm.2089
98. Santra S, Liao HX, Zhang R, Muldoon M, Watson S, Fischer W, et al. Mosaic vaccines elicit CD8+ T lymphocyte responses that confer enhanced immune coverage of diverse HIV strains in monkeys. *Nat Med* (2010) **16**(3):324–8. doi:10.1038/nm.2108
99. Santra S, Muldoon M, Watson S, Buzby A, Balachandran H, Carlson KR, et al. Breadth of cellular and humoral immune responses elicited in rhesus monkeys by multi-valent mosaic and consensus immunogens. *Virology* (2012) **428**(2):121–7. doi:10.1016/j.virol.2012.03.012
100. Ondondo BO. Fallen angels or risen apes? A tale of the intricate complexities of imbalanced immune responses in the pathogenesis and progression of immune-mediated and viral cancers. *Front Immunol* (2014) **5**:90. doi:10.3389/fimmu.2014.00090
101. Dietze KK, Zelinskyy G, Liu J, Kretzmer F, Schimmer S, Dittmer U. Combining regulatory T cell depletion and inhibitory receptor blockade improves reactivation of exhausted virus-specific CD8+ T cells and efficiently reduces chronic retroviral loads. *PLoS Pathog* (2013) **9**(12):e1003798. doi:10.1371/journal.ppat.1003798
102. Palmer BE, Neff CP, Lecureux J, Ehler A, Dsouza M, Remling-Mulder L, et al. In vivo blockade of the PD-1 receptor suppresses HIV-1 viral loads and improves CD4+ T cell levels in humanized mice. *J Immunol* (2013) **190**(1):211–9. doi:10.4049/jimmunol.1201108
103. Deeks SG, Walker BD. Human immunodeficiency virus controllers: mechanisms of durable virus control in the absence of antiretroviral therapy. *Immunity* (2007) **27**(3):406–16. doi:10.1016/j.immuni.2007.08.010
104. Migueles SA, Sabbaghian MS, Shupert WL, Bettinotti MP, Marincola FM, Martino L, et al. HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. *Proc Natl Acad Sci U S A* (2000) **97**(6):2709–14. doi:10.1073/pnas.050567397
105. Fellay J, Shianna KV, Ge D, Colombo S, Ledergerber B, Weale M, et al. A whole-genome association study of major determinants for host control of HIV-1. *Science* (2007) **317**(5840):944–7. doi:10.1126/science.1143767
106. Carrington M, O'Brien SJ. The influence of HLA genotype on AIDS. *Annu Rev Med* (2003) **54**:535–51. doi:10.1146/annurev.med.54.101601.152346
107. Fellay J, Frahm N, Shianna KV, Cirulli ET, Casimiro DR, Robertson MN, et al. Host genetic determinants of T cell responses to the MRKAd5 HIV-1 gag/pol/nef vaccine in the step trial. *J Infect Dis* (2011) **203**(6):773–9. doi:10.1093/infdis/jiq125
108. Dean M, Carrington M, Winkler C, Huttley GA, Smith MW, Allikmets R, et al. Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. *Science* (1996) **273**(5283):1856–62.
109. Magierowska M, Theodorou I, Debre P, Sanson F, Autran B, Riviere Y, et al. Combined genotypes of CCR5, CCR2, SDF1, and HLA genes can predict the long-term nonprogressor status in human immunodeficiency virus-1-infected individuals. *Blood* (1999) **93**(3):936–41.
110. Liu R, Paxton WA, Choe S, Ceradini D, Martin SR, Horuk R, et al. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell* (1996) **86**(3):367–77. doi:10.1016/S0092-8674(00)80110-5
111. Alter G, Altfeld M. NK cells in HIV-1 infection: evidence for their role in the control of HIV-1 infection. *J Intern Med* (2009) **265**(1):29–42. doi:10.1111/j.1365-2796.2008.02045.x
112. Alter G, Martin MP, Teigen N, Carr WH, Suscovich TJ, Schneidewind A, et al. Differential natural killer cell-mediated inhibition of HIV-1 replication based on distinct KIR/HLA subtypes. *J Exp Med* (2007) **204**(12):3027–36. doi:10.1084/jem.20070695
113. Alter G, Teigen N, Ahern R, Streeck H, Meier A, Rosenberg ES, et al. Evolution of innate and adaptive effector cell functions during acute HIV-1 infection. *J Infect Dis* (2007) **195**(10):1452–60. doi:10.1086/513878
114. Boulet S, Kleyman M, Kim JY, Kamya P, Sharafi S, Simic N, et al. A combined genotype of KIR3DL1 high expressing alleles and HLA-B*57 is associated with a reduced risk of HIV infection. *AIDS* (2008) **22**(12):1487–91. doi:10.1097/QAD.0b013e3282ffde7e
115. Boulet S, Sharafi S, Simic N, Bruneau J, Routy JP, Tsoukas CM, et al. Increased proportion of KIR3DS1 homozygotes in HIV-exposed uninfected individuals. *AIDS* (2008) **22**(5):595–9. doi:10.1097/QAD.0b013e3282f56b23
116. Jennes W, Verheyden S, Demanet C, Adje-Toure CA, Vuylsteke B, Nkengasong JN, et al. Cutting edge: resistance to HIV-1 infection among African female sex workers is associated with inhibitory KIR in the absence of their HLA ligands. *J Immunol* (2006) **177**(10):6588–92. doi:10.4049/jimmunol.177.10.6588
117. Hutter G, Nowak D, Mossner M, Ganepola S, Mussig A, Allers K, et al. Long-term control of HIV by CCR5 Delta32/Delta32 stem-cell transplantation. *N Engl J Med* (2009) **360**(7):692–8. doi:10.1056/NEJMoa0802905
118. Allers K, Hutter G, Hofmann J, Loddenkemper C, Rieger K, Thiel E, et al. Evidence for the cure of HIV infection by CCR5Delta32/Delta32 stem cell transplantation. *Blood* (2011) **117**(10):2791–9. doi:10.1182/blood-2010-09-309591
119. Yukl SA, Boritz E, Busch M, Bentsen C, Chun TW, Douek D, et al. Challenges in detecting HIV persistence during potentially curative interventions: a study of the Berlin patient. *PLoS Pathog* (2013) **9**(5):e1003347. doi:10.1371/journal.ppat.1003347

120. Hutter G, Ganepola S. Eradication of HIV by transplantation of CCR5-deficient hematopoietic stem cells. *ScientificWorldJournal* (2011) **11**:1068–76. doi:10.1100/tsw.2011.102
121. Biasin M, Piacentini L, Lo Caputo S, Kanari Y, Magri G, Trabattoni D, et al. Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G: a possible role in the resistance to HIV of HIV-exposed seronegative individuals. *J Infect Dis* (2007) **195**(7):960–4. doi:10.1086/511988
122. Malim MH, Bieniasz PD. HIV restriction factors and mechanisms of evasion. *Cold Spring Harb Perspect Med* (2012) **2**(5):a006940. doi:10.1101/cshperspect.a006940
123. Laguette N, Sobhian B, Casartelli N, Ringeard M, Chable-Bessia C, Segeral E, et al. SAMHD1 is the dendritic- and myeloid-cell-specific HIV-1 restriction factor counteracted by Vpx. *Nature* (2011) **474**(7353):654–7. doi:10.1038/nature10117
124. Jin X, Brooks A, Chen H, Bennett R, Reichman R, Smith H. APOBEC3G/CEM15 (hA3G) mRNA levels associate inversely with human immunodeficiency virus viremia. *J Virol* (2005) **79**(17):11513–6. doi:10.1128/JVI.79.17.11513-11516.2005
125. Price H, Lacap P, Tuff J, Wachihi C, Kimani J, Ball TB, et al. A TRIM5alpha exon 2 polymorphism is associated with protection from HIV-1 infection in the Pumwani sex worker cohort. *AIDS* (2010) **24**(12):1813–21. doi:10.1097/QAD.0b013e32833b5256
126. McDermott AB, Koup RA. CD8(+) T cells in preventing HIV infection and disease. *AIDS* (2012) **26**(10):1281–92. doi:10.1097/QAD.0b013e328353bcaf
127. Saez-Cirion A, Sinet M, Shin SY, Urrutia A, Versmisse P, Lacabaratz C, et al. Heterogeneity in HIV suppression by CD8 T cells from HIV controllers: association with Gag-specific CD8 T cell responses. *J Immunol* (2009) **182**(12):7828–37. doi:10.4049/jimmunol.0803928
128. Addo MM, Draenert R, Rathod A, Verrill CL, Davis BT, Gandhi RT, et al. Fully differentiated HIV-1 specific CD8+ T effector cells are more frequently detectable in controlled than in progressive HIV-1 infection. *PLoS One* (2007) **2**(3):e321. doi:10.1371/journal.pone.0000321
129. Saez-Cirion A, Lacabaratz C, Lambotte O, Versmisse P, Urrutia A, Boufassa F, et al. 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* (2007) **104**(16):6776–81. doi:10.1073/pnas.0611244104
130. Betts MR, Nason MC, West SM, De Rosa SC, Migueles SA, Abraham J, et al. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. *Blood* (2006) **107**(12):4781–9. doi:10.1182/blood-2005-12-4818
131. Migueles SA, Laborico AC, Shupert WL, Sabbaghian MS, Rabin R, Hallahan CW, et al. HIV-specific CD8+ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors. *Nat Immunol* (2002) **3**(11):1061–8. doi:10.1038/ni845
132. Kiepiela P, Ngumbela K, Thobakgale C, Ramduth D, Honeyborne I, Moodley E, et al. CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. *Nat Med* (2007) **13**(1):46–53. doi:10.1038/nm1520
133. Edwards BH, Bansal A, Sabbaj S, Bakari J, Mulligan MJ, Goepfert PA. Magnitude of functional CD8+ T-cell responses to the gag protein of human immunodeficiency virus type 1 correlates inversely with viral load in plasma. *J Virol* (2002) **76**(5):2298–305. doi:10.1128/jvi.76.5.2298-2305.2002
134. Klein MR, van Baalen CA, Holwerda AM, Kerkhof Garde SR, Bende RJ, Keet IP, et al. Kinetics of Gag-specific cytotoxic T lymphocyte responses during the clinical course of HIV-1 infection: a longitudinal analysis of rapid progressors and long-term asymptomatics. *J Exp Med* (1995) **181**(4):1365–72. doi:10.1084/jem.181.4.1365
135. Cao Y, Qin L, Zhang L, Safrit J, Ho DD. Virologic and immunologic characterization of long-term survivors of human immunodeficiency virus type 1 infection. *N Engl J Med* (1995) **332**(4):201–8. doi:10.1056/NEJM199501263320401
136. Chun TW, Justement JS, Moir S, Hallahan CW, Ehler LA, Liu S, et al. Suppression of HIV replication in the resting CD4+ T cell reservoir by autologous CD8+ T cells: implications for the development of therapeutic strategies. *Proc Natl Acad Sci U S A* (2001) **98**(1):253–8. doi:10.1073/pnas.98.1.253
137. Barker E, Bossart KN, Locher CP, Patterson BK, Levy JA. CD8+ cells from asymptomatic human immunodeficiency virus-infected individuals suppress superinfection of their peripheral blood mononuclear cells. *J Gen Virol* (1996) **77**(Pt 12):2953–62. doi:10.1099/0022-1317-77-12-2953
138. Migueles SA, Osborne CM, Royce C, Compton AA, Joshi RP, Weeks KA, et al. Lytic granule loading of CD8+ T cells is required for HIV-infected cell elimination associated with immune control. *Immunity* (2008) **29**(6):1009–21. doi:10.1016/j.immuni.2008.10.010
139. Killian MS, Johnson C, Teque F, Fujimura S, Levy JA. Natural suppression of human immunodeficiency virus type 1 replication is mediated by transitional memory CD8+ T cells. *J Virol* (2011) **85**(4):1696–705. doi:10.1128/JVI.01120-10
140. Akinsiku OT, Bansal A, Sabbaj S, Heath SL, Goepfert PA. Interleukin-2 production by polyfunctional HIV-1-specific CD8 T cells is associated with enhanced viral suppression. *J Acquir Immune Defic Syndr* (2011) **58**(2):132–40. doi:10.1097/QAI.0b013e318224d2e9
141. Hersperger AR, Pereyra F, Nason M, Demers K, Sheth P, Shin LY, et al. Perforin expression directly ex vivo by HIV-specific CD8 T-cells is a correlate of HIV elite control. *PLoS Pathog* (2010) **6**(5):e1000917. doi:10.1371/journal.ppat.1000917
142. Pereyra F, Addo MM, Kaufmann DE, Liu Y, Miura T, Rathod A, et al. Genetic and immunologic heterogeneity among persons who control HIV infection in the absence of therapy. *J Infect Dis* (2008) **197**(4):563–71. doi:10.1086/526786
143. Shan L, Deng K, Shroff NS, Durand CM, Rabi SA, Yang HC, et al. Stimulation of HIV-1-specific cytolytic T lymphocytes facilitates elimination of latent viral reservoir after virus reactivation. *Immunity* (2012) **36**(3):491–501. doi:10.1016/j.immuni.2012.01.014
144. Pereyra F, Palmer S, Miura T, Block BL, Wiegand A, Rothchild AC, et al. Persistent low-level viremia in HIV-1 elite controllers and relationship to immunologic parameters. *J Infect Dis* (2009) **200**(6):984–90. doi:10.1086/605446
145. Graf EH, Mexas AM, Yu JJ, Shaheen F, Liszewski MK, Di Mascio M, et al. Elite suppressors harbor low levels of integrated HIV DNA and high levels of 2-LTR circular HIV DNA compared to HIV+ patients on and off HAART. *PLoS Pathog* (2011) **7**(2):e1001300. doi:10.1371/journal.ppat.1001300
146. Blankson JN, Bailey JR, Thayil S, Yang HC, Lassen K, Lai J, et al. Isolation and characterization of replication-competent human immunodeficiency virus type 1 from a subset of elite suppressors. *J Virol* (2007) **81**(5):2508–18. doi:10.1128/JVI.02165-06
147. Buckheit RW III, Siliciano RF, Blankson JN. Primary CD8+ T cells from elite suppressors effectively eliminate non-productively HIV-1 infected resting and activated CD4+ T cells. *Retrovirology* (2013) **10**:68. doi:10.1186/1742-4690-10-68
148. Buckheit RW III, Salgado M, Silciano RF, Blankson JN. Inhibitory potential of subpopulations of CD8+ T cells in HIV-1-infected elite suppressors. *J Virol* (2012) **86**(24):13679–88. doi:10.1128/JVI.02439-12
149. O'Connell KA, Bailey JR, Blankson JN. Elucidating the elite: mechanisms of control in HIV-1 infection. *Trends Pharmacol Sci* (2009) **30**(12):631–7. doi:10.1016/j.tips.2009.09.005
150. Ndhlovu ZM, Proudfoot J, Cesa K, Alvino DM, McMullen A, Vine S, et al. Elite controllers with low to absent effector CD8+ T cell responses maintain highly functional, broadly directed central memory responses. *J Virol* (2012) **86**(12):6959–69. doi:10.1128/JVI.00531-12
151. van Grevenynghe J, Procopio FA, He Z, Chomont N, Riou C, Zhang Y, et al. Transcription factor FOXO3a controls the persistence of memory CD4(+) T cells during HIV infection. *Nat Med* (2008) **14**(3):266–74. doi:10.1038/nm1728
152. Borrow P, Bhardwaj N. Innate immune responses in primary HIV-1 infection. *Curr Opin HIV AIDS* (2008) **3**(1):36–44. doi:10.1097/COH.0b013e3282f2bce7
153. Montoya CJ, Velilla PA, Chougnet C, Landay AL, Rugeles MT. Increased IFN-gamma production by NK and CD3+/CD56+ cells in sexually HIV-1-exposed but uninfected individuals. *Clin Immunol* (2006) **120**(2):138–46. doi:10.1016/j.clim.2006.02.008
154. Ravet S, Scott-Algara D, Bonnet E, Tran HK, Tran T, Nguyen N, et al. Distinctive NK-cell receptor repertoires sustain high-level constitutive NK-cell activation in HIV-exposed uninfected individuals. *Blood* (2007) **109**(10):4296–305. doi:10.1182/blood-2006-08-040238
155. Scott-Algara D, Truong LX, Versmisse P, David A, Luong TT, Nguyen NV, et al. Cutting edge: increased NK cell activity in HIV-1-exposed but uninfected Vietnamese intravascular drug users. *J Immunol* (2003) **171**(11):5663–7. doi:10.4049/jimmunol.171.11.5663
156. Boulet S, Song R, Kamya P, Bruneau J, Shoukry NH, Tsoukas CM, et al. HIV protective KIR3DL1 and HLA-B genotypes influence NK cell function following stimulation with HLA-devoid cells. *J Immunol* (2010) **184**(4):2057–64. doi:10.4049/jimmunol.0902621

157. Martin MP, Gao X, Lee JH, Nelson GW, Detels R, Goedert JJ, et al. Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. *Nat Genet* (2002) **31**(4):429–34.
158. Nuvor SV, van der Sande M, Rowland-Jones S, Whittle H, Jaye A. Natural killer cell function is well preserved in asymptomatic human immunodeficiency virus type 2 (HIV-2) infection but similar to that of HIV-1 infection when CD4 T-cell counts fall. *J Virol* (2006) **80**(5):2529–38. doi:10.1128/JVI.80.5.2529-2538.2006
159. Iqbal SM, Ball TB, Kimani J, Kiama P, Thottingal P, Embree JE, et al. Elevated T cell counts and RANTES expression in the genital mucosa of HIV-1-resistant Kenyan commercial sex workers. *J Infect Dis* (2005) **192**(5):728–38. doi:10.1086/432482
160. Heeney JL, Dalgleish AG, Weiss RA. Origins of HIV and the evolution of resistance to AIDS. *Science* (2006) **313**(5786):462–6. doi:10.1126/science.1123016
161. Cocchi F, DeVico AL, Garzino-Demo A, Arya SK, Gallo RC, Lusso P. Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. *Science* (1995) **270**(5243):1811–5. doi:10.1126/science.270.5243.1811
162. Amella CA, Sherry B, Shepp DH, Schmidtmayerova H. Macrophage inflammatory protein 1alpha inhibits postentry steps of human immunodeficiency virus type 1 infection via suppression of intracellular cyclic AMP. *J Virol* (2005) **79**(9):5625–31. doi:10.1128/JVI.79.9.5625-5631.2005
163. Saunders KO, Ward-Caviness C, Schutte RJ, Freel SA, Overman RG, Thielman NM, et al. Secretion of MIP-1beta and MIP-1alpha by CD8(+) T-lymphocytes correlates with HIV-1 inhibition independent of coreceptor usage. *Cell Immunol* (2011) **266**(2):154–64. doi:10.1016/j.cellimm.2010.09.011
164. Iqbal SM, Kaul R. Mucosal innate immunity as a determinant of HIV susceptibility. *Am J Reprod Immunol* (2008) **59**(1):44–54. doi:10.1111/j.1600-0897.2007.00563.x
165. Sibeko S, Makvandi-Nejad S. From the laboratory to clinical trials and back again: lessons learned from HIV prevention trials. *Am J Reprod Immunol* (2013) **69**(Suppl 1):106–15. doi:10.1111/aji.12045
166. Miyazawa M, Lopalco L, Mazzotta F, Lo Caputo S, Veas F, Clerici M, et al. The "immunologic advantage" of HIV-exposed seronegative individuals. *AIDS* (2009) **23**(2):161–75. doi:10.1097/QAD.0b013e32832d74ca
167. Erickson AL, Willberg CB, McMahan V, Liu A, Buchbinder SP, Grohskopf LA, et al. Potentially exposed but uninfected individuals produce cytotoxic and polyfunctional human immunodeficiency virus type 1-specific CD8(+) T-cell responses which can be defined to the epitope level. *Clin Vaccine Immunol* (2008) **15**(11):1745–8. doi:10.1128/CVI.00247-08
168. Clerici M, Levin JM, Kessler HA, Harris A, Berzofsky JA, Landay AL, et al. HIV-specific T-helper activity in seronegative health care workers exposed to contaminated blood. *JAMA* (1994) **271**(1):42–6. doi:10.1001/jama.271.1.42
169. Pinto LA, Sullivan J, Berzofsky JA, Clerici M, Kessler HA, Landay AL, et al. ENV-specific cytotoxic T lymphocyte responses in HIV seronegative health care workers occupationally exposed to HIV-contaminated body fluids. *J Clin Invest* (1995) **96**(2):867–76. doi:10.1172/JCI118133
170. Pallikkuth S, Wanchu A, Bhatnagar A, Sachdeva RK, Sharma M. Human immunodeficiency virus (HIV) gag antigen-specific T-helper and granule-dependent CD8 T-cell activities in exposed but uninfected heterosexual partners of HIV type 1-infected individuals in North India. *Clin Vaccine Immunol* (2007) **14**(9):1196–202. doi:10.1128/CVI.0488-06
171. Ritchie AJ, Campion SL, Kopycinski J, Moodie Z, Wang ZM, Pandya K, et al. Differences in HIV-specific T cell responses between HIV-exposed and -unexposed HIV-seronegative individuals. *J Virol* (2011) **85**(7):3507–16. doi:10.1128/JVI.02444-10
172. Pala P, Serwanga J, Watera C, Ritchie AJ, Moodie Z, Wang M, et al. Quantitative and qualitative differences in the T cell response to HIV in uninfected Ugandans exposed or unexposed to HIV-infected partners. *J Virol* (2013) **87**(16):9053–63. doi:10.1128/JVI.00721-13
173. Kaul R, Plummer FA, Kimani J, Dong T, Kiama P, Rostron T, et al. HIV-1-specific mucosal CD8+ lymphocyte responses in the cervix of HIV-1-resistant prostitutes in Nairobi. *J Immunol* (2000) **164**(3):1602–11. doi:10.4049/jimmunol.164.3.1602
174. Critchfield JW, Young DH, Hayes TL, Braun JV, Garcia JC, Pollard RB, et al. Magnitude and complexity of rectal mucosa HIV-1-specific CD8+ T-cell responses during chronic infection reflect clinical status. *PLoS One* (2008) **3**(10):e3577. doi:10.1371/journal.pone.0003577
175. Ferre AL, Hunt PW, McConnell DH, Morris MM, Garcia JC, Pollard RB, et al. HIV controllers with HLA-DRB1*13 and HLA-DQB1*06 alleles have strong, polyfunctional mucosal CD4+ T-cell responses. *J Virol* (2010) **84**(21):11020–9. doi:10.1128/JVI.00980-10
176. Nguyen M, Pean P, Lopalco L, Nouhin J, Phoung V, Ly N, et al. HIV-specific antibodies but not t-cell responses are associated with protection in seronegative partners of HIV-1-infected individuals in Cambodia. *J Acquir Immune Defic Syndr* (2006) **42**(4):412–9. doi:10.1097/01.qai.0000222289.97825.35
177. Lopalco L, Barassi C, Paolucci C, Breda D, Brunelli D, Nguyen M, et al. Predictive value of anti-cell and anti-human immunodeficiency virus (HIV) humoral responses in HIV-1-exposed seronegative cohorts of European and Asian origin. *J Gen Virol* (2005) **86**(Pt 2):339–48. doi:10.1099/vir.0.80585-0
178. Devito C, Hinkula J, Kaul R, Kimani J, Kiama P, Lopalco L, et al. Cross-clade HIV-1-specific neutralizing IgA in mucosal and systemic compartments of HIV-1-exposed, persistently seronegative subjects. *J Acquir Immune Defic Syndr* (2002) **30**(4):413–20. doi:10.1097/00042560-200208010-00007
179. Devito C, Hinkula J, Kaul R, Lopalco L, Bwayo JJ, Plummer F, et al. Mucosal and plasma IgA from HIV-exposed seronegative individuals neutralize a primary HIV-1 isolate. *AIDS* (2000) **14**(13):1917–20. doi:10.1097/00002030-200009080-00006
180. Alfsen A, Iniguez P, Bouguyon E, Bomsel M. Secretory IgA specific for a conserved epitope on gp41 envelope glycoprotein inhibits epithelial transcytosis of HIV-1. *J Immunol* (2001) **166**(10):6257–65. doi:10.4049/jimmunol.166.10.6257
181. Devito C, Broliden K, Kaul R, Svensson L, Johansen K, Kiama P, et al. Mucosal and plasma IgA from HIV-1-exposed uninfected individuals inhibit HIV-1 transcytosis across human epithelial cells. *J Immunol* (2000) **165**(9):5170–6. doi:10.4049/jimmunol.165.9.5170
182. Hladik F, McElrath MJ. Setting the stage: host invasion by HIV. *Nat Rev Immunol* (2008) **8**(6):447–57. doi:10.1038/nri2302
183. Tudor D, Derrien M, Diomede L, Drillet AS, Houimel M, Moog C, et al. HIV-1 gp41-specific monoclonal mucosal IgAs derived from highly exposed but IgG-seronegative individuals block HIV-1 epithelial transcytosis and neutralize CD4(+) cell infection: an IgA gene and functional analysis. *Mucosal Immunol* (2009) **2**(5):412–26. doi:10.1038/mi.2009.89
184. Clerici M, Barassi C, Devito C, Pastori C, Piconi S, Trabattoni D, et al. Serum IgA of HIV-exposed uninfected individuals inhibit HIV through recognition of a region within the alpha-helix of gp41. *AIDS* (2002) **16**(13):1731–41. doi:10.1097/00002030-200209060-00004
185. Belec L, Ghys PD, Hocini H, Nkengasong JN, Tranchot-Diallo J, Diallo MO, et al. Cervicovaginal secretory antibodies to human immunodeficiency virus type 1 (HIV-1) that block viral transcytosis through tight epithelial barriers in highly exposed HIV-1-seronegative African women. *J Infect Dis* (2001) **184**(11):1412–22. doi:10.1086/324375
186. Mazzoli S, Lopalco L, Salvi A, Trabattoni D, Lo Caputo S, Semplici F, et al. Human immunodeficiency virus (HIV)-specific IgA and HIV neutralizing activity in the serum of exposed seronegative partners of HIV-seropositive persons. *J Infect Dis* (1999) **180**(3):871–5. doi:10.1086/314934
187. Mazzoli S, Trabattoni D, Lo Caputo S, Piconi S, Ble C, Meacci F, et al. HIV-specific mucosal and cellular immunity in HIV-seronegative partners of HIV-seropositive individuals. *Nat Med* (1997) **3**(11):1250–7. doi:10.1038/nm1197-1250
188. Kaul R, Plummer F, Clerici M, Bomsel M, Lopalco L, Broliden K. Mucosal IgA in exposed, uninfected subjects: evidence for a role in protection against HIV infection. *AIDS* (2001) **15**(3):431–2. doi:10.1097/00002030-200102160-00026
189. Lo Caputo S, Trabattoni D, Vichi F, Piconi S, Lopalco L, Villa ML, et al. Mucosal and systemic HIV-1-specific immunity in HIV-1-exposed but uninfected heterosexual men. *AIDS* (2003) **17**(4):531–9. doi:10.1097/00002030-200303070-00008
190. Choi RY, Levinson P, Guthrie BL, Lohman-Payne B, Bosire R, Liu AY, et al. Cervicovaginal HIV-1-neutralizing immunoglobulin A detected among HIV-1-exposed seronegative female partners in HIV-1-discordant couples. *AIDS* (2012) **26**(17):2155–63. doi:10.1097/QAD.0b013e328359b99b
191. Migueles SA, Connors M. Long-term nonprogressive disease among untreated HIV-infected individuals: clinical implications of understanding immune control of HIV. *JAMA* (2010) **304**(2):194–201. doi:10.1001/jama.2010.925

192. Alexander R, Mestecky J. Neutralizing antibodies in mucosal secretions: IgG or IgA? *Curr HIV Res* (2007) **5**(6):588–93. doi:10.2174/157016207782418452
193. Priddy FH, Brown D, Kublin J, Monahan K, Wright DP, Lalezari J, et al. Safety and immunogenicity of a replication-incompetent adenovirus type 5 HIV-1 clade B gag/pol/nef vaccine in healthy adults. *Clin Infect Dis* (2008) **46**(11):1769–81. doi:10.1086/587993
194. Shin H, Iwasaki A. A vaccine strategy that protects against genital herpes by establishing local memory T cells. *Nature* (2012) **491**(7424):463–7. doi:10.1038/nature11522
195. Jespers V, Harandi AM, Hinkula J, Medaglini D, Le Grand R, Stahl-Hennig C, et al. Assessment of mucosal immunity to HIV-1. *Expert Rev Vaccines* (2010) **9**(4):381–94. doi:10.1586/erv.10.21
196. Spentzou A, Bergin P, Gill D, Cheeseman H, Ashraf A, Kaltsidis H, et al. Viral inhibition assay: a CD8 T cell neutralization assay for use in clinical trials of HIV-1 vaccine candidates. *J Infect Dis* (2010) **201**(5):720–9. doi:10.1086/650492
197. Martins MA, Wilson NA, Reed JS, Ahn CD, Klimentidis YC, Allison DB, et al. T-cell correlates of vaccine efficacy after a heterologous simian immunodeficiency virus challenge. *J Virol* (2010) **84**(9):4352–65. doi:10.1128/JVI.02365-09
198. Yamamoto T, Johnson MJ, Price DA, Wolinsky DI, Almeida JR, Petrovas C, et al. Virus inhibition activity of effector memory CD8(+) T cells determines simian immunodeficiency virus load in vaccinated monkeys after vaccine breakthrough infection. *J Virol* (2012) **86**(10):5877–84. doi:10.1128/JVI.00315-12
199. Slichter CK, Friedrich DP, Smith RJ, Walsh PN, Mize G, Czartoski JL, et al. Measuring inhibition of HIV replication by ex vivo CD8 T cells. *J Immunol Methods* (2013) **404**:71–80. doi:10.1016/j.jim.2013.12.006
200. Naarding MA, Fernandez N, Kappes JC, Hayes P, Ahmed T, Icyuz M, et al. Development of a luciferase based viral inhibition assay to evaluate vaccine induced CD8 T-cell responses. *J Immunol Methods* (2013). doi:10.1016/j.jim.2013.11.021
201. Yang H, Wu H, Hancock G, Clutton G, Sande N, Xu X, et al. Antiviral inhibitory capacity of CD8+ T cells predicts the rate of CD4+ T-cell decline in HIV-1 infection. *J Infect Dis* (2012) **206**(4):552–61. doi:10.1093/infdis/jis379
202. Staprans SI, Barry AP, Silvestri G, Safrit JT, Kozyr N, Sumpter B, et al. Enhanced SIV replication and accelerated progression to AIDS in macaques primed to mount a CD4 T cell response to the SIV envelope protein. *Proc Natl Acad Sci U S A* (2004) **101**(35):13026–31. doi:10.1073/pnas.0404739101
203. Tenbusch M, Ignatius R, Temchura V, Nabi G, Tippler B, Stewart-Jones G, et al. Risk of immunodeficiency virus infection may increase with vaccine-induced immune response. *J Virol* (2012) **86**(19):10533–9. doi:10.1128/JVI.00796-12
204. Celum CL. The interaction between herpes simplex virus and human immunodeficiency virus. *Herpes* (2004) **11**(Suppl 1):36A–45A.
205. Van de Perre P, Segondy M, Foulongne V, Ouedraogo A, Konate I, Huraux JM, et al. Herpes simplex virus and HIV-1: deciphering viral synergy. *Lancet Infect Dis* (2008) **8**(8):490–7. doi:10.1016/S1473-3099(08)70181-6
206. Freeman EE, Weiss HA, Glynn JR, Cross PL, Whitworth JA, Hayes RJ. Herpes simplex virus 2 infection increases HIV acquisition in men and women: systematic review and meta-analysis of longitudinal studies. *AIDS* (2006) **20**(1):73–83. doi:10.1097/01.aids.0000198081.09337.a7
207. Rebbapragada A, Wachihi C, Pettengell C, Sunderji S, Huibner S, Jaoko W, et al. Negative mucosal synergy between Herpes simplex type 2 and HIV in the female genital tract. *AIDS* (2007) **21**(5):589–98. doi:10.1097/QAD.0b013e328012b896
208. Pantaleo G, Menzo S, Vaccarezza M, Graziosi C, Cohen OJ, Demarest JF, et al. Studies in subjects with long-term nonprogressive human immunodeficiency virus infection. *N Engl J Med* (1995) **332**(4):209–16. doi:10.1056/NEJM199501263320402
209. Miller CJ, Lu FX. Anti-HIV and -SIV immunity in the vagina. *Int Rev Immunol* (2003) **22**(1):65–76. doi:10.1080/08830180305230
210. Scamurra RW, Nelson DB, Lin XM, Miller DJ, Silverman GJ, Kappel T, et al. Mucosal plasma cell repertoire during HIV-1 infection. *J Immunol* (2002) **169**(7):4008–16. doi:10.4049/jimmunol.169.7.4008
211. Piantadosi A, Panteleeff D, Blish CA, Baeten JM, Jaoko W, McClelland RS, et al. Breadth of neutralizing antibody response to human immunodeficiency virus type 1 is affected by factors early in infection but does not influence disease progression. *J Virol* (2009) **83**(19):10269–74. doi:10.1128/JVI.01149-09
212. Simek MD, Rida W, Priddy FH, Pung P, Carrow E, Laufer DS, et al. Human immunodeficiency virus type 1 elite neutralizers: individuals with broad and potent neutralizing activity identified by using a high-throughput neutralization assay together with an analytical selection algorithm. *J Virol* (2009) **83**(14):7337–48. doi:10.1128/JVI.00110-09
213. Euler Z, van Gils MJ, Bunnik EM, Phung P, Schweighardt B, Wrin T, et al. Cross-reactive neutralizing humoral immunity does not protect from HIV type 1 disease progression. *J Infect Dis* (2010) **201**(7):1045–53. doi:10.1086/651144
214. Bailey JR, Lassen KG, Yang HC, Quinn TC, Ray SC, Blankson JN, et al. Neutralizing antibodies do not mediate suppression of human immunodeficiency virus type 1 in elite suppressors or selection of plasma virus variants in patients on highly active antiretroviral therapy. *J Virol* (2006) **80**(10):4758–70. doi:10.1128/JVI.80.10.4758-4770.2006
215. Harrer T, Harrer E, Kalams SA, Elbeik T, Staprans SI, Feinberg MB, et al. Strong cytotoxic T cell and weak neutralizing antibody responses in a subset of persons with stable nonprogressing HIV type 1 infection. *AIDS Res Hum Retroviruses* (1996) **12**(7):585–92. doi:10.1089/aid.1996.12.585
216. Walker LM, Huber M, Doores KJ, Falkowska E, Pejchal R, Julien JP, et al. Broad neutralization coverage of HIV by multiple highly potent antibodies. *Nature* (2011) **477**(7365):466–70. doi:10.1038/nature10373
217. Veazey RS, Shattock RJ, Pope M, Kirijan JC, Jones J, Hu Q, et al. Prevention of virus transmission to macaque monkeys by a vaginally applied monoclonal antibody to HIV-1 gp120. *Nat Med* (2003) **9**(3):343–6. doi:10.1038/nm833
218. Hessell AJ, Rakasz EG, Tehrani DM, Huber M, Weisgrau KL, Landucci G, et al. Broadly neutralizing monoclonal antibodies 2F5 and 4E10 directed against the human immunodeficiency virus type 1 gp41 membrane-proximal external region protect against mucosal challenge by simian-human immunodeficiency virus SHIVBa-L. *J Virol* (2010) **84**(3):1302–13. doi:10.1128/JVI.01272-09
219. Hessell AJ, Poignard P, Hunter M, Hangartner L, Tehrani DM, Bleeker WK, et al. Effective, low-titer antibody protection against low-dose repeated mucosal SHIV challenge in macaques. *Nat Med* (2009) **15**(8):951–4. doi:10.1038/nm.1974
220. Hessell AJ, Rakasz EG, Poignard P, Hangartner L, Landucci G, Forthal DN, et al. Broadly neutralizing human anti-HIV antibody 2G12 is effective in protection against mucosal SHIV challenge even at low serum neutralizing titers. *PLoS Pathog* (2009) **5**(5):e1000433. doi:10.1371/journal.ppat.1000433
221. Conley AJ, Kessler JA II, Boots LJ, McKenna PM, Schleif WA, Emini EA, et al. The consequence of passive administration of an anti-human immunodeficiency virus type 1 neutralizing monoclonal antibody before challenge of chimpanzees with a primary virus isolate. *J Virol* (1996) **70**(10):6751–8.
222. Moldt B, Rakasz EG, Schultz N, Chan-Hui PY, Swiderek K, Weisgrau KL, et al. Highly potent HIV-specific antibody neutralization in vitro translates into effective protection against mucosal SHIV challenge in vivo. *Proc Natl Acad Sci U S A* (2012) **109**(46):18921–5. doi:10.1073/pnas.1214785109
223. Ruprecht RM. Passive immunization with human neutralizing monoclonal antibodies against HIV-1 in macaque models: experimental approaches. *Methods Mol Biol* (2009) **525**:559–66, xiv. doi:10.1007/978-1-59745-554-1_31
224. Pace CS, Song R, Ochsenbauer C, Andrews CD, Franco D, Yu J, et al. Bispecific antibodies directed to CD4 domain 2 and HIV envelope exhibit exceptional breadth and picomolar potency against HIV-1. *Proc Natl Acad Sci U S A* (2013) **110**(33):13540–5. doi:10.1073/pnas.1304985110
225. Trkola A, Kuster H, Rusert P, Joos B, Fischer M, Leemann C, et al. Delay of HIV-1 rebound after cessation of antiretroviral therapy through passive transfer of human neutralizing antibodies. *Nat Med* (2005) **11**(6):615–22. doi:10.1038/nm1244
226. Mehandru S, Vcelar B, Wrin T, Stiegler G, Joos B, Mohri H, et al. Adjunctive passive immunotherapy in human immunodeficiency virus type 1-infected individuals treated with antiviral therapy during acute and early infection. *J Virol* (2007) **81**(20):11016–31. doi:10.1128/JVI.01739-06
227. Spearman P, Lally MA, Elizaga M, Montefiori D, Tomaras GD, McElrath MJ, et al. A trimeric, V2-deleted HIV-1 envelope glycoprotein vaccine elicits potent neutralizing antibodies but limited breadth of neutralization in human volunteers. *J Infect Dis* (2011) **203**(8):1165–73. doi:10.1093/infdis/jiq175
228. Srivastava IK, Stamatatos L, Kan E, Vajdy M, Lian Y, Hilt S, et al. Purification, characterization, and immunogenicity of a soluble trimeric envelope protein containing a partial deletion of the V2 loop derived from SF162, an R5-tropic human immunodeficiency virus type 1 isolate. *J Virol* (2003) **77**(20):11244–59. doi:10.1128/JVI.77.20.11244-11259.2003

229. Barnett SW, Srivastava IK, Ulmer JB, Donnelly JJ, Rappuoli R. Development of V2-deleted trimeric envelope vaccine candidates from human immunodeficiency virus type 1 (HIV-1) subtypes B and C. *Microbes Infect* (2005) **7**(14):1386–91. doi:10.1016/j.micinf.2005.07.018
230. Czerkinsky C, Holmgren J. Mucosal delivery routes for optimal immunization: targeting immunity to the right tissues. *Curr Top Microbiol Immunol* (2012) **354**:1–18. doi:10.1007/82_2010_112
231. Masopust D, Vezys V, Marzo AL, Lefrancois L. Preferential localization of effector memory cells in nonlymphoid tissue. *Science* (2001) **291**(5512):2413–7. doi:10.1126/science.1058867
232. Kiyono H, Fukuyama S. NALT- versus Peyer's-patch-mediated mucosal immunity. *Nat Rev Immunol* (2004) **4**(9):699–710. doi:10.1038/nri1439
233. Ranasinghe C, Turner SJ, McArthur C, Sutherland DB, Kim JH, Doherty PC, et al. Mucosal HIV-1 pox virus prime-boost immunization induces high-avidity CD8+ T cells with regime-dependent cytokine/granzyme B profiles. *J Immunol* (2007) **178**(4):2370–9. doi:10.4049/jimmunol.178.12.8221
234. Yuki Y, Nochi T, Kiyono H. Progress towards an AIDS mucosal vaccine: an overview. *Tuberculosis* (2007) **87**(Suppl 1):S35–44. doi:10.1016/j.tube.2007.05.005
235. Hutchinson E. Vaccination: oral vaccine induces genitorectal immunity. *Nat Rev Immunol* (2012) **12**(9):619. doi:10.1038/nri3283
236. Petrarca C, Lazzarin F, Pannellini T, Iezzi M, Braga M, Mistrello G, et al. Monomeric allergoid intragastric administration induces local and systemic tolerogenic response involving IL-10-producing CD4(+)CD25(+) T regulatory cells in mice. *Int J Immunopathol Pharmacol* (2010) **23**(4):1021–31.
237. Wu HY, Nguyen HH, Russell MW. Nasal lymphoid tissue (NALT) as a mucosal immune inductive site. *Scand J Immunol* (1997) **46**(5):506–13. doi:10.1046/j.1365-3083.1997.d01-159.x
238. Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. *Nat Med* (2005) **11**(4 Suppl):S45–53. doi:10.1038/nm1213
239. Holmgren J, Czerkinsky C, Eriksson K, Mharandi A. Mucosal immunisation and adjuvants: a brief overview of recent advances and challenges. *Vaccine* (2003) **21**(Suppl 2):S89–95. doi:10.1016/S0264-410X(03)00206-8
240. Wang SW, Bertley FM, Kozlowski PA, Herrmann L, Manson K, Mazzara G, et al. An SHIV DNA/MVA rectal vaccination in macaques provides systemic and mucosal virus-specific responses and protection against AIDS. *AIDS Res Hum Retroviruses* (2004) **20**(8):846–59. doi:10.1089/0889222041725253
241. Vajdy M, Singh M, Kazzaz J, Soenawan E, Ugozzoli M, Zhou F, et al. Mucosal and systemic anti-HIV responses in rhesus macaques following combinations of intranasal and parenteral immunizations. *AIDS Res Hum Retroviruses* (2004) **20**(11):1269–81. doi:10.1089/aid.2004.20.1269
242. Vajdy M. Current efforts on generation of optimal immune responses against HIV through mucosal immunisations. *Drugs R D* (2006) **7**(5):267–88. doi:10.2165/00126839-200607050-00001
243. Barnett SW, Srivastava IK, Kan E, Zhou F, Goodsell A, Cristillo AD, et al. Protection of macaques against vaginal SHIV challenge by systemic or mucosal and systemic vaccinations with HIV-envelope. *AIDS* (2008) **22**(3):339–48. doi:10.1097/QAD.0b013e3282f3ca57
244. Albu DI, Jones-Trower A, Woron AM, Stellrecht K, Broder CC, Metzger DW. Intranasal vaccination using interleukin-12 and cholera toxin subunit B as adjuvants to enhance mucosal and systemic immunity to human immunodeficiency virus type 1 glycoproteins. *J Virol* (2003) **77**(10):5589–97. doi:10.1128/JVI.77.10.5589-5597.2003
245. Devito C, Zuber B, Schroder U, Benthin R, Okuda K, Broliden K, et al. Intranasal HIV-1-gp160-DNA/gp41 peptide prime-boost immunization regimen in mice results in long-term HIV-1 neutralizing humoral mucosal and systemic immunity. *J Immunol* (2004) **173**(11):7078–89. doi:10.4049/jimmunol.173.11.7078
246. Marinaro M, Riccomi A, Rappuoli R, Pizza M, Fiorelli V, Tripiciano A, et al. Mucosal delivery of the human immunodeficiency virus-1 Tat protein in mice elicits systemic neutralizing antibodies, cytotoxic T lymphocytes and mucosal IgA. *Vaccine* (2003) **21**(25–26):3972–81. doi:10.1016/S0264-410X(03)00295-0
247. VanCott TC, Kaminski RW, Mascola JR, Kalyanaraman VS, Wassef NM, Alving CR, et al. HIV-1 neutralizing antibodies in the genital and respiratory tracts of mice intranasally immunized with oligomeric gp160. *J Immunol* (1998) **160**(4):2000–12.
248. Klavinskis LS, Barnfield C, Gao L, Parker S. Intranasal immunization with plasmid DNA-lipid complexes elicits mucosal immunity in the female genital and rectal tracts. *J Immunol* (1999) **162**(1):254–62.
249. Wang X, Uto T, Akagi T, Akashi M, Baba M. Induction of potent CD8+ T-cell responses by novel biodegradable nanoparticles carrying human immunodeficiency virus type 1 gp120. *J Virol* (2007) **81**(18):10009–16. doi:10.1128/JVI.00489-07
250. Miyake A, Akagi T, Enose Y, Ueno M, Kawamura M, Horiuchi R, et al. Induction of HIV-specific antibody response and protection against vaginal SHIV transmission by intranasal immunization with inactivated SHIV-capturing nanospheres in macaques. *J Med Virol* (2004) **73**(3):368–77. doi:10.1002/jmv.20100
251. Akagi T, Kawamura M, Ueno M, Hiraishi K, Adachi M, Serizawa T, et al. Mucosal immunization with inactivated HIV-1-capturing nanospheres induces a significant HIV-1-specific vaginal antibody response in mice. *J Med Virol* (2003) **69**(2):163–72. doi:10.1002/jmv.10279
252. Manrique M, Kozlowski PA, Cobo-Molinos A, Wang SW, Wilson RL, Martinez-Viedma Mdel P, et al. Resistance to infection, early and persistent suppression of simian immunodeficiency virus SIVmac251 viremia, and significant reduction of tissue viral burden after mucosal vaccination in female rhesus macaques. *J Virol* (2014) **88**(1):212–24. doi:10.1128/JVI.02523-13
253. Manrique M, Kozlowski PA, Cobo-Molinos A, Wang SW, Wilson RL, Montefiori DC, et al. Immunogenicity of a vaccine regimen composed of simian immunodeficiency virus DNA, rMVA, and viral particles administered to female rhesus macaques via four different mucosal routes. *J Virol* (2013) **87**(8):4738–50. doi:10.1128/JVI.03531-12
254. Manrique M, Kozlowski PA, Cobo-Molinos A, Wang SW, Wilson RL, Montefiori DC, et al. Long-term control of simian immunodeficiency virus mac251 viremia to undetectable levels in half of infected female rhesus macaques nasally vaccinated with simian immunodeficiency virus DNA/recombinant modified vaccinia virus Ankara. *J Immunol* (2011) **186**(6):3581–93. doi:10.4049/jimmunol.1002594
255. Manrique M, Kozlowski PA, Wang SW, Wilson RL, Micewicz E, Montefiori DC, et al. Nasal DNA-MVA SIV vaccination provides more significant protection from progression to AIDS than a similar intramuscular vaccination. *Mucosal Immunol* (2009) **2**(6):536–50. doi:10.1038/mi.2009.103
256. Manrique M, Micewicz E, Kozlowski PA, Wang SW, Aurora D, Wilson RL, et al. DNA-MVA vaccine protection after X4 SHIV challenge in macaques correlates with day-of-challenge antiviral CD4+ cell-mediated immunity levels and postchallenge preservation of CD4+ T cell memory. *AIDS Res Hum Retroviruses* (2008) **24**(3):505–19. doi:10.1089/aid.2007.0191
257. Li Z, Zhang M, Zhou C, Zhao X, Iijima N, Frankel FR. Novel vaccination protocol with two live mucosal vectors elicits strong cell-mediated immunity in the vagina and protects against vaginal virus challenge. *J Immunol* (2008) **180**(4):2504–13. doi:10.4049/jimmunol.180.4.2504
258. Reinhardt RL, Khoruts A, Merica R, Zell T, Jenkins MK. Visualizing the generation of memory CD4 T cells in the whole body. *Nature* (2001) **410**(6824):101–5. doi:10.1038/35065111
259. Garcia-Sastre A, Palese P. Influenza virus vectors. *Biologicals* (1995) **23**(2):171–8. doi:10.1006/biol.1995.0028
260. Palese P, Zavala F, Muster T, Nussenzweig RS, Garcia-Sastre A. Development of novel influenza virus vaccines and vectors. *J Infect Dis* (1997) **176**(Suppl 1):S45–9. doi:10.1086/514175
261. Li J, Arevalo MT, Zeng M. Engineering influenza viral vectors. *Bioengineered* (2013) **4**(1):9–14. doi:10.4161/bioe.21950
262. Li S, Polonis V, Isobe H, Zaghouani H, Guinea R, Moran T, et al. Chimeric influenza virus induces neutralizing antibodies and cytotoxic T cells against human immunodeficiency virus type 1. *J Virol* (1993) **67**(11):6659–66.
263. Muster T, Steindl F, Purtscher M, Trkola A, Klima A, Himmler G, et al. A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. *J Virol* (1993) **67**(11):6642–7.
264. Muster T, Guinea R, Trkola A, Purtscher M, Klima A, Steindl F, et al. Cross-neutralizing activity against divergent human immunodeficiency virus type 1 isolates induced by the gp41 sequence ELDKWAS. *J Virol* (1994) **68**(6):4031–4.
265. Muster T, Ferko B, Klima A, Purtscher M, Trkola A, Schulz P, et al. Mucosal model of immunization against human immunodeficiency virus type 1 with a chimeric influenza virus. *J Virol* (1995) **69**(11):6678–86.
266. Sexton A, De Rose R, Reece JC, Alcantara S, Loh L, Moffat JM, et al. Evaluation of recombinant influenza virus-simian immunodeficiency virus vaccines in macaques. *J Virol* (2009) **83**(15):7619–28. doi:10.1128/JVI.00470-09
267. Gherardi MM, Najera JL, Perez-Jimenez E, Guerra S, Garcia-Sastre A, Esteban M. Prime-boost immunization schedules based on influenza virus and vaccinia virus vectors potentiate cellular immune responses against human

immunodeficiency virus Env protein systemically and in the genitorectal draining lymph nodes. *J Virol* (2003) **77**(12):7048–57. doi:10.1128/JVI.77.12.7048-7057.2003

268. Gherardi MM, Esteban M. Recombinant poxviruses as mucosal vaccine vectors. *J Gen Virol* (2005) **86**(Pt 11):2925–36. doi:10.1099/vir.0.81181-0
269. Gherardi MM, Esteban M. Mucosal and systemic immune responses induced after oral delivery of vaccinia virus recombinants. *Vaccine* (1999) **17**(9–10):1074–83. doi:10.1016/S0264-410X(98)00324-7
270. Belyakov IM, Derby MA, Ahlers JD, Kelsall BL, Earl P, Moss B, et al. Mucosal immunization with HIV-1 peptide vaccine induces mucosal and systemic cytotoxic T lymphocytes and protective immunity in mice against intrarectal recombinant HIV-vaccinia challenge. *Proc Natl Acad Sci U S A* (1998) **95**(4):1709–14. doi:10.1073/pnas.95.4.1709
271. Bertley FM, Kozlowski PA, Wang SW, Chappelle J, Patel J, Sonuyi O, et al. Control of simian/human immunodeficiency virus viremia and disease progression after IL-2-augmented DNA-modified vaccinia virus Ankara nasal vaccination in nonhuman primates. *J Immunol* (2004) **172**(6):3745–57. doi:10.4049/jimmunol.172.6.3745
272. Stevceva L, Alvarez X, Lackner AA, Tryniszewska E, Kelsall B, Nacsa J, et al. Both mucosal and systemic routes of immunization with the live, attenuated NYVAC/simian immunodeficiency virus SIV(gpe) recombinant vaccine result in gag-specific CD8(+) T-cell responses in mucosal tissues of macaques. *J Virol* (2002) **76**(22):11659–76. doi:10.1128/JVI.76.22.11659-11676.2002
273. Kaufman DR, Liu J, Carville A, Mansfield KG, Havenga MJ, Goudsmit J, et al. Trafficking of antigen-specific CD8+ T lymphocytes to mucosal surfaces following intramuscular vaccination. *J Immunol* (2008) **181**(6):4188–98. doi:10.4049/jimmunol.181.6.4188
274. Tatsis N, Lin SW, Harris-McCoy K, Garber DA, Feinberg MB, Ertl HC. Multiple immunizations with adenovirus and MVA vectors improve CD8+ T cell functionality and mucosal homing. *Virology* (2007) **367**(1):156–67. doi:10.1016/j.virol.2007.05.028
275. Baig J, Levy DB, McKay PF, Schmitz JE, Santra S, Subbramanian RA, et al. Elicitation of simian immunodeficiency virus-specific cytotoxic T lymphocytes in mucosal compartments of rhesus monkeys by systemic vaccination. *J Virol* (2002) **76**(22):11484–90. doi:10.1128/JVI.76.22.11484-11490.2002
276. Barouch DH, Liu J, Li H, Maxfield LF, Abbink P, Lynch DM, et al. Vaccine protection against acquisition of neutralization-resistant SIV challenges in rhesus monkeys. *Nature* (2012) **482**(7383):89–93. doi:10.1038/nature10766
277. Yuki Y, Kiyono H. New generation of mucosal adjuvants for the induction of protective immunity. *Rev Med Virol* (2003) **13**(5):293–310. doi:10.1002/rmv.398
278. Belyakov IM, Hel Z, Kelsall B, Kuznetsov VA, Ahlers JD, Nacsa J, et al. Mucosal AIDS vaccine reduces disease and viral load in gut reservoir and blood after mucosal infection of macaques. *Nat Med* (2001) **7**(12):1320–6. doi:10.1038/nm1201-1320
279. Gherardi MM, Perez-Jimenez E, Najera JL, Esteban M. Induction of HIV immunity in the genital tract after intranasal delivery of a MVA vector: enhanced immunogenicity after DNA prime-modified vaccinia virus Ankara boost immunization schedule. *J Immunol* (2004) **172**(10):6209–20. doi:10.4049/jimmunol.172.10.6209
280. Bradney CP, Sempowski GD, Liao HX, Haynes BF, Staats HF. Cytokines as adjuvants for the induction of anti-human immunodeficiency virus peptide immunoglobulin G (IgG) and IgA antibodies in serum and mucosal secretions after nasal immunization. *J Virol* (2002) **76**(2):517–24. doi:10.1128/JVI.76.2.517-524.2002
281. Horner AA, Datta SK, Takabayashi K, Belyakov IM, Hayashi T, Cinman N, et al. Immunostimulatory DNA-based vaccines elicit multifaceted immune responses against HIV at systemic and mucosal sites. *J Immunol* (2001) **167**(3):1584–91. doi:10.4049/jimmunol.167.3.1584
282. Dumais N, Patrick A, Moss RB, Davis HL, Rosenthal KL. Mucosal immunization with inactivated human immunodeficiency virus plus CpG oligodeoxynucleotides induces genital immune responses and protection against intravaginal challenge. *J Infect Dis* (2002) **186**(8):1098–105. doi:10.1086/344232
283. Daftarian P, Ali S, Sharan R, Lacey SF, La Rosa C, Longmate J, et al. Immunization with Th-CTL fusion peptide and cytosine-phosphate-guanine DNA in transgenic HLA-A2 mice induces recognition of HIV-infected T cells and clears vaccinia virus challenge. *J Immunol* (2003) **171**(8):4028–39. doi:10.4049/jimmunol.171.8.4028
284. Jiang JQ, Patrick A, Moss RB, Rosenthal KL. CD8+ T-cell-mediated cross-clade protection in the genital tract following intranasal immunization with inactivated human immunodeficiency virus antigen plus CpG oligodeoxynucleotides. *J Virol* (2005) **79**(1):393–400. doi:10.1128/JVI.79.1.393-400.2005
285. Courtney AN, Nehete PN, Nehete BP, Thapa P, Zhou D, Sastry KJ. Alpha-galactosylceramide is an effective mucosal adjuvant for repeated intranasal or oral delivery of HIV peptide antigens. *Vaccine* (2009) **27**(25–26):3335–41. doi:10.1016/j.vaccine.2009.01.083
286. Moser C, Amacker M, Kammer AR, Rasi S, Westerfeld N, Zurbriggen R. Influenza virosomes as a combined vaccine carrier and adjuvant system for prophylactic and therapeutic immunizations. *Expert Rev Vaccines* (2007) **6**(5):711–21. doi:10.1586/14760584.6.5.711
287. Leroux-Roels G, Maes C, Clement F, van Engelenburg F, van den Dobbelsteen M, Adler M, et al. Randomized phase I: safety, immunogenicity and mucosal antiviral activity in young healthy women vaccinated with HIV-1 Gp41 P1 peptide on virosomes. *PLoS One* (2013) **8**(2):e55438. doi:10.1371/journal.pone.0055438
288. Azizi A, Ghunaim H, Diaz-Mitoma F, Mestecky J. Mucosal HIV vaccines: a holy grail or a dud? *Vaccine* (2010) **28**(24):4015–26. doi:10.1016/j.vaccine.2010.04.018
289. Mora JR, von Andrian UH. T-cell homing specificity and plasticity: new concepts and future challenges. *Trends Immunol* (2006) **27**(5):235–43. doi:10.1016/j.it.2006.03.007
290. Zabel BA, Agace WW, Campbell JJ, Heath HM, Parent D, Roberts AI, et al. Human G protein-coupled receptor GPR-9-6/CC chemokine receptor 9 is selectively expressed on intestinal homing T lymphocytes, mucosal lymphocytes, and thymocytes and is required for thymus-expressed chemokine-mediated chemotaxis. *J Exp Med* (1999) **190**(9):1241–56. doi:10.1084/jem.190.9.1241
291. Svensson M, Marsal J, Ericsson A, Carramolino L, Broden T, Marquez G, et al. CCL25 mediates the localization of recently activated CD8alphabeta(+) lymphocytes to the small-intestinal mucosa. *J Clin Invest* (2002) **110**(8):1113–21. doi:10.1172/JCI0215988
292. Dullaers M, Li D, Xue Y, Ni L, Gayet I, Morita R, et al. A T cell-dependent mechanism for the induction of human mucosal homing immunoglobulin A-secreting plasmablasts. *Immunity* (2009) **30**(1):120–9. doi:10.1016/j.immuni.2008.11.008
293. Kunkel EJ, Campbell JJ, Haraldsen G, Pan J, Boisvert J, Roberts AI, et al. Lymphocyte CC chemokine receptor 9 and epithelial thymus-expressed chemokine (TECK) expression distinguish the small intestinal immune compartment: epithelial expression of tissue-specific chemokines as an organizing principle in regional immunity. *J Exp Med* (2000) **192**(5):761–8. doi:10.1084/jem.192.5.761
294. Papadakis KA, Prehn J, Nelson V, Cheng L, Binder SW, Ponath PD, et al. The role of thymus-expressed chemokine and its receptor CCR9 on lymphocytes in the regional specialization of the mucosal immune system. *J Immunol* (2000) **165**(9):5069–76. doi:10.4049/jimmunol.165.9.5069
295. von Andrian UH, Mackay CR. T-cell function and migration. Two sides of the same coin. *N Engl J Med* (2000) **343**(14):1020–34. doi:10.1056/NEJM200010053431407
296. Wagner N, Lohler J, Kunkel EJ, Ley K, Leung E, Krissansen G, et al. Critical role for beta7 integrins in formation of the gut-associated lymphoid tissue. *Nature* (1996) **382**(6589):366–70. doi:10.1038/382366a0
297. Berlin C, Berg EL, Briskin MJ, Andrew DP, Kilshaw PJ, Holzmann B, et al. Alpha 4 beta 7 integrin mediates lymphocyte binding to the mucosal vascular addressin MAdCAM-1. *Cell* (1993) **74**(1):185–95. doi:10.1016/0092-8674(93)90305-A
298. Hamann A, Andrew DP, Jablonski-Westrich D, Holzmann B, Butcher EC. Role of alpha 4-integrins in lymphocyte homing to mucosal tissues in vivo. *J Immunol* (1994) **152**(7):3282–93.
299. Rose JR, Williams MB, Rott LS, Butcher EC, Greenberg HB. Expression of the mucosal homing receptor alpha4beta7 correlates with the ability of CD8+ memory T cells to clear rotavirus infection. *J Virol* (1998) **72**(1):726–30.
300. Mavigner M, Cazabat M, Dubois M, L'Faqihi FE, Requena M, Pasquier C, et al. Altered CD4+ T cell homing to the gut impairs mucosal immune reconstitution in treated HIV-infected individuals. *J Clin Invest* (2012) **122**(1):62–9. doi:10.1172/JCI59011

301. Veazey RS, DeMaria M, Chalifoux LV, Shvetz DE, Pauley DR, Knight HL, et al. Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection. *Science* (1998) **280**(5362):427–31. doi:10.1126/science.280.5362.427
302. Schon MP, Arya A, Murphy EA, Adams CM, Strauch UG, Agace WW, et al. Mucosal T lymphocyte numbers are selectively reduced in integrin alpha E (CD103)-deficient mice. *J Immunol* (1999) **162**(11):6641–9.
303. Shacklett BL, Cox CA, Sandberg JK, Stollman NH, Jacobson MA, Nixon DF. Trafficking of human immunodeficiency virus type 1-specific CD8+ T cells to gut-associated lymphoid tissue during chronic infection. *J Virol* (2003) **77**(10):5621–31. doi:10.1128/JVI.77.10.5621-5631.2003
304. Shacklett BL, Cu-Uvin S, Beadle TJ, Pace CA, Fast NM, Donahue SM, et al. Quantification of HIV-1-specific T-cell responses at the mucosal cervicovaginal surface. *AIDS* (2000) **14**(13):1911–5. doi:10.1097/00002030-200009080-00005
305. Inoue M, Ogawa H, Miyata M, Shiozaki H, Tanizawa O. Expression of E-cadherin in normal, benign, and malignant tissues of female genital organs. *Am J Clin Pathol* (1992) **98**(1):76–80.
306. Kiravu A, Gumbi P, Mkhize NN, Olivier A, Denny L, Passmore JA. Evaluation of CD103 (alphaEbeta7) integrin expression by CD8 T cells in blood as a surrogate marker to predict cervical T cell responses in the female genital tract during HIV infection. *Clin Immunol* (2011) **141**(2):143–51. doi:10.1016/j.clim.2011.06.008
307. Mora JR. Homing imprinting and immunomodulation in the gut: role of dendritic cells and retinoids. *Inflamm Bowel Dis* (2008) **14**(2):275–89. doi:10.1002/ibd.20280
308. Mora JR, Bono MR, Manjunath N, Weninger W, Cavanagh LL, Rosemblatt M, et al. Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. *Nature* (2003) **424**(6944):88–93. doi:10.1038/nature01726
309. Mora JR, Cheng G, Picarella D, Briskin M, Buchanan N, von Andrian UH. Reciprocal and dynamic control of CD8 T cell homing by dendritic cells from skin- and gut-associated lymphoid tissues. *J Exp Med* (2005) **201**(2):303–16. doi:10.1084/jem.20041645
310. Campbell DJ, Butcher EC. Rapid acquisition of tissue-specific homing phenotypes by CD4(+) T cells activated in cutaneous or mucosal lymphoid tissues. *J Exp Med* (2002) **195**(1):135–41. doi:10.1084/jem.20011502
311. Mora JR, Iwata M, Eksteen B, Song SY, Junt T, Senman B, et al. Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. *Science* (2006) **314**(5802):1157–60. doi:10.1126/science.1132742
312. Shacklett BL. Cell-mediated immunity to HIV in the female reproductive tract. *J Reprod Immunol* (2009) **83**(1–2):190–5. doi:10.1016/j.jri.2009.07.012
313. Wira CR, Patel MV, Ghosh M, Mukura L, Fahey JV. Innate immunity in the human female reproductive tract: endocrine regulation of endogenous antimicrobial protection against HIV and other sexually transmitted infections. *Am J Reprod Immunol* (2011) **65**(3):196–211. doi:10.1111/j.1600-0897.2011.00970.x
314. Shukair SA, Allen SA, Cianci GC, Stieh DJ, Anderson MR, Baig SM, et al. Human cervicovaginal mucus contains an activity that hinders HIV-1 movement. *Mucosal Immunol* (2013) **6**(2):427–34. doi:10.1038/mi.2012.87
315. Poonia B, Walter L, Dufour J, Harrison R, Marx PA, Veazey RS. Cyclic changes in the vaginal epithelium of normal rhesus macaques. *J Endocrinol* (2006) **190**(3):829–35. doi:10.1677/joe.1.06873
316. Vishwanathan SA, Guenthner PC, Lin CY, Dobard C, Sharma S, Adams DR, et al. High susceptibility to repeated, low-dose, vaginal SHIV exposure late in the luteal phase of the menstrual cycle of pigtail macaques. *J Acquir Immune Defic Syndr* (2011) **57**(4):261–4. doi:10.1097/QAI.0b013e318220ebd3
317. Veazey RS, Shattock RJ, Klasse PJ, Moore JP. Animal models for microbicide studies. *Curr HIV Res* (2012) **10**(1):79–87. doi:10.2174/157016212799304715
318. Heffron R, Donnell D, Rees H, Celum C, Mugo N, Were E, et al. Use of hormonal contraceptives and risk of HIV-1 transmission: a prospective cohort study. *Lancet Infect Dis* (2012) **12**(1):19–26. doi:10.1016/S1473-3099(11)70247-X
319. Pudney J, Quayle AJ, Anderson DJ. Immunological microenvironments in the human vagina and cervix: mediators of cellular immunity are concentrated in the cervical transformation zone. *Biol Reprod* (2005) **73**(6):1253–63. doi:10.1095/biolreprod.105.043133
320. Xu H, Wang X, Veazey RS. Mucosal immunology of HIV infection. *Immunol Rev* (2013) **254**(1):10–33. doi:10.1111/imr.12072
321. Kasturi SP, Skountzou I, Albrecht RA, Koutsonanos D, Hua T, Nakaya HI, et al. Programming the magnitude and persistence of antibody responses with innate immunity. *Nature* (2011) **470**(7335):543–7. doi:10.1038/nature09737
322. Tregoning JS, Buffa V, Oszmiana A, Klein K, Walters AA, Shattock RJ. A "prime-pull" vaccine strategy has a modest effect on local and systemic antibody responses to HIV gp140 in mice. *PLoS One* (2013) **8**(11):e80559. doi:10.1371/journal.pone.0080559

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.

Received: 10 March 2014; accepted: 23 April 2014; published online: 08 May 2014.
*Citation: Chanzu N and Ondondo B (2014) Induction of potent and long-lived antibody and cellular immune responses in the genitorectal mucosa could be the critical determinant of HIV vaccine efficacy. Front. Immunol. **5**:202. doi: 10.3389/fimmu.2014.00202*
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology.
Copyright © 2014 Chanzu and Ondondo. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 03 July 2014
doi: 10.3389/fmicb.2014.00329

Challenges to the development of vaccines to hepatitis C virus that elicit neutralizing antibodies

Heidi E. Drummer [1,2,3] *

[1] *Viral Fusion Laboratory, Centre for Biomedical Research, Burnet Institute, Melbourne, VIC, Australia.*
[2] *Department of Microbiology, Monash University, Clayton, VIC, Australia*
[3] *Department of Microbiology and Immunology, The Peter Doherty Institute for Infection and Immunity, The University of Melbourne, Parkville, VIC, Australia.*

Edited by:
Eric J. Gowans, The University of Adelaide, Australia

Reviewed by:
Dirk Dittmer, University of North Carolina at Chapel Hill, USA
Janet Mans, University of Pretoria, South Africa

****Correspondence:***
Heidi E. Drummer, Viral Fusion Laboratory, Centre for Biomedical Research, Burnet Institute, 85 Commercial Road, Melbourne, VIC 3004, Australia
e-mail: hdrummer@burnet.edu.au

Despite 20 years of research, a vaccine to prevent hepatitis C virus (HCV) infection has not been developed. A vaccine to prevent HCV will need to induce broadly reactive immunity able to prevent infection by the 7 genetically and antigenically distinct genotypes circulating world-wide. HCV encodes two surface exposed glycoproteins, E1 and E2 that function as a heterodimer to mediate viral entry. Neutralizing antibodies (NAbs) to both E1 and E2 have been described with the major NAb target being E2. The function of E2 is to attach virions to host cells via cell surface receptors that include, but is not limited to, the tetraspanin CD81 and scavenger receptor class B type 1. However, E2 has developed a number of immune evasion strategies to limit the effectiveness of the NAb response and possibly limit the ability of the immune system to generate potent NAbs in natural infection. Hypervariable regions that shield the underlying core domain, subdominant neutralization epitopes and glycan shielding combine to make E2 a difficult target for the immune system. This review summarizes recent information on the role of NAbs to prevent HCV infection, the targets of the NAb response and structural information on glycoprotein E2 in complex with neutralizing antibodies. This new information should provide a framework for the rational design of new vaccine candidates that elicit highly potent broadly reactive NAbs to prevent HCV infection.

Keywords: glycoprotein E2, neutralizing antibody, CD81, immune evasion, viral entry

ARTICLE

The most cost effective means of controlling infectious disease is through vaccination, however, a prophylactic or therapeutic vaccine for HCV is not available. HCV is a small, enveloped positive sense RNA virus within the Flaviviridae family. HCV is classified into seven major genotypes that display up to 33 % nucleotide variation and >100 subtypes that display up to 20% nucleotide variation. The genotypes of HCV have a geographical distribution with genotypes 1–3 prevalent world-wide. Individuals infected with HCV harbor a swarm of closely related viruses referred to as a quasispecies. The degree of sequence variation observed for HCV exceeds that for HIV and influenza, posing a major challenge to vaccine development. Essential to the success of an HCV vaccine will be an ability to confer broad protection against the circulating strains of HCV.

THE ROLE OF ANTIBODY IN HCV INFECTION

The effectiveness of all current licensed viral vaccines relies on the production of neutralizing antibody (**NAb**; Lambert et al., 2005). Strong evidence now exists that NAbs play a major role in clearance of HCV infections. Longitudinal analysis of HCV infection cohorts reveals that broadly specific NAb (**brNAb**) elicited early in infection correlates with viral clearance (Lavillette et al., 2005; Pestka et al., 2007; Dowd et al., 2009). By contrast, people who failed to make NAbs progressed to chronic infection. Sequence analysis of the structural region during chronic infection reveals that the development of a NAb response correlates with sequence evolution, likely to be a result of immune escape (von Hahn et al., 2007; Liu et al., 2010). In a single case study, Raghuraman et al. (2012) examined the NAb and cellular immune responses in a patient with long term chronic HCV who spontaneously cleared their virus after 62 weeks of infection. Viral clearance correlated with the appearance of NAb at 48 weeks and a reversal of T cell exhaustion. Neutralizing antibodies also play an important role in people who have previously cleared their HCV infection but become infected on reexposure to HCV. Neutralizing antibodies were detected during the acute phase of infection in 60% of reinfected participants who went on to clear their reinfection but was not detected in 2 patients who progressed to chronic infection (Osburn et al., 2010). Overall, the findings of these studies suggest that the development of a NAb response during the early phase of acute infection is a strong correlate of viral clearance. Consistent with these observations, passive transfer of broadly neutralizing monoclonal or polyclonal NAbs to experimental animals protects them against viral challenge (Law et al., 2008; Vanwolleghem et al., 2008; Morin et al., 2012). However, administration of a sterilizing dose of antibody must precede infection, otherwise the antibody is not effective at preventing infection, but can reduce viral loads (Meuleman et al., 2008; Morin et al., 2012).

HCV GLYCOPROTEINS E1 AND E2

Hepatitis C virus encodes two glycoproteins, E1, and E2 that are cleaved from the viral polyprotein by signal peptidases. E1/E2 function as a heterodimer to mediate viral entry. E2 mediates attachment to cellular receptors CD81 and scavenger receptor class B type 1 (**SR-B1**) via sequences within its RBD (Pileri et al.,

1998; Scarselli et al., 2002). The functional properties of E1/E2 and the ability of E1/E2-specific antibodies to prevent viral entry can be studied using retroviruses pseudotyped with E1/E2 (**HCVpp**; Bartosch et al., 2003; Drummer et al., 2003; Hsu et al., 2003) or cell culture derived HCV (**HCVcc**) made by transfecting full-length HCV RNA into Huh 7 cells (Lindenbach et al., 2005; Wakita et al., 2005; Yi et al., 2006).

Glycoprotein E1 is essential for viral entry as HCVpp lacking E1 is non-infectious but its function in entry is unknown (Drummer et al., 2003). E1 contains a C-terminal transmembrane domain (TMD) that anchors the ectodomain to the virion and contains four or five glycosylation sites (**Figure 1A**). Relatively few NAbs have been described for E1 (Keck et al., 2004b; Meunier et al., 2008) suggesting it is a subdominant immunogen in natural infection. Glycoprotein E1 is essential for the correct assembly and stability of the E1/E2 heterodimer in HCVcc and allosterically modulates the structure of E2 and its ability to bind cellular receptors (McCaffrey et al., 2011; Wahid et al., 2013).

Glycoprotein E2 comprises ∼ 11 largely conserved N-linked glycosylation sites and 18 conserved cysteines (**Figure 1A**). The receptor binding domain, residues 384–661 (**RBD**), folds independently of other E1/E2 sequences. The RBD is linked through a conserved C-terminal stem to the **TMD** (Drummer and Poumbourios, 2004; **Figure 1A**). The binding site for CD81, the major cellular receptor for all HCV strains, comprises highly conserved segments within the E2 RBD (Drummer et al., 2002; Roccasecca et al., 2003; Zhang et al., 2004; Drummer et al., 2006; Owsianka et al., 2006; **Figure 1A**). These regions of E2 interact with the large extracellular loop (**LEL**) of CD81 through Ile182, Asn184, Phe186, and Leu162 on the head subdomain (Higginbottom et al., 2000; Drummer et al., 2002).

VARIABLE REGIONS OF THE E2 GLYCOPROTEIN

Located within the RBD are four variable regions (**Figure 1A**). The N-terminal hypervariable region 1 (HVR1) is 27 amino acids in length and resides outside the core domain of E2, and no structural information is available (Weiner et al., 1991; Kato et al., 1992). Despite the high degree of sequence variation, the overall basic charge of this region is preserved, possibly to maintain interactions with SR-B1 (Penin et al., 2001; Dao Thi et al., 2011). Deletion of HVR1 from the E2 RBD abolishes the interaction with SR-B1, while deletion of HVR1 in the context of HCVpp abolishes high-density lipoprotein (HDL)-mediated enhancement of viral entry, thereby increasing the effectiveness of NAb (Dao Thi et al., 2011). Originally, hypervariable region 2, HVR2, was described as a nine amino acid sequence (Kato et al., 1992) downstream of HVR1. Further analysis across different HCV genotypes suggested a more extensive area of variation (res. 461–481) flanked by conserved cysteine residues that form a surface exposed disulfide bonded loop, not essential for folding of the E2 RBD core (McCaffrey et al., 2007; Kong et al., 2013). Sequence identity within HVR2 ranges from 39% in genotypes 1a and b to 93% in genotype 5a (McCaffrey et al., 2007). An additional cysteine-flanked variable region (**igVR**, res. 570–580) was described that is relatively conserved within a genotype but exhibits a high degree of intergenotypic variation in both length (10–15 res) and sequence (McCaffrey et al., 2007). Deletion of either HVR2 or the igVR in the context of E1/E2 incorporated into HCVpp is not tolerated as E2 fails to form heterodimers with E1 and the resultant HCVpp are not infectious (McCaffrey et al., 2011). These data suggest that HVR2 and the igVR are essential to the virion incorporated E1/E2 structure. There is no evidence in the literature to suggest that HVR2 or the igVR are targets of the antibody response and so are unlikely to be under immune pressure. McCaffrey et al. (2007) have shown that HVR1, HVR2, and the igVR can be simultaneously deleted from the RBD without disrupting its native fold and receptor binding ability, indicating that they are outside a conserved core domain (McCaffrey et al., 2007). Analysis of sequence variation within patient isolates revealed a third hypervariable region **(HVR3)** within the sequence 434–450, that appears to be under immune selection pressure (Troesch et al., 2006; Torres-Puente et al., 2008).

THE THREE DIMENSIONAL STRUCTURE OF THE E2 CORE DOMAIN

Recently, X-ray crystallography and electron microscopy have provided the first insights into the structure of glycoprotein E2. The structure provided by Kong et al. (2013) was obtained for an E2 core domain containing residues 412–645 that lacked two glycosylation sites at Asn448 and Asn576, and HVR2 residues 460–485 were replaced with a Gly-Ser-Ser-Gly linker (**Figure 1B**). The second structure was for an E2 core comprising residues 456–656 (Khan et al., 2014). The structures were largely similar and revealed that unlike its related glycoprotein E flavivirus counterparts HCV E2 does not have a three domain architecture reminiscent of other class II fusion proteins. Instead, E2 core adopts a compact globular immunoglobulin-like fold comprising a central β-sandwich surrounded by short front and back layers comprising loops, short helices and β sheets (Kong et al., 2013; Khan et al., 2014; **Figures 1B,C**). Verification that E2 adopts a compact globular fold when expressed as the complete E2 ectodomain was provided by electron microscopy (Kong et al., 2013). The immunoglobulin fold resembles domain III of the class II fusion proteins, the only common structural element with the alpha and flavivirus fusion proteins. In the core domain of HCV E2, 8 disulfide bonds were formed but many regions lacked regular secondary structure including the regions between residues 412–420 and 454–491 surrounding the truncated HVR2 region and a loop at 586–596 (Kong et al., 2013). Six of the N-linked glycans also were also largely disordered at Asn417, Asn423, Asn532, Asn540, Asn623, and Asn556 (Kong et al., 2013). The immunoglobulin sandwich is formed by 4 β strands that form an inner sheet and two solvent exposed β strands that comprise the outer sheet. A loop connects the inner sheet to the outer sheet and contains many of the key CD81 binding residues and is adjacent to the front layer where additional CD81 contact residues are found that are surface exposed (**Figures 1D,E**). The igVR is within a flexible region spanning 567–596 and the back layer is formed by two short α-helices and 4 β sheets.

TARGETS OF THE ANTIBODY RESPONSE TO HCV

ANTIBODY RESPONSE TO E1

Neutralizing monoclonal antibodies specific to E1 or epitopes that are targets of NAbs have been described although they are relatively limited in number. This may in part be due to the inability to

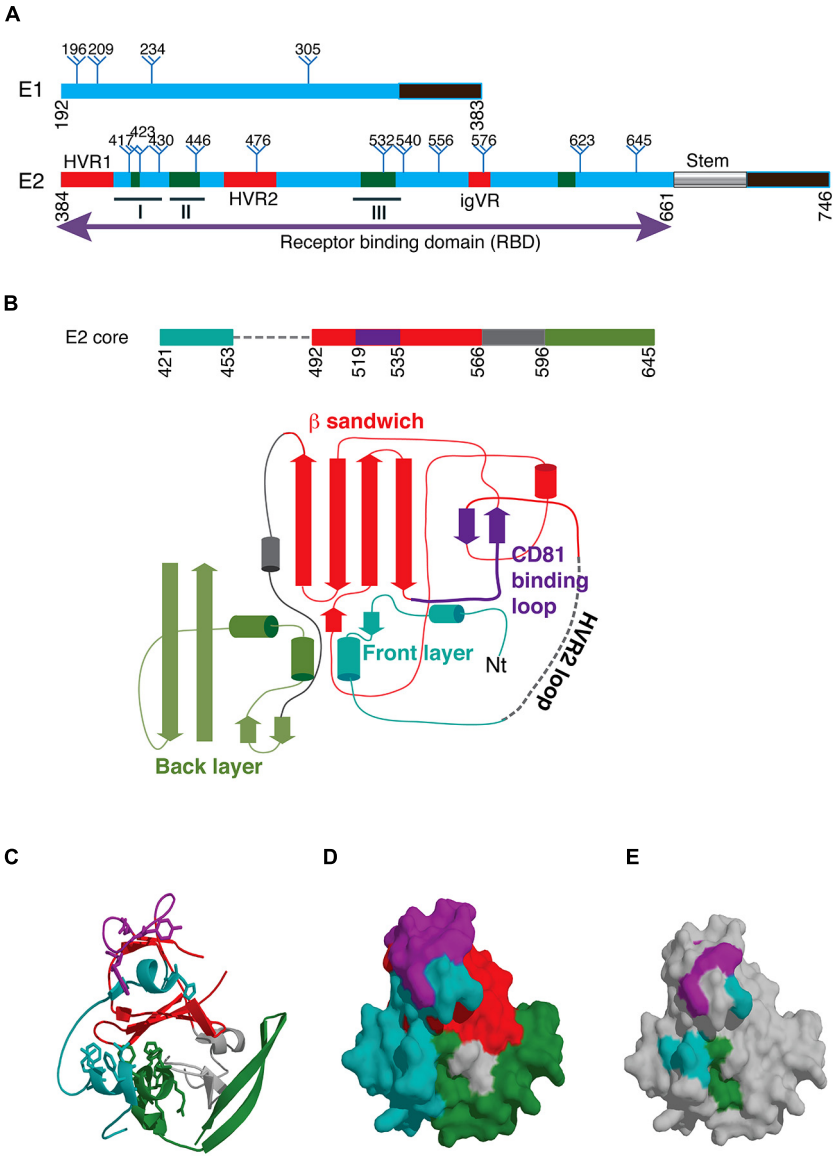

FIGURE 1 | (A) Schematic of hepatitis C virus glycoproteins E1 and E2 showing the location of conserved glycosylation sites (trees). The transmembrane domains are shown in black and the E2 stem region is indicated with a cylinder. The location of hypervariable regions in E2 is shown in red and the three immunogenic epitopes designated epitopes I, II, and III are underlined. Regions involved in CD81 binding are shown in green with specific contact residues shown in **Table 1**. **(B)** Schematic of the E2 core domain according to the designations of Kong et al. (2013). **(C)** Structure of the E2 core domain colored according to **B**. **(D)** Surface representation of **C**. Purple and teal regions represent the neutralizing face of E2 and overlap with CD81 contact residues shown in **E**. **(E)** Surface representation of the E2 core domain with those residues involved in CD81 with their locations colored according to **B**.

express a recombinant form of E1 that is a known mimic for the structure of E1 in virions and the likelihood that E1 is largely occluded by E2 in virion incorporated heterodimers. Antibody H-111 binds an epitope at the N-terminus of E1, immunoprecipitates E1/E2 heterodimers and has cross-neutralizing potential (Keck et al., 2004b). Two MAbs isolated from a human who had cleared their genotype 1b infection, IGH505 and IGH526 recognize overlapping but distinct epitopes within the sequence 307–340, and neutralize genotypes 1a, 1b, 4a, 5a, and 6a viruses, but not 2b or 3a. Interestingly, only 2/31 MAbs isolated by Meunier et al. (2008) were specific to E1 neutralized virus suggesting that such antibodies may be rarely elicited. Additional epitopes recognized by human serum have been discovered using overlapping peptides or using mass spectrometry and include epitopes at 264–318 (Kachko et al., 2011) and 308–325 (Grollo et al., 2006).

THE ANTIBODY RESPONSE TO E2

Thus far, two specificities of NAb elicited in natural infection and following vaccination with E2 derived immunogens have been identified. Neutralizing antibodies directed toward the N-terminal

HVR1 are immunodominant in natural infection (Kato et al., 1993, 1994; Zibert et al., 1995). Immune serum raised to HVR1 has the ability to protect chimpanzees from experimental infection with a homologous strain of virus but are type-specific with limited ability to neutralize immune escape variants within the HVR1 sequence (Farci et al., 1996). As a result antibodies are believed to have a major role in driving immune escape (Edwards et al., 2012) and HVR1 has been proposed to be an immune decoy (Mondelli et al., 2001). Analysis of the evolution of HCV sequences in people infected with HCV reveals that in the absence of NAb activity, the HVR1 sequence remains stable but rapidly evolves under NAb pressure (Liu et al., 2010). Analysis of HVR1 specific antibody responses in HCV-infected people revealed that those who cleared virus spontaneously were more likely to have detectable anti-HVR1 antibodies in their serum within the first 6 months of infection (Zibert et al., 1997) and suggests that induction of HVR1 specific antibodies in some circumstances may favor viral clearance.

While HVR1 specific antibodies are largely type-specific, evidence that some anti-HVR1 antibodies can be cross reactive was obtained by vaccinating rabbits with a panel of HVR1 peptides (Shang et al., 1999). The subsequent antibody response was able to recognize diverse HVR1 sequences, capture HCV from patient serum and prevent HCV binding to cells. A monoclonal antibody specific to HVR1 was also shown to be cross reactive and contained conserved amino acids within its epitope (Li et al., 2001). While neutralization assays were not available at the time these studies were preformed the data suggest that HVR1 cannot be entirely discounted as a desirable component of an HCV vaccine.

The second major specificity of NAb is directed toward sequences involved in CD81 binding. Antibodies specific to the CD81 binding site located on the surface of E2 are frequently cross neutralizing due to the high degree of sequence conservation (Keck et al., 2004a; Johansson et al., 2007; Law et al., 2008). Residues involved in binding CD81 have been mapped by performing mutagenesis of E2 RBD E1/E2 and E1/E2 incorporated into HCVpp and is summarized in **Table 1**. A striking feature of these contact residues is that they are mostly aromatic and hydrophobic amino acids and they are highly conserved between genotypes or the substitutions are conservative; in the case of Trp437, Phe is more frequently observed in genotypes 1b and 2-7, and for residues Leu438 and Phe442 alternative amino acids are I, V or M and L, M or I, respectively. The major NAb specificities elicited toward E2 that block CD81 binding in infected humans recognize epitopes that contain amino acids within the regions spanning residues 411–428 and 429–448 and 523–549 and thus directly overlap with the regions of E2 involved in CD81 binding. These regions involved in CD81 binding overlap with neutralization epitopes and this area represents the neutralizing face of E2 (**Figures 1D,E**).

A subset of antibodies directed to these regions can bind to their epitopes in the context of synthetic peptide analogs of 411–428 (epitope I) and 429–448 (epitope II; **Figure 1A**). Examples of human monoclonal NAbs that recognize these regions include HCV1, and 95-2 (epitope I), and 84-1, 84-25, 85-26 and 84-27 (epitope II). Antibodies that bind their epitope within the 523–549 sequence, referred to as epitope III, have also been described and include e20, (1:7) and A8 but appear to be conformation dependent (Allander et al., 2000; Johansson et al., 2007; Mancini et al., 2009; Edwards et al., 2012). Another subset of antibodies recognize discontinuous epitopes containing amino acids from one or more of these regions and only bind folded E2 in the context of the recombinant RBD or virion incorporated E2 such as CBH-5, CBH-7, AR3A, AR3B, AR3C, AR3D, and Fab e137 (Hadlock et al., 2000; Law et al., 2008; Owsianka et al., 2008; Perotti et al., 2008; Edwards et al., 2012).

Table 1 | Residues of E2 involved in binding to CD81.

Residue[a]	Epitope[b]	Alternative amino acids[c]	Reference
Trp420	I	–	Owsianka et al. (2006)
His421	I	–	Boo et al. (2012)
Trp437	II	Phe	Drummer et al. (2006)
Leu438	II	Ile,Val, Met	Drummer et al. (2006)
Leu441	II	–	Drummer et al. (2006)
Phe442	II	Leu, Met, Ile	Drummer et al. (2006)
Tyr527	III	–	Owsianka et al. (2006)
Trp529	III	Phe	Owsianka et al. (2006)
Gly530	III	–	Owsianka et al. (2006)
Asp535	III	–	Owsianka et al. (2006)
Y^{613}RLWHY[d]		–	Roccasecca et al. (2003)

[a] *Analysis was performed on genotype 1a isolates.* [b] *Location of amino acid within an antigenic region.* [c] *Alternative amino acids observed in other isolates of HCV. A dash indicates conserved.* [d] *Data derived by replacing this region with SAASAS.*

Recently, X-ray crystal structures have been determined for neutralizing MAbs in complex with synthetic peptides analogs of epitopes I and II and of the region encompassing epitope III in the context of the E2 core domain. The structures reveal important information about the conformation of these epitopes, the mode of binding and the presence of a neutralizing face on the surface of the E2 molecule.

EPITOPE I

The region encompassing epitope I is absent from the available structures of the E2 core domain but two structures of MAbs in complex with synthetic peptides reveal important structural information about this region. The murine antibody AP33 was first discovered in 2001 and is a brNAb specific to epitope I (Owsianka et al., 2001, 2005). Its binding site comprises a discontinuous sequence within this region and includes amino acids Leu413, Asn415, Gly418, and Trp420 with contribution by Asn417. These amino acids are highly conserved between HCV isolates and explain its broad cross reactivity (Tarr et al., 2006). However, this specificity of antibody does not appear to be frequently elicited in patients with HCV as it was detected in only 2.5% of serum samples collected from acute and chronic phases of HCV (Tarr et al., 2007). The X-ray structure of AP33 with epitope I reveals that the peptide forms a type 1 β-hairpin structure that is sandwiched between the heavy and light chain of antibody.

Hydrogen bonds formed with Asn416 and Gly418 and hydrophobic interactions between the side chains of Leu413 and Trp420 form the major interactions and residues from all CDR loops except L2 are involved in binding to the peptide (Kong et al., 2012a).

The human MAb HCV1 is specific to epitope I and is able to neutralize genotype 1a, 1b, 2b, 3a, and 4a and binds its epitope at nanomolar affinity (Broering et al., 2009). Administration of HCV1 to chimpanzees protects against HCV infection and can be used to treat acutely infected chimpanzees (Morin et al., 2012). As a result of these promising results, HCV1 is currently being evaluated in clinical trials for its ability to prevent reinfection of the liver following transplantation. The major contact residues are Leu413 and Trp420 (Broering et al., 2009) although NAb escape mutants can be selected that possess the mutation Asn415Asp/Lys. The crystal structure of HCV1 in complex with a peptide containing HCV residues 412-423 again reveals a type 1 β hairpin structure for the peptide in contact with the heavy chain CDR2 and CDR3 loops and the CDR3 loop of the light chain (Kong et al., 2012b; **Figure 2A**). The ability of HCV1 to mediate broad neutralization is further explained by the conservation of its contact residues and the solvent accessibility of this region on the surface of E2. Additional alanine scanning performed in the context of E1/E2 heterodimer revealed that Asn415 and Gly418 were essential for HCV1 binding because these residues are critical to the formation of the β hairpin turn and the conformation of epitope I. Mutagenesis studies revealed that substitution of Asn415 with glutamine, glutamate or lysine preserved the β turn architecture and maintained viral fitness while significantly reducing the effectiveness of HCV1 to neutralize virus, and thus provides a potential mechanism for *in vivo* escape (Kong et al., 2012b).

EPITOPE II

The region encompassing epitope II overlaps extensively with key CD81 contact residues (Drummer et al., 2006) and brNAb that bind this region have only recently been described (Law et al., 2008; Keck et al., 2012). Antibodies to this region are particularly interesting as passage of cell culture derived virus with a neutralizing amount of antibody fails to select for escape mutants suggesting that fit viruses with escape mutations cannot be selected (Keck et al., 2012). Two antibodies, 84-1 and 84-27 recognize a synthetic peptide analog and their crystal structures have been determined (Krey et al., 2013). In both cases, the peptide adopts the same conformation suggesting it reflects that observed in the native structure of E2, and not an induced fit conferred by the antibody. The peptide adopts a 1.5 α-helical turn spanning Trp437-Phe442 with Tyr438-Lys446 adopting an extended conformation. In the case of 84-1, E2 amino acids Leu441 and Phe442 make extensive hydrophobic contact with the antibody heavy chain that is further stabilized by a hydrogen bonding network that includes Lys446 and Trp443 (**Figure 2B**). Analysis of the Los Alamos HCV database revealed that Thr435, Gly436, Ala439, Leu441, and Tyr443 are highly conserved while Gln444 and His445 are less conserved. The key contact residue Phe442, conserved in 60% of sequences, can only be replaced with bulky hydrophobic amino acids. The conservation of Leu441 and Phe442 suggest a strict structural constraint at this position to maintain glycoprotein function, possibly explaining why neutralization escape mutants could not be selected with these antibodies. This immunogenic domain of E2 overlaps with HVR3 and suggests that the region is under immune selection pressure suggesting that some antibodies to this region, elicited in natural infection, may not be able to prevent immune escape *in vivo*, as was observed for 84-1 *in vitro*.

EPITOPE III

The E2 structure provided by Kong et al. (2013) was obtained by co-crystallization of E2 with neutralizing human MAb AR3C whose epitope contains residues from epitope III at positions 530, 535, 538, and 540 and one residue from epitope I at position 424 (Law et al., 2008; **Figure 2C**). In the co-crystal structure, AR3C buries 828 Å of E2 surface area and 161 Å of E2 glycan surface area and additional points of contact with E2 were revealed (Kong et al., 2013). 86% of the buried E2 surface comprises residues that are 80–100% conserved across all E2 sequences. The epitope of AR3C comprises most of the front layer, some amino acids between 421–446 and the CD81 binding loop. The majority of contacts with E2 are mediated by the heavy chain CDR3 (44% of total buried surface). The binding site for AR3C overlaps directly with the CD81 binding site and is a surface exposed, hydrophobic region, relatively unobscured by carbohydrate (Kong et al., 2013). A vaccine with the ability to elicit such antibody specificities is therefore highly desirable as they are likely to have broad neutralization potential.

WHY HAVE VACCINES FOR HCV NOT BEEN DEVELOPED?

Unlike its flavivirus counterparts, expression of the HCV glycoproteins E1 and E2 does not result in the secretion of subviral particles, the component of all flaviviral vaccines. The ability to culture HCV was developed in 2005 but remains limited to a handful of isolates and titres remain low making large-scale production of an inactivated vaccine unlikely. Nevertheless, Akazawa et al. (2013) have successfully demonstrated that sufficient HCVcc can be produced from cell culture and the inactivated HCVcc were used to immunize mice. The antibodies were able to neutralize genotypes 1a, 1b, and 2a viruses and prevented the infection of human liver transplanted onto uPa-SCID mice but only at the lowest homologous challenge dose of virus (Akazawa et al., 2013). *In vivo* heterologous neutralization was not examined. The immune serum from mice vaccinated with the inactivated HCVcc particles were more efficient at neutralizing homologous virus than immune serum from mice vaccinated with recombinant E2 alone or recombinant E1 and E2, suggesting intact particles are likely to be more immunogenic than the isolated recombinant glycoproteins.

Only one HCV vaccine candidate aimed at eliciting NAb has been tested in humans. The vaccine initially developed by Chiron Corporation was based on recombinant HCV E1 and E2 glycoproteins purified from mammalian cells as a non-disulfide linked heterodimer. In preclinical studies conducted in chimpanzees, the vaccine induced high titres of E1 and E2 specific antibodies and prevented five chimpanzees from becoming infected with a homologous challenge of virus and protection correlated with the

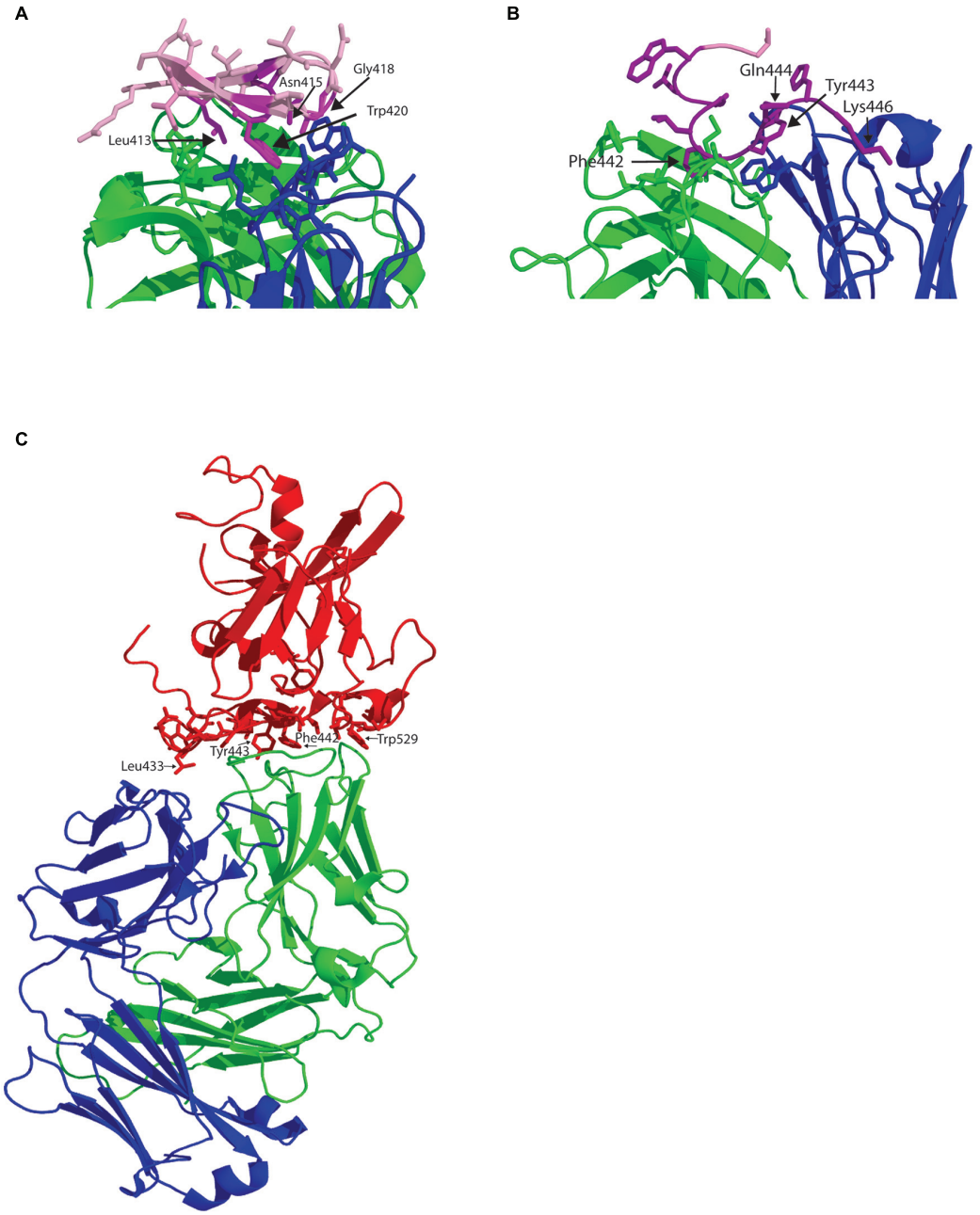

FIGURE 2 | Structure of human MAbs in complex with their epitopes. (A) Structure of the paratope of MAb HCV1 in complex with synthetic peptide spanning residues 412–423 within E2 epitope I (pink). Amino acids side chains within the paratope in contact with the antibody epitope are shown as sticks. The antibody heavy chain is colored green and the light chain is in blue and major contact residues within the peptide shown as purple sticks. **(B)** Structure of the paratope of HC84-1 with its epitope spanning 435–446. Labeled as for **A**. **(C)** Structure of the E2 core domain in complex with neutralizing antibody AR3C. E2 is colored in red with the side chains of the amino acid in contact with the antibody shown as sticks. Heavy chain is green and light chain is blue.

presence of high titre anti-E2 antibodies (Choo et al., 1994). Challenge of the chimpanzees with a closely related heterologous virus strain resulted in infection in all cases; all but one vaccinee did not progress to chronic infection, suggesting the vaccine does induce a degree of protective but not sterilizing immunity (Houghton and Abrignani, 2005).

The recombinant E1/E2 vaccine was trialed in healthy human volunteers and adjuvanted with MF59. The initial results of this trial revealed that 15/41 subjects had antibody reactive to E1 region 313-327, 21/41 subjects had anti-HVR1 specific antibodies, 23/41 had epitope I reactive antibodies, and 13/41 had epitope II reactive antibodies, where reactivity was defined as a greater than twofold

higher optical density compared to pre-immune or placebo samples (Ray et al., 2010). Nine, 11, 10, and 5 samples with E1, HVR1, epitope I, or epitope II reactive antibodies, respectively, neutralized virus, although it is not entirely clear what level of neutralization was achieved. The higher proportions of epitope I reactive antibodies compared to that reported in natural infection is a promising outcome of this vaccine trial if it can be correlated with an increase in cross-neutralizing activity in these sera. In a subsequent study, using a more extensive panel of HCVcc from each of the 7 genotypes revealed that of the three subjects tested, one subject elicited sera with the ability to mediate ∼25 to 80% neutralization of HCVcc at a 1/50 dilution of serum with the highest neutralization observed against genotype 6 and the lowest to genotype 7 (Law et al., 2013). These results should provide encouragement to the field of vaccine research that cross neutralizing antibodies can be elicited by recombinant E1/E2 based vaccines and warrant further study. A major limitation of the studies described above are the relatively low titres of NAbs elicited, their limited cross neutralizing potential, and their modest level of neutralization. A number of mechanisms whereby the HCV glycoproteins can evade the immune response have been illuminated and suggest that alternative approaches to vaccine design may be required, similar to the approach currently being used for HIV and influenza (Nabel, 2012).

INTERFERENCE OF NEUTRALIZATION BY VARIABLE REGIONS OF E2

The three variable regions of E2 have been implicated in immune evasion by HCV. HVR1 contains the epitopes of NAbs that block E2 binding to SR-B1 but not to CD81 (Sabo et al., 2011). Instead, HVR1 antibodies can allosterically shield the CD81 binding site and conserved NAb epitopes therein providing an additional mechanism of neutralization escape (Bankwitz et al., 2010). Roccasecca et al. (2003) examined the effect of chimerization of HVR1 and HVR2 in closely related strains of HCV and the effect of HVR1 deletion on CD81 binding. The efficiency of CD81 binding was found to be strain dependent and deletion of HVR1 enhanced CD81 binding when performed in the context of recombinant E2 RBD and recombinant CD81 LEL and RBD binding to CD81 on the surface of Molt-4 cells suggesting that HVR1 may at least partially occlude the CD81 binding site. When HVR1 or HVR2 was swapped between E2 strains, no difference in CD81 binding was observed. However, when both HVR1 and HVR2 of strain H were interchanged with those of the N2 strain, a fourfold reduction in CD81 binding was observed. The reciprocal chimera resulted in an increase in CD81 binding (Roccasecca et al., 2003). These results suggest that CD81 binding is modulated by the presence of HVR1 and the sequence of HVR2 implying that these regions are flexible surface exposed domains that may provide a means to shield the underlying CD81 binding site, occluding it from immune surveillance.

The effect of the variable regions on CD81 binding was further explored by McCaffrey et al. (2007). In this study, HVR1, HVR2, and the igVR were deleted from the E2 RBD individually and in combination to identify the first E2 core domain (McCaffrey et al., 2007). While all three variable regions could be deleted without affecting CD81 binding, deletion of two of the three variable regions modulated CD81 binding function. Deletion of HVR2 and the igVR, or HVR1 and the igVR resulted in similar levels of CD81 binding as wild-type E2 RBD. However, deletion of HVR1 and HVR2 from the E2 RBD resulted in an approximately 50% reduction in CD81 binding. These results indicate that the presence of the igVR interferes with CD81 binding function when HVR1 and HVR2 is absent and point to a functional interaction between the igVR and HVR1 and/or HVR2 such that the CD81 binding site is properly formed or becomes fully accessible to the receptor. The available crystal structures of E2 are of a monomer. Absent from these structures is information about the E2 stem region, E1 and detailed structural information about HVR1, epitope I and HVR2. It is likely that the structure of intact E2 on the surface of a virion as a heterodimer with E1 differs from the structures of the E2 core thus far available. Indeed, all fusion proteins exist as either dimers of heterodimers or trimers of heterodimers and this is likely to be the case for HCV as well. Thus it is possible that in these higher order structures of virion incorporated E2, the variable regions may form additional contacts with the opposing E1 or E2 protomers providing additional mechanisms whereby HVR1, HVR2, and the igVR can interfere with antibody recognition and CD81 binding.

Finally, the reason why HVR2 and igVR sequences evolve rapidly in infected patients remains unresolved. Neither region has been implicated as being targets of the antibody response suggesting that a direct mechanism of immune evasion is not likely. However, it is possible that the sequence of HVR2 and the igVR could alter their ability to shield the E2 core from neutralizing antibodies or alters E1/E2 conformation while retaining function providing an allosteric mechanism of immune escape and require further studies.

NEUTRALIZING AND NON-NEUTRALIZING ANTIBODIES

A subset of epitope II directed non-neutralizing antibodies have been reported to interfere with the capacity of epitope I antibodies to neutralize virus (Zhang et al., 2009) while other epitope II-directed NAbs act cooperatively with epitope I specific NAbs (Keck et al., 2012, 2013). The ability of neutralizing antibodies directed toward epitope II to prevent neutralization escape suggests that they are a highly desirable component of the antibody response to any HCV vaccine but it is not clear what proportions of antibodies specific to epitope II have neutralizing/non-neutralizing activity in natural infection or in vaccination. Analysis of the fine specificity of the antibody response in natural infection is necessary to dissect these effects and examine their neutralization capacity.

GLYCOSYLATION OF E2 AND ITS EFFECT ON NEUTRALIZATION

E2 contains 11 sites for N-linked glycosylation that are largely conserved in different HCV isolates suggesting they play important roles in glycoprotein structure and function. Helle et al. (2007) found that Asn417, Asn532, and Asn623 obscured E2 from CD81 binding and interfered with the ability of antibody to neutralize virus in the context of HCVpp. In HCVcc, an additional two glycosylation sites impacted on neutralization sensitivity at Asn423 and

Asn446 (Helle et al., 2010). All five glycosylation sites are directly adjacent to CD81 contact residues suggesting that they may restrict the ability to elicit NAbs toward these CD81 binding regions or impact on accessibility of underlying epitopes to antibody neutralization. In genotype 3a viruses, an additional glycosylation site at position 495 reduces the sensitivity of HCV to neutralization using pooled HCV positive immune serum, and suggests that in some viruses, additional mechanisms of glycan mediated immune escape may evolve (Anjum et al., 2013). The structure of E2 indicates that 7/11 glycans shield the neutralizing face of the E2 core domain confirming that glycans potentially have the ability to restrict the generation of antibodies to these regions in natural infection and vaccination and /or restrict antibody access to epitopes.

IMPACT ON VACCINE DESIGN

While recombinant E2 provides an obvious vaccine candidate as it can be produced in large amounts, the above information suggests its use requires caution. Variable regions within E2 have the potential to occlude the underlying CD81 binding site, HVR1 is immunodominant and drives immune escape and the extensive glycan shield protects the neutralizing face of E2. In addition, the use of isolated E2 may promote the elicitation of antibodies to the non-neutralizing face highly exposed in monomeric E2. The lack of neutralizing ability of antibodies directed to this non-neutralizing face of E2 is likely to be explained by its occlusion in virion incorporated E1/E2 heterodimers or its occlusion in the higher order arrangement of E1/E2 heterodimers in the virion, e.g., dimers or trimers of heterodimers. These caveats on the use of E2 as an immunogen are highly reminiscent of the HIV-1 gp120 monomer that contains five variable regions, extensive glycan shielding, and an immunodominant non-neutralizing face.

The low frequency of antibodies elicited to epitope I in natural infection suggest it is subdominant and engineering of the E2 protein may be required to increase the titre of antibodies specific to this region in vaccine candidates. Similar studies investigating the titre and prevalence of antibodies directed toward epitopes II or III are required to understand whether these specificities are elicited in native forms of E2 or whether reengineering of E2 is required to make these epitopes immunodominant. Finally, key to successful vaccine design is an understanding of the correlates of protective immunity. Future studies aimed at delineating the fine specificity of the antibody response in people who successfully clear their HCV infection will provide essential information to guide the rational design of an HCV vaccine.

ACKNOWLEDGMENT

Heidi E. Drummer is supported by NHMRC senior research fellowship 1041897 and its Independent Research Institute Infrastructure Support Scheme. The authors gratefully acknowledge the contribution to this work of the Victorian Operational Infrastructure Support Program received by the Burnet Institute.

REFERENCES

Akazawa, D., Moriyama, M., Yokokawa, H., Omi, N., Watanabe, N., Date, T., et al. (2013). Neutralizing antibodies induced by cell culture-derived hepatitis C virus protect against infection in mice. *Gastroenterology* 145, 447–55.e1–4. doi: 10.1053/j.gastro.2013.05.007

Allander, T., Drakenberg, K., Beyene, A., Rosa, D., Abrignani, S., Houghton, M., et al. (2000). Recombinant human monoclonal antibodies against different conformational epitopes of the E2 envelope glycoprotein of hepatitis C virus that inhibit its interaction with CD81. *J. Gen. Virol.* 81, 2451–2459.

Anjum, S., Wahid, A., Afzal, M. S., Albecka, A., Alsaleh, K., Ahmad, T., et al. (2013). Additional glycosylation within a specific hypervariable region of subtype 3a of hepatitis C virus protects against virus neutralization. *J. Infect. Dis.* 208, 1888–1897. doi: 10.1093/infdis/jit376

Bankwitz, D., Steinmann, E., Bitzegeio, J., Ciesek, S., Friesland, M., Herrmann, E., et al. (2010). Hepatitis C virus hypervariable region 1 modulates receptor interactions, conceals the CD81 binding site, and protects conserved neutralizing epitopes. *J. Virol.* 84, 5751–5763. doi: 10.1128/JVI.02200-09

Bartosch, B., Dubuisson, J., and Cosset, F. L. (2003). Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. *J. Exp. Med.* 197, 633–642. doi: 10.1084/jem.20021756

Boo, I., teWierik, K., Douam, F., Lavillette, D., Poumbourios, P., and Drummer, H. E. (2012). Distinct roles in folding, CD81 receptor binding and viral entry for conserved histidine residues of hepatitis C virus glycoprotein E1 and E2. *Biochem. J.* 443, 85–94. doi: 10.1042/BJ20110868

Broering, T. J., Garrity, K. A., Boatright, N. K., Sloan, S. E., Sandor, F., Thomas, W. D., et al. (2009). Identification and characterization of broadly neutralizing human monoclonal antibodies directed against the E2 envelope glycoprotein of hepatitis C virus. *J. Virol.* 83, 12473–12482. doi: 10.1128/JVI.01138-09

Choo, Q. L., Kuo, G., Ralston, R., Weiner, A., Chien, D., Van Nest, G., et al. (1994). Vaccination of chimpanzees against infection by the hepatitis C virus. *Proc. Natl. Acad. Sci. U.S.A.* 91, 1294–1298. doi: 10.1073/pnas.91.4.1294

Dao Thi, V. L., Dreux, M., and Cosset, F. L. (2011). Scavenger receptor class B type I and the hypervariable region-1 of hepatitis C virus in cell entry and neutralisation. *Expert Rev. Mol. Med.* 13:e13. doi: 10.1017/S1462399411001785

Dowd, K. A., Netski, D. M., Wang, X. H., Cox, A. L., and Ray, S. C. (2009). Selection pressure from neutralizing antibodies drives sequence evolution during acute infection with hepatitis C virus. *Gastroenterology* 136, 2377–2386. doi: 10.1053/j.gastro.2009.02.080

Drummer, H. E., Boo, I., Maerz, A. L., and Poumbourios, P. (2006). A conserved Gly436-Trp-Leu-Ala-Gly-Leu-Phe-Tyr motif in hepatitis C virus glycoprotein E2 is a determinant of CD81 binding and viral entry. *J. Virol.* 80, 7844–7853. doi: 10.1128/JVI.00029-06

Drummer, H. E., Maerz, A., and Poumbourios, P. (2003). Cell surface expression of functional hepatitis C virus E1 and E2 glycoproteins. *FEBS Lett.* 546, 385–390. doi: 10.1016/S0014-5793(03)00635-5

Drummer, H. E., and Poumbourios, P. (2004). Hepatitis C virus glycoprotein E2 contains a membrane-proximal heptad repeat sequence that is essential for E1/E2 glycoprotein heterodimerization and viral entry. *J. Biol. Chem.* 279, 30066–30072. doi: 10.1074/jbc.M405098200

Drummer, H. E., Wilson, K. A., and Poumbourios, P. (2002). Identification of the hepatitis C virus e2 glycoprotein binding site on the large extracellular loop of CD81. *J. Virol.* 76, 11143–11147. doi: 10.1128/JVI.76.21.11143-11147.2002

Edwards, V. C., Tarr, A. W., Urbanowicz, R. A., and Ball, J. K. (2012). The role of neutralizing antibodies in hepatitis C virus infection. *J. Gen. Virol.* 93, 1–19. doi: 10.1099/vir.0.035956-0

Farci, P., Shimoda, A., Wong, D., Cabezon, T., De Gioannis, D., Strazzera, A., et al. (1996). Prevention of hepatitis C virus infection in chimpanzees by hyperimmune serum against the hypervariable region 1 of the envelope 2 protein. *Proc. Natl. Acad. Sci. U.S.A.* 93, 15394–15399. doi: 10.1073/pnas.93.26.15394

Grollo, L., Torresi, J., Drummer, H., Zeng, W., Williamson, N., and Jackson, D. C. (2006). Exploiting information inherent in binding sites of virus-specific antibodies: design of an HCV vaccine candidate cross-reactive with multiple genotypes. *Antivir. Ther.* 11, 1005–1014.

Hadlock, K. G., Lanford, R. E., Perkins, S., Rowe, J., Yang, Q., Levy, S., et al. (2000). Human monoclonal antibodies that inhibit binding of hepatitis C virus E2 protein to CD81 and recognize conserved conformational epitopes. *J. Virol.* 74, 10407–10416. doi: 10.1128/JVI.74.22.10407-10416.2000

Helle, F., Goffard, A., Morel, V., Duverlie, G., McKeating, J., Keck, Z. Y., et al. (2007). The neutralizing activity of anti-hepatitis C virus antibodies is modulated by specific glycans on the E2 envelope protein. *J. Virol.* 81, 8101–8111. doi: 10.1128/JVI.00127-07

Helle, F., Vieyres, G., Elkrief, L., Popescu, C. I., Wychowski, C., Descamps, V., et al. (2010). Role of N-linked glycans in the functions of hepatitis C virus envelope proteins incorporated into infectious virions. *J. Virol.* 84, 11905–11915. doi: 10.1128/JVI.01548-10

Higginbottom, A., Quinn, E. R., Kuo, C. C., Flint, M., Wilson, L. H., Bianchi, E., et al. (2000). Identification of amino acid residues in CD81 critical for interaction with hepatitis C virus envelope glycoprotein E2. *J. Virol.* 74, 3642–3649. doi: 10.1128/JVI.74.8.3642-3649.2000

Houghton, M., and Abrignani, S. (2005). Prospects for a vaccine against the hepatitis C virus. *Nature* 436, 961–966. doi: 10.1038/nature04081

Hsu, M., Zhang, J., Flint, M., Logvinoff, C., Cheng-Mayer, C., Rice, C. M., et al. (2003). Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. *Proc. Natl. Acad. Sci. U.S.A.* 100, 7271–7276. doi: 10.1073/pnas.0832180100

Johansson, D. X., Voisset, C., Tarr, A. W., Aung, M., Ball, J. K., Dubuisson, J., et al. (2007). Human combinatorial libraries yield rare antibodies that broadly neutralize hepatitis C virus. *Proc. Natl. Acad. Sci. U.S.A.* 104, 16269–16274. doi: 10.1073/pnas.0705522104

Kachko, A., Kochneva, G., Sivolobova, G., Grazhdantseva, A., Lupan, T., Zubkova, I., et al. (2011). New neutralizing antibody epitopes in hepatitis C virus envelope glycoproteins are revealed by dissecting peptide recognition profiles. *Vaccine* 30, 69–77. doi: 10.1016/j.vaccine.2011.10.045

Kato, N., Nakazawa, T., Ootsuyama, Y., Sugiyama, K., Ohkoshi, S., and Shimotohno, K. (1994). Virus isolate-specific antibodies against hypervariable region 1 of the hepatitis C virus second envelope protein, gp70. *Jpn. J. Cancer Res.* 85, 987–991. doi: 10.1111/j.1349-7006.1994.tb02894.x

Kato, N., Ootsuyama, Y., Ohkoshi, S., Nakazawa, T., Sekiya, H., Hijikata, M., et al. (1992). Characterization of hypervariable regions in the putative envelope protein of hepatitis C virus. *Biochem. Biophys. Res. Commun.* 189, 119–127. doi: 10.1016/0006-291X(92)91533-V

Kato, N., Sekiya, H., Ootsuyama, Y., Nakazawa, T., Hijikata, M., Ohkoshi, S., et al. (1993). Humoral immune response to hypervariable region 1 of the putative envelope glycoprotein (gp70) of hepatitis C virus. *J. Virol.* 67, 3923–3930.

Keck, Z., Wang, W., Wang, Y., Lau, P., Carlsen, T. H., Prentoe, J., et al. (2013). Cooperativity in virus neutralization by human monoclonal antibodies to two adjacent regions located at the amino terminus of hepatitis C virus E2 glycoprotein. *J. Virol.* 87, 37–51. doi: 10.1128/JVI.01941-12

Keck, Z. Y., Op De Beeck, A., Hadlock, K. G., Xia, J., Li, T. K., Dubuisson, J., et al. (2004a). Hepatitis C virus e2 has three immunogenic domains containing conformational epitopes with distinct properties and biological functions. *J. Virol.* 78, 9224–9232. doi: 10.1128/JVI.78.17.9224-9232.2004

Keck, Z. Y., Sung, V. M., Perkins, S., Rowe, J., Paul, S., Liang, T. J., et al. (2004b). Human monoclonal antibody to hepatitis C virus E1 glycoprotein that blocks virus attachment and viral infectivity. *J. Virol.* 78, 7257–7263. doi: 10.1128/JVI.78.13.7257-7263.2004

Keck, Z. Y., Xia, J., Wang, Y., Wang, W., Krey, T., Prentoe, J., et al. (2012). Human monoclonal antibodies to a novel cluster of conformational epitopes on HCV E2 with resistance to neutralization escape in a genotype 2a isolate. *PLoS Pathog.* 8:e1002653. doi: 10.1371/journal.ppat.1002653

Khan, A. G., Whidby, J., Miller, M. T., Scarborough, H., Zatorski, A. V., Cygan, A., et al. (2014). Structure of the core ectodomain of the hepatitis C virus envelope glycoprotein 2. *Nature* 509, 381–384. doi: 10.1038/nature13117

Kong, L., Giang, E., Nieusma, T., Kadam, R. U., Cogburn, K. E., Hua, Y., et al. (2013). Hepatitis C virus e2 envelope glycoprotein core structure. *Science* 342, 1090–1094. doi: 10.1126/science.1243876

Kong, L., Giang, E., Nieusma, T., Robbins, J. B., Deller, M. C., Stanfield, R. L., et al. (2012a). Structure of hepatitis C virus envelope glycoprotein E2 antigenic site 412 to 423 in complex with antibody AP33. *J. Virol.* 86, 13085–13088. doi: 10.1128/JVI.01939-12

Kong, L., Giang, E., Robbins, J. B., Stanfield, R. L., Burton, D. R., Wilson, I. A., et al. (2012b). Structural basis of hepatitis C virus neutralization by broadly neutralizing antibody HCV1. *Proc. Natl. Acad. Sci. U.S.A.* 109, 9499–9504. doi: 10.1073/pnas.1202924109

Krey, T., Meola, A., Keck, Z. Y., Damier-Piolle, L., Foung, S. K., and Rey, F. A. (2013). Structural basis of HCV neutralization by human monoclonal antibodies resistant to viral neutralization escape. *PLoS Pathog.* 9:e1003364. doi: 10.1371/journal.ppat.1003364

Lambert, P. H., Liu, M., and Siegrist, C. A. (2005). Can successful vaccines teach us how to induce efficient protective immune responses? *Nat. Med.* 11(Suppl. 4), S54–S62. doi: 10.1038/nm1216

Lavillette, D., Morice, Y., Germanidis, G., Donot, P., Soulier, A., Pagkalos, E., et al. (2005). Human serum facilitates hepatitis C virus infection, and neutralizing responses inversely correlate with viral replication kinetics at the acute phase of hepatitis C virus infection. *J. Virol.* 79, 6023–6034. doi: 10.1128/JVI.79.10.6023-6034.2005

Law, J. L., Chen, C., Wong, J., Hockman, D., Santer, D. M., Frey, S. E., et al. (2013). A hepatitis C virus (HCV) vaccine comprising envelope glycoproteins gpE1/gpE2 derived from a single isolate elicits broad cross-genotype neutralizing antibodies in humans. *PLoS ONE* 8:e59776. doi: 10.1371/journal.pone.0059776

Law, M., Maruyama, T., Lewis, J., Giang, E., Tarr, A. W., Stamataki, Z., et al. (2008). Broadly neutralizing antibodies protect against hepatitis C virus quasispecies challenge. *Nat. Med.* 14, 25–27. doi: 10.1038/nm1698

Li, C., Candotti, D., and Allain, J. P. (2001). Production and characterization of monoclonal antibodies specific for a conserved epitope within hepatitis C virus hypervariable region 1. *J. Virol.* 75, 12412–12420. doi: 10.1128/JVI.75.24.12412-12420.2001

Lindenbach, B. D., Evans, M. J., Syder, A. J., Wolk, B., Tellinghuisen, T. L., Liu, C. C., et al. (2005). Complete replication of hepatitis C virus in cell culture. *Science* 309, 623–626. doi: 10.1126/science.1114016

Liu, L., Fisher, B. E., Dowd, K. A., Astemborski, J., Cox, A. L., and Ray, S. C. (2010). Acceleration of hepatitis C virus envelope evolution in humans is consistent with progressive humoral immune selection during the transition from acute to chronic infection. *J. Virol.* 84, 5067–5077. doi: 10.1128/JVI.02265-09

Mancini, N., Diotti, R. A., Perotti, M., Sautto, G., Clementi, N., Nitti, G., et al. (2009). Hepatitis C virus (HCV) infection may elicit neutralizing antibodies targeting epitopes conserved in all viral genotypes. *PLoS ONE* 4:e8254. doi: 10.1371/journal.pone.0008254

McCaffrey, K., Boo, I., Poumbourios, P., and Drummer, H. E. (2007). Expression and characterization of a minimal hepatitis C virus glycoprotein E2 core domain that retains CD81 binding. *J. Virol.* 81, 9584–9590. doi: 10.1128/JVI.02782-06

McCaffrey, K., Gouklani, H., Boo, I., Poumbourios, P., and Drummer, H. E. (2011). The variable regions of hepatitis C virus glycoprotein E2 have an essential structural role in glycoprotein assembly and virion infectivity. *J. Gen. Virol.* 92, 112–121. doi: 10.1099/vir.0.026385-0

Meuleman, P., Hesselgesser, J., Paulson, M., Vanwolleghem, T., Desombere, I., Reiser, H., et al. (2008). Anti-CD81 antibodies can prevent a hepatitis C virus infection in vivo. *Hepatology* 48, 1761–1768. doi: 10.1002/hep.22547

Meunier, J. C., Russell, R. S., Goossens, V., Priem, S., Walter, H., Depla, E., et al. (2008). Isolation and characterization of broadly neutralizing human monoclonal antibodies to the e1 glycoprotein of hepatitis C virus. *J. Virol.* 82, 966–973. doi: 10.1128/JVI.01872-07

Mondelli, M. U., Cerino, A., Segagni, L., Meola, A., Cividini, A., Silini, E., et al. (2001). Hypervariable region 1 of hepatitis C virus: immunological decoy or biologically relevant domain? *Antiviral Res.* 52, 153–159. doi: 10.1016/S0166-3542(01)00180-2

Morin, T. J., Broering, T. J., Leav, B. A., Blair, B. M., Rowley, K. J., Boucher, E. N., et al. (2012). Human monoclonal antibody HCV1 effectively prevents and treats HCV infection in chimpanzees. *PLoS Pathog.* 8:e1002895. doi: 10.1371/journal.ppat.1002895

Nabel, G. J. (2012). Rational design of vaccines for AIDS and influenza. *Trans. Am. Clin. Climatol. Assoc.* 123, 9–15; discussion 15–16.

Osburn, W. O., Fisher, B. E., Dowd, K. A., Urban, G., Liu, L., Ray, S. C., et al. (2010). Spontaneous control of primary hepatitis C virus infection and immunity against persistent reinfection. *Gastroenterology* 138, 315–324. doi: 10.1053/j.gastro.2009.09.017

Owsianka, A., Clayton, R. F., Loomis-Price, L. D., McKeating, J. A., and Patel, A. H. (2001). Functional analysis of hepatitis C virus E2 glycoproteins and virus-like particles reveals structural dissimilarities between different forms of E2. *J. Gen. Virol.* 82, 1877–1883.

Owsianka, A., Tarr, A. W., Juttla, V. S., Lavillette, D., Bartosch, B., Cosset, F. L., et al. (2005). Monoclonal antibody AP33 defines a broadly neutralizing epitope on the hepatitis C virus E2 envelope glycoprotein. *J. Virol.* 79, 11095–11104. doi: 10.1128/JVI.79.17.11095-11104.2005

Owsianka, A. M., Tarr, A. W., Keck, Z. Y., Li, T. K., Witteveldt, J., Adair, R., et al. (2008). Broadly neutralizing human monoclonal antibodies to the hepatitis C virus E2 glycoprotein. *J. Gen. Virol.* 89, 653–659. doi: 10.1099/vir.0.83386-0

Owsianka, A. M., Timms, J. M., Tarr, A. W., Brown, R. J., Hickling, T. P., Szwejk, A., et al. (2006). Identification of conserved residues in the E2 envelope glycoprotein of the hepatitis C virus that are critical for CD81 binding. *J. Virol.* 80, 8695–8704. doi: 10.1128/JVI.00271-06

Penin, F., Combet, C., Germanidis, G., Frainais, P. O., Deleage, G., and Pawlotsky, J. M. (2001). Conservation of the conformation and positive charges of hepatitis C virus E2 envelope glycoprotein hypervariable region 1 points to a role in cell attachment. *J. Virol.* 75, 5703–5710. doi: 10.1128/JVI.75.12.5703-5710.2001

Perotti, M., Mancini, N., Diotti, R. A., Tarr, A. W., Ball, J. K., Owsianka, A., et al. (2008). Identification of a broadly cross-reacting and neutralizing human monoclonal antibody directed against the hepatitis C virus E2 protein. *J. Virol.* 82, 1047–1052. doi: 10.1128/JVI.01986-07

Pestka, J. M., Zeisel, M. B., Blaser, E., Schurmann, P., Bartosch, B., Cosset, F. L., et al. (2007). Rapid induction of virus-neutralizing antibodies and viral clearance in a single-source outbreak of hepatitis C. *Proc. Natl. Acad. Sci. U.S.A.* 104, 6025–6030. doi: 10.1073/pnas.0607026104

Pileri, P., Uematsu, Y., Campagnoli, S., Galli, G., Falugi, F., Petracca, R., et al. (1998). Binding of hepatitis C virus to CD81. *Science* 282, 938–941. doi: 10.1126/science.282.5390.938

Raghuraman, S., Park, H., Osburn, W. O., Winkelstein, E., Edlin, B. R., and Rehermann, B. (2012). Spontaneous clearance of chronic hepatitis C virus infection is associated with appearance of neutralizing antibodies and reversal of T-cell exhaustion. *J. Infect. Dis.* 205, 763–771. doi: 10.1093/infdis/jir835

Ray, R., Meyer, K., Banerjee, A., Basu, A., Coates, S., Abrignani, S., et al. (2010). Characterization of antibodies induced by vaccination with hepatitis C virus envelope glycoproteins. *J. Infect. Dis.* 202, 862–866. doi: 10.1086/655902

Roccasecca, R., Ansuini, H., Vitelli, A., Meola, A., Scarselli, E., Acali, S., et al. (2003). Binding of the hepatitis C virus E2 glycoprotein to CD81 is strain specific and is modulated by a complex interplay between hypervariable regions 1 and 2. *J. Virol.* 77, 1856–1867. doi: 10.1128/JVI.77.3.1856-1867.2003

Sabo, M. C., Luca, V. C., Prentoe, J., Hopcraft, S. E., Blight, K. J., Yi, M., et al. (2011). Neutralizing monoclonal antibodies against hepatitis C virus E2 protein bind discontinuous epitopes and inhibit infection at a postattachment step. *J. Virol.* 85, 7005–7019. doi: 10.1128/JVI.00586-11

Scarselli, E., Ansuini, H., Cerino, R., Roccasecca, R. M., Acali, S., Filocamo, G., et al. (2002). The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. *EMBO J.* 21, 5017–5025. doi: 10.1093/emboj/cdf529

Shang, D., Zhai, W., and Allain, J. P. (1999). Broadly cross-reactive, high-affinity antibody to hypervariable region 1 of the hepatitis C virus in rabbits. *Virology* 258, 396–405. doi: 10.1006/viro.1999.9730

Tarr, A. W., Owsianka, A. M., Jayaraj, D., Brown, R. J., Hickling, T. P., Irving, W. L., et al. (2007). Determination of the human antibody response to the epitope defined by the hepatitis C virus-neutralizing monoclonal antibody AP33. *J. Gen. Virol.* 88, 2991–3001. doi: 10.1099/vir.0.83065-0

Tarr, A. W., Owsianka, A. M., Timms, J. M., McClure, C. P., Brown, R. J., Hickling, T. P., et al. (2006). Characterization of the hepatitis C virus E2 epitope defined by the broadly neutralizing monoclonal antibody AP33. *Hepatology* 43, 592–601. doi: 10.1002/hep.21088

Torres-Puente, M., Cuevas, J. M., Jimenez-Hernandez, N., Bracho, M. A., Garcia-Robles, I., Wrobel, B., et al. (2008). Using evolutionary tools to refine the new hypervariable region 3 within the envelope 2 protein of hepatitis C virus. *Infect. Genet. Evol.* 8, 74–82. doi: 10.1016/j.meegid.2007.10.005

Troesch, M., Meunier, I., Lapierre, P., Lapointe, N., Alvarez, F., Boucher, M., et al. (2006). Study of a novel hypervariable region in hepatitis C virus (HCV) E2 envelope glycoprotein. *Virology* 352, 357–367. doi: 10.1016/j.virol.2006.05.015

Vanwolleghem, T., Bukh, J., Meuleman, P., Desombere, I., Meunier, J. C., Alter, H., et al. (2008). Polyclonal immunoglobulins from a chronic hepatitis C virus patient protect human liver-chimeric mice from infection with a homologous hepatitis C virus strain. *Hepatology* 47, 1846–1855. doi: 10.1002/hep.22244

von Hahn, T., Yoon, J. C., Alter, H., Rice, C. M., Rehermann, B., Balfe, P., et al. (2007). Hepatitis C virus continuously escapes from neutralizing antibody and T-cell responses during chronic infection in vivo. *Gastroenterology* 132, 667–678. doi: 10.1053/j.gastro.2006.12.008

Wahid, A., Helle, F., Descamps, V., Duverlie, G., Penin, F., and Dubuisson, J. (2013). Disulfide bonds in hepatitis C virus glycoprotein e1 control the assembly and entry functions of e2 glycoprotein. *J. Virol.* 87, 1605–1617. doi: 10.1128/JVI.02659-12

Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., et al. (2005). Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat. Med.* 11, 791–796. doi: 10.1038/nm1268

Weiner, A. J., Brauer, M. J., Rosenblatt, J., Richman, K. H., Tung, J., Crawford, K., et al. (1991). Variable and hypervariable domains are found in the regions of HCV corresponding to the flavivirus envelope and NS1 proteins and the pestivirus envelope glycoproteins. *Virology* 180, 842–848. doi: 10.1016/0042-6822(91)90104-J

Yi, M., Villanueva, R. A., Thomas, D. L., Wakita, T., and Lemon, S. M. (2006). Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. *Proc. Natl. Acad. Sci. U.S.A.* 103, 2310–2315. doi: 10.1073/pnas.0510727103

Zhang, J., Randall, G., Higginbottom, A., Monk, P., Rice, C. M., and McKeating, J. A. (2004). CD81 is required for hepatitis C virus glycoprotein-mediated viral infection. *J. Virol.* 78, 1448–1455. doi: 10.1128/JVI.78.3.1448-1455.2004

Zhang, P., Zhong, L., Struble, E. B., Watanabe, H., Kachko, A., Mihalik, K., et al. (2009). Depletion of interfering antibodies in chronic hepatitis C patients and vaccinated chimpanzees reveals broad cross-genotype neutralizing activity. *Proc. Natl. Acad. Sci. U.S.A.* 106, 7537–7541. doi: 10.1073/pnas.0902749106

Zibert, A., Meisel, H., Kraas, W., Schulz, A., Jung, G., and Roggendorf, M. (1997). Early antibody response against hypervariable region 1 is associated with acute self-limiting infections of hepatitis C virus. *Hepatology* 25, 1245–1249. doi: 10.1002/hep.510250530

Zibert, A., Schreier, E., and Roggendorf, M. (1995). Antibodies in human sera specific to hypervariable region 1 of hepatitis C virus can block viral attachment. *Virology* 208, 653–661. doi: 10.1006/viro.1995.1196

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 06 May 2014; paper pending published: 04 June 2014; accepted: 16 June 2014; published online: 03 July 2014.

Citation: Drummer HE (2014) Challenges to the development of vaccines to hepatitis C virus that elicit neutralizing antibodies. Front. Microbiol. ***5****:329. doi: 10.3389/fmicb.2014.00329*

This article was submitted to Microbial Immunology, a section of the journal Frontiers in Microbiology.

Copyright © 2014 Drummer. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 11 September 2014
doi: 10.3389/fmicb.2014.00477

Targeting regulatory T cells to improve vaccine immunogenicity in early life

Jorjoh Ndure [1] ***and Katie L. Flanagan*** [2] *******

[1] *Infant Immunology Group, Vaccinology Theme, Medical Research Council Laboratories, Fajara, The Gambia*
[2] *Vaccine and Infectious Diseases Laboratory, Department of Immunology, Monash University, Melbourne, VIC, Australia*

Edited by:
Shuo Li, Burnet Institute, Australia

Reviewed by:
Diego A. Vargas-Inchaustegui, National Cancer Institute, USA
Lenny Moise, EpiVax, USA

****Correspondence:***
Katie L. Flanagan, Vaccine and Infectious Diseases Laboratory, Department of Immunology, Monash University, Level 2, AMREP Building, 89 Commercial Road, Melbourne, VIC 3004, Australia
e-mail: katie.flanagan@dhhs.tas.gov.au

Human newborns and infants are bombarded with multiple pathogens on leaving the sterile intra-uterine environment, and yet have suboptimal innate immunity and limited immunological memory, thus leading to increased susceptibility to infections in early life. They are thus the target age group for a host of vaccines against common bacterial and viral pathogens. They are also the target group for many vaccines in development, including those against tuberculosis (TB), malaria, and HIV infection. However, neonatal and infant responses too many vaccines are suboptimal, and in the case of the polysaccharide vaccines, it has been necessary to develop the alternative conjugated formulations in order to induce immunity in early life. Immunoregulatory factors are an intrinsic component of natural immunity necessary to dampen or control immune responses, with the caveat that they may also decrease immunity to infections or lead to chronic infection. This review explores the key immunoregulatory factors at play in early life, with a particular emphasis on regulatory T cells (Tregs). It goes on to explore the role that Tregs play in limiting vaccine immunogenicity, and describes animal and human studies in which Tregs have been depleted in order to enhance vaccine responses. A deeper understanding of the role that Tregs play in limiting or controlling vaccine-induced immunity would provide strategies to improve vaccine immunogenicity in this critical age group. New adjuvants and drugs are being developed that can transiently suppress Treg function, and their use as part of human vaccination strategies against infections is becoming a real prospect for the future.

Keywords: regulatory T cells, vaccines, infants, neonates, immunogenicity, immune modulation, adjuvants

INTRODUCTION

The infant immune system is uniquely adapted to meet the challenges of early life (Kollmann et al., 2012). The newborn emerges from an immune-protected environment into a world where they constantly encounter new antigens. There is therefore a need to have a series of immunoregulatory mechanisms in place in order to prevent excessive inflammation and tissue damage. At the same time the infant needs to develop immune memory upon pathogen encounter in order to be protected against future challenge. The newborn has little immunological memory, and neonates and infants are heavily reliant on innate immunity to protect them against antigenic challenge as discussed in a series of comprehensive review articles (Levy, 2007; Ghazal et al., 2013; Levy and Wynn, 2014).

In this review we discuss the regulatory factors that infants employ to suppress or control their developing immunity. We will focus on regulatory T cells (Tregs) in particular, and the potential role they play in suppressing or controlling vaccine-induced immunity in early life. We will explore the mechanisms of action used by Tregs to confer suppression, and differences in the phenotypic and functional characteristics between Tregs in infants and adults. We will discuss the role of Tregs in malaria, HIV, and hepatitis C virus (HCV) infections; and briefly describe the results of clinical trials in human infants of vaccines against these three infections. A detailed understanding of the immunoregulatory factors controlling vaccine immunogenicity in early life may provide potential strategies for improving vaccine efficacy in this vulnerable age group. We will discuss immunotherapeutic agents and vaccine adjuvants developed for use in humans that can down-modulate Treg activity and thus enhance vaccine efficacy, demonstrating that this approach is a viable option for the future.

THE INFANT IMMUNE SYSTEM

INNATE IMMUNITY

The innate immune system which acts as the first line of defense against infection is suboptimal at birth, and does not reach full capacity until teenage years. Innate cells express pattern recognition receptors (PRRs) which detect highly conserved pattern associate molecular patterns (PAMPs) expressed by invading pathogens or vaccines, including Toll-like receptors (TLRs) and NOD-like receptors (NLRs). Newborns and young infants have similar levels of expression of these PRRs as adults (Kollmann et al., 2012), however, responses to PRR stimulation are low at birth in part due to diminished innate signaling pathways such as IRF7 translocation (Danis et al., 2008) and TLR3 and 4 signaling (Aksoy et al., 2007). Reactivity to certain PRR agonists, e.g., TLR4 and TLR5 are acquired rapidly, and reactivity of the viral ssRNA sensing TLR7 and TLR8 receptors is robust from birth (Burl et al., 2011), hence TLR7/8 agonists are being investigated

as possible adjuvants to boost immune responses to neonatal vaccines (Dowling et al., 2013). Th2 (IL-6, IL-10) and Th17 (IL-6, IL-23) polarizing cytokines dominate the innate response in early life, while TNF-α and IL-1β responses rise in the first few years of life as the former cytokines decline (Belderbos et al., 2009; Kollmann et al., 2009; Nguyen et al., 2010; Burl et al., 2011). Infant dendritic cell (DC) function is also suboptimal (De Wit et al., 2004; Goriely et al., 2004; Aksoy et al., 2007), and NK cells (Guilmot et al., 2011) and neutrophil functions (Carr, 2000) are less potent than in adults. Low complement levels in neonatal plasma are thought to increase susceptibility to certain bacterial infections, and lead to impaired adaptive immunity (Levy, 2007).

ADAPTIVE IMMUNITY IN INFANCY

Infant T cell immunity

The adaptive immune system is characterized by minimal immunological memory at birth, since the newborn has been relatively protected from antigenic exposure *in utero*, and most of their T cells are of a naïve phenotype. Furthermore, high levels of TGF-β, progesterone and prostaglandin E2 *in utero* required to prevent the mother developing Th1 alloreactivity to her fetus (Philbin and Levy, 2009), alongside poor innate Th1 support (Langrish et al., 2002), result in the newborn having intrinsically skewed Th2-type immunity from birth. Additionally, the Th17 biased innate immunity in infants also results in a Th17 adaptive bias. This bias against Th1 immunity results in an increased vulnerability to microbial infections and suboptimal reactivity to many vaccines. Despite this, infants have been shown to stimulate adult level Th1 type immune responses to BCG vaccination (Marchant et al., 1999) and are thus capable of robust Th1 immunity. However, neonatal BCG vaccination results in a Th17 biased mycobacterial response compared to those receiving BCG at $4\frac{1}{2}$ months of age (Burl et al., 2010), in keeping with the Th17 bias described above.

Infant B cell immunity

Newborn infants acquire IgG antibodies transplacentally from their mothers which provide protection against infections encountered in early life, while the other immunoglobulin subclasses are unable to cross the maternal–placenta interface. The maternally acquired antibody (MAb) levels wane over the first 6 months of life and are usually absent by 1 year of age. Several studies suggest that MAbs inhibit humoral responses to infant vaccines; including live measles vaccine (Albrecht et al., 1977) and oral poliomyelitis vaccine, and non-live vaccines including pertussis (Burstyn et al., 1983; Englund et al., 1995), tetanus and diphtheria toxoids (Bjorkholm et al., 1995), Hib conjugate vaccine (Claesson et al., 1989; Daum et al., 1991) and hepatitis A vaccine (Kanra et al., 2000); while other studies report no influence of MAbs on responses to these vaccines (Gans et al., 1998; Siegrist et al., 1998; Sallusto et al., 1999). Responses may still be protective even if MAb inhibition occurs (Jones et al., 2014), and while MAbs may interfere with the generation of humoral responses to vaccination, T cell responses do not seem to be similarly affected (Siegrist, 2003).

Human neonatal antibody responses are delayed in onset, of shorter duration, achieve lower peak levels, and have lower affinity than adults. The isotype distribution also differs, with IgG1 and IgG2 levels peaking at ∼3–4 years of age, and IgG4 only reaching adult levels at 4–6 years of age, while IgG3 is stable from birth (Ngamphaiboon et al., 1998). Histological studies of infant splenic tissue show that the marginal zone does not reach full development until 2 years of age, which alongside low complement levels (Zandvoort and Timens, 2002; Kruetzmann et al., 2003) and low expression of CD21 (complement receptor 2; Griffioen et al., 1993), would account for the delayed antibody response to T cell independent glycoproteins and polysaccharide antigens, including encapsulated bacteria such as *Streptococcus pneumoniae* and *Haemophilus influenzae*, and thus poor reactivity to polysaccharide vaccines (Adkins et al., 2004).

IMMUNOREGULATORY FACTORS IN NEONATAL AND INFANT PLASMA

Neonatal and infant plasma contain a number of immunoregulatory factors that serve to maintain Th2 polarization, and limit pro-inflammatory innate and adaptive immunity. Newborns and infants have high levels of plasma adenosine, an endogenous purine metabolite with immunosuppressive properties. Adenosine causes mononuclear cells to produce cAMP, which acts as a second messenger to inhibit TLR-stimulated production of pro-inflammatory cytokines while polarizing toward IL-10 and Th17 cytokine production (Levy et al., 2006; Power Coombs et al., 2011; Philbin et al., 2012). Neonatal monocytes have increased sensitivity to these effects of adenosine via their adenosine A3 receptors, thus modulation of this system could potentially be used to enhance innate and therefore adaptive pro-inflammatory responses.

Several studies have shown that there are high levels of the immunosuppressive cytokine IL-10 in cord blood (CB; De Wit et al., 2004; Belderbos et al., 2009; Nguyen et al., 2010). IL-10 can be produced by most cell types of the immune system, including antigen presenting cells (APCs), granulocytes, and Th1, Th2 and many regulatory T cell subsets. IL-10 acts at a number of stages of an immune response in order to control inflammation. It inhibits the production of pro-inflammatory cytokines and chemokines by monocytes, macrophages and DCs, leading to increased IL-10 production by various T cell subsets. It suppresses both Th1 and the more recently described "Th1+Th17" cells, while enhancing $CD4^+FOXP3^+$ (forkhead box P3) regulatory T cell survival and activity, and promoting IgG and IgA class switching by B cells (Banchereau et al., 2012).

HUMAN REGULATORY T CELL SUBTYPES AND THEIR MODES OF ACTION

Regulatory T cells are unique subpopulations of T cells that play a major role in immune homeostasis and tolerance (Sakaguchi, 2000; Belkaid et al., 2002; Mills and Mcguirk, 2004; Belkaid, 2007). Although Tregs have been shown to be beneficial in preventing an over-exuberant response and immune pathology following encounter with pathogens (Belkaid, 2008; Belkaid and Tarbell, 2009), they have also been shown to limit the favorable effector responses required for sterilizing immunity, thus allowing pathogen persistence (Kao et al., 2010).

THYMUS DERIVED AND PERIPHERAL CD4$^+$FOXP3$^+$ Tregs

The Treg field was invigorated with the discovery of the transcription factor FOXP3 which is vital for the development, function and homeostasis of Tregs (Fontenot et al., 2003; Hori et al., 2003), and is thus considered the master regulator of Tregs. Its importance is further highlighted by patients with mutations in FOXP3 who develop a severe fatal disorder known as immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome (Bennett and Ochs, 2001; Gambineri et al., 2003).

Recently a group of experts in the Treg field introduced a consensus nomenclature for FOXP3$^+$ Tregs. They suggest replacing previously used terms which they describe as being to some extent "inaccurate and ambiguous" (Abbas et al., 2013). They recommend that the subset of FOXP3$^+$ Tregs of thymic origin, which are also known as natural Tregs (nTregs), should be called thymus-derived Tregs (tTregs); while FOXP3$^+$ Tregs that differentiate in the periphery should be called peripherally derived Tregs (pTreg) rather than the previous term inducible Tregs (iTregs). The pTregs are induced in the periphery in response to antigenic stimulation, and possess identical characteristics to tTregs, and therefore both subsets will be described together.

Phenotype of human CD4$^+$ FOXP3$^+$ Tregs

CD4$^+$FOXP3$^+$ Tregs are the most widely studied Treg subset. They were first described as a subset of CD4$^+$ T cells which constitutively express the interleukin 2 (IL-2) receptor alpha-chains (CD25) and can prevent the development of autoimmunity in mice (Sakaguchi et al., 1995).

Determining the precise phenotype of human CD4$^+$FOXP3$^+$ Tregs has proved difficult with many conflicting studies. Since CD25 is transiently expressed on conventional T cells (Hatakeyama et al., 1989), the CD25^{hi} subset is described as a more reliable marker of CD4$^+$FOXP3$^+$ Tregs in humans (Schmetterer et al., 2012). Human FOXP3$^+$ Tregs also tend to express low levels of the IL-7 receptor CD127 (Liu et al., 2006; Seddiki et al., 2006). Therefore the most commonly analyzed phenotypes in human studies are CD4$^+$CD25$^+$FOXP3$^+$ or CD4$^+$CD25$^+$CD127^{lo}. Furthermore, human CD4$^+$FOXP3$^+$ Tregs generally express high levels of the co-inhibitory receptor cytotoxic T lymphocyte antigen 4 (CTLA4; Sansom and Walker, 2006). More recently the chemokine markers CCR4, CCR6, CXCR3, and CXCR10 have been proposed to define four distinct populations of human tTregs, each with distinct functional characteristics (Duhen et al., 2012). Each of these four Treg subsets are thought to co-localize *in vivo* with and regulate a distinct Th subset (Th1, Th2, Th17, Th22) expressing the same chemokine receptors.

CD45RA expression can be used to distinguish tTregs that are naïve or resting (rTregs; FOXP3^{lo}CD45RA$^+$) from the memory subset described as activated Tregs (aTregs; FOXP3^{hi}CD45RA$^-$; Miyara et al., 2009). The memory Tregs can be further subdivided into central memory (Treg$_{CM}$) and effector memory (Treg$_{EM}$) similarly to Th cells, based on the expression of chemokine receptor 7 (CCR7; Sallusto et al., 1999; Tosello et al., 2008).

Certain subpopulations among CD4$^+$FOXP3$^+$ Tregs are more suppressive than others. For example, Tregs expressing the tumor necrosis factor receptor 2 (TNFRII) are thought to represent a highly suppressive CD4$^+$FOXP3$^+$ Treg subset (Minigo et al., 2009). Those CD4$^+$CD25$^+$FOXP3$^+$ Tregs expressing the transmembrane cyclic ADP ribose hydrolase CD38 (mainly thymic derived and in the spleen) have particularly high suppressive activity in a murine model (Patton et al., 2011). CD38 is part of a cascade involved in the production of the immunosuppressive factor adenosine from NAD$^+$ (Horenstein et al., 2013) which can have immunoregulatory properties as discussed earlier. Interestingly, the majority of infant T cells express CD38 (Scalzo-Inguanti and Plebanski, 2011), and as previously mentioned infants also have high plasma levels of adenosine, but a link between these two factors has yet to be explored in neonates and infants.

Mechanisms of CD4$^+$ FOXP3$^+$ Treg mediated suppression

CD4$^+$FOXP3$^+$ Tregs can suppress the proliferation and activation of a multitude of immune cell types including T cells, NK and NKT cells, monocytes, macrophages, B cells, DCs, and eosinophils. They employ a variety of mechanisms to mediate this suppression, and are thought to be flexible in this respect by adapting their mechanism according to their local environment (reviewed by Wing and Sakaguchi, 2012). Both IL-2 and CTLA-4-dependent mechanisms have been described, with CD25 and CTLA-4 knockout mice having a similar phenotype to Foxp3 deficient mice (Wing and Sakaguchi, 2012). It is thought that the constitutive expression of CD25 by CD4$^+$FOXP3$^+$ Tregs allows them to consume the available IL-2, depriving effector T cells (Teffs) and leading to effector cell death (De La Rosa et al., 2004). Those tTregs expressing CTLA-4 can suppress T cell responses via down-modulation of CD28 signaling (Walker, 2013), and reduced co-stimulatory capacity of CD80/86 expressed by DCs (Wing et al., 2011).

A commonly used mechanism of Treg action is the production of soluble inhibitory factors, including either membrane bound or released immunosuppressive cytokines IL-10, TGF-β, and IL-35 (Collison et al., 2007). FOXP3$^+$ Tregs can also generate high concentrations of adenosine (Mandapathil et al., 2010) which binds to the A2a receptor on immune cells activating an immunoinhibitory loop (Sitkovsky and Ohta, 2005) which results in inhibition of T cell proliferation and cytokine production (Raskovalova et al., 2005).

TYPE 1 REGULATORY T CELLS (Tr1)

The Tr1 Tregs are a unique Treg subset that do not rely on the expression of high levels of CD25 or FOXP3 for their function (Levings and Roncarolo, 2000). They are activated in the periphery following antigenic stimulation in the presence of IL-10 (Groux et al., 1997; Vieira et al., 2004). Recently, lymphocyte-activation gene 3 (LAG3) and CD49b have been described to represent specific markers for Tr1 cells (Gagliani et al., 2013).

The Tr1 Tregs are known to produce high levels of the immunosuppressive cytokines IL-10 and TGF-β, some IL-5, low levels of IFN-γ and IL-2, and no IL-4 (Groux et al., 1997). The secretion of IL-10 is the main mechanism by which Tr1 cells are thought to mediate suppression. The IL-10 can be either free or membrane bound, and has been shown to suppress Teff proliferation/activation both directly

and indirectly via a modulation of APC function (Roncarolo et al., 2006). They have also been shown to use cell–cell contact mechanisms (Gregori et al., 2012) and the production of granzyme B and perforin (Gregori et al., 2010) to mediate suppression.

T HELPER TYPE 3 CELLS (Th3)

This unique subset of TGF-β producing Tregs was identified in early studies investigating oral tolerance. They have been shown to suppress the proliferation and activation of Th1 cells and suppress the development of autoimmunity in the mouse model of multiple sclerosis (Chen et al., 1994). They become activated in the periphery upon encounter with a specific antigen, and suppress via the production of the inhibitory cytokine TGF-β. Some studies show that Th3 cells may have a role to play in controlling autoimmunity and allergy in humans (Andersson et al., 2002; Perez-Machado et al., 2003), but the role that this subset plays in the maintenance of immune tolerance in humans is still not clearly defined.

$CD8^+$ Tregs

While $CD4^+$ Tregs have been widely studied in humans, $CD8^+$ Tregs have not received the same attention. However, there is increasing evidence that subsets of $CD8^+$ Tregs also play important immunoregulatory roles, and impaired $CD8^+$ Treg function may lead to autoimmunity (Hu et al., 2004; Lu et al., 2008). The most widely described phenotype for $CD8^+$ Tregs is $CD25^+CD28^-$ (Ciubotariu et al., 1998; Filaci et al., 2004). Other markers include CD122, CTLA-4, GITR, CD38, CD103, and CD8αα (Uss et al., 2006; Simone et al., 2008; Smith and Kumar, 2008; Liu et al., 2014); a host of different CD8 Treg subsets have been described in humans expressing various combinations of these markers (Suzuki et al., 2012). While FOXP3 expression has been described in many CD8 Treg subsets, it may also represent an activation marker rather than acting as a regulatory factor since $CD8^+FOXP3^+$ cells have been found to be minimally suppressive in some studies (Mayer et al., 2011). Mechanisms of action of $CD8^+$ Tregs that have been reported include cell–cell contact mediated suppression, secretion of the suppressive cytokines IL-10 and TGF-β, and induction of APC energy (Suzuki et al., 2008). $CD8^+CD45RA^+CCR7^+FOXP3^+$ cells may represent a discrete subset of CD8 Tregs which interfere with the TCR signaling cascade (Suzuki et al., 2012). More extensive work is required to better understand the origin and role of $CD8^+$ Tregs in immunoregulation and autoimmunity, particularly in humans.

PHENOTYPIC AND FUNCTIONAL DIFFERENCES BETWEEN Tregs IN INFANTS COMPARED TO ADULTS

Distinct qualitative and quantitative differences have been identified between the Tregs in adults and those of infants. Most of the studies in infants have analyzed Tregs in neonatal CB for comparison with adults, and different phenotypic markers have been used to characterize the Tregs in these studies contributing to some discrepancies in the results.

$FOXP3^+$ Tregs have been found in much higher levels at birth compared to adults, whether defined as $CD4^+CD25^+CD127^{lo}$ (Nettenstrom et al., 2013) or $CD4^+CD25^+FOXP3^+$ (Flanagan et al., 2010). Preterm infants have been shown to have higher levels still (Luciano et al., 2014). However, a study comparing $CD4^+CD25^+CD127^{lo}$ Tregs at different age groups, showed slight increases in Treg frequencies with age: 6.10% in CB; 7.22% in adults aged 20–25 years; and 7.5% in adults over the age of 60 years (Santner-Nanan et al., 2008); and another study found that neonates had similar number of cells expressing FOXP3 compared to their mothers, and a lower number of $CD4^+CD25^{bright}$ cells (Ly et al., 2009). The reason for these conflicting results is not known.

Cord blood Tregs have been shown to be predominantly of the $CD45RA^+CD45RO^-$ naïve phenotype in several studies (Kanegane et al., 1991; Wing et al., 2002; Takahata et al., 2004; Ly et al., 2009; Flanagan et al., 2010). Other phenotypic differences between cord and adult Tregs include the observation that CB Tregs are mostly $CD27^+$ and thus at an earlier differentiation state than their mothers; they have a lower apoptotic potential as evidenced by lower CD95/Fas expression than their mothers; and less CD62L suggesting less of a $Treg_{CM}$ lymph node homing phenotype (Flanagan et al., 2010). CB Tregs also express less CCR6 than their matched mothers, which is the chemokine receptor that characterizes the Th17- and Th22-like Tregs (Duhen et al., 2012). Since infants are Th2 biased then their Tregs should be predominantly of a $CCR4^+CCR6^-CXCR3^-$ Th2 Treg phenotype in keeping with the classification discussed previously (Duhen et al., 2012), although this has not been investigated in infants.

In vitro Treg suppression assays are difficult to perform in infants due to the lack of availability of large volumes of blood, combined with the low Treg frequencies in peripheral blood. Studies using CB are easier since large volumes are available for study. Several studies have shown that newborn CB Tregs are highly functional whereby they suppress T cell proliferation and IFN-γ production, further deviating from a Th1 response (Godfrey et al., 2005; Wing et al., 2005). Fan et al. (2012) found that $CD4^+CD25^+$ CB Tregs had a stronger immunosuppressive function than adult blood Tregs following two cycles of polyclonal stimulation. Mayer et al. (2012) found that CB $CD4^+CD25^{hi}$ cells failed to suppress upon TCR activation whereas those freshly purified from adult blood did, but CB Tregs became strongly suppressive after antigen-specific stimulation. Another study found that low FOXP3 expression levels by CB Tregs correlated with minimal suppressive activity, but following expansion there was a significant increase in the suppressive activity of these CB Tregs with a shift from the $CD45RA^+$ to the $CD45RA^-$ phenotype (Fujimaki et al., 2008). It has recently been shown that CB Tregs can be expanded more easily than adult peripheral blood Tregs, and CB Tregs are better suppressors in allogeneic mixed lymphocyte reactions than their adult counterparts (Lin et al., 2014).

Taken together these studies suggest distinct differences between infant and adult Tregs. Overall they seem to be present in higher frequencies than in adults, are more naïve and less differentiated, and are highly suppressive; all supporting an active immunoregulatory role in early life.

THE ROLE OF Tregs IN REGULATING IMMUNITY TO MALARIA, HIV, AND HEPATITIS C VIRUS

Regulatory T cells have been implicated with an immunoregulatory role in both murine and human malaria infections (Scholzen et al., 2010). In mice, ablation of $Foxp3^+$ Tregs led to increased T cell activation and decreased parasitaemia (Abel et al., 2012). *In vivo* depletion of Tregs protected mice from experimental cerebral malaria in a *Plasmodium berghei* model of infection (Wu et al., 2010). A FOXP3 promoter polymorphism in children has been associated with significant parasitaemia in a Congolese study suggesting a Treg role (Koukouikila-Koussounda et al., 2013). Malaria infected red blood cells (iRBCs) induced $CD4^+CD25^{hi}FOXP3^+$ Tregs *in vitro* in healthy human volunteers (Scholzen et al., 2009). In human malaria sporozoite challenge experiments, Tregs have been shown to be induced rapidly after infection, and linked to lower pro-inflammatory cytokines and increased TGF-β production (Walther et al., 2005). Another study showed that malaria antigens can activate latent TGF-β on the surface of aTregs (Clemente et al., 2011). A study of 112 subjects in Kenya (infants to adults) found a correlation between the frequency of $CD4^+CD25^{hi}$ T cells and increased risk of clinical malaria, suggesting Tregs may negatively affect natural immunity to malaria in humans (Todryk et al., 2008). In naturally exposed Gambians $CD4^+FOXP3^+CD127^{lo}$ Tregs during acute infection were inversely correlated with memory responses at 28 days, suggesting suppression of immune memory. In the same study, a $CD4^+FOXP3^-CD45RO^+$ T cell population co-producing IFN-γ and IL-10 was more prevalent among children with uncomplicated malaria than those with severe disease, suggesting a beneficial immunoregulatory role for this IL-10 producing subset, presumably by limiting excessive inflammation (Walther et al., 2009). A role for the highly suppressive $TNFRII^+$ Tregs in malaria parasite survival has been implicated in a study of Indonesian school children (Wammes et al., 2013). Overall, these data support an induction of Tregs during acute malaria infection which can limit the generation of immune memory and increase susceptibility to infection, but also control immunopathology and disease severity.

The role of Tregs in HIV infection remains poorly understood and the data are conflicting, in part due to the different phenotypes used to define Tregs in the various studies. However, the studies do support a regulatory role. For example, combination anti-retroviral therapy (cART) non-responders with persistent CD4 counts <200 cells/μL on therapy had higher peripheral blood Tregs and aTregs than cART responders, with higher $IL\text{-}10^+$ Tregs and lower FOXP3 in lymphoid tissue (Gaardbo et al., 2014). Another longitudinal study also found higher numbers of Tregs associated with immunological non-responders defined as CD4 <500/μL (Saison et al., 2014). A study analyzing multiple Treg phenotypes in HIV infected individuals found evidence of Treg redistribution depending on HIV status (Serana et al., 2014). Untreated viraemic patients with stable CD4 counts had higher proportions of naïve Tregs with decreased $Treg_{CM}$ compared to those on cART and healthy controls (Serana et al., 2014). The study suggests that effective cART restores Treg homeostasis since Treg subpopulations in the cART group were similar to those of healthy donors. Increased proportions and decreased numbers of Tregs associate with progression of HIV (Wang et al., 2013). Treg depletion in a murine chronic retrovirus infection model resulted in reduced viral loads (Dietze et al., 2013). In combination, the data suggest that Tregs may suppress HIV-specific immunity leading to lower CD4 counts and viral persistence.

Hepatitis C virus is characterized by its ability to establish chronic infection in the majority of those infected, and an immunoregulatory role for Tregs in this process has been well described. Chronic HCV patients have increased levels of $CD4^+$ and Tr1 Tregs in peripheral blood which are thought to suppress anti-viral T cell responses leading to viral persistence (Chang, 2007). Certain HCV epitope variants have been shown to induce Tregs in HCV-infected patients (Cusick et al., 2011). Chronic HCV patients have more serum IL-10 than those with resolved infection, which is proposed to play a role in the induction of $CD4^+FOXP3^+$ Tregs in chronic HCV infection (Macdonald et al., 2002; Cusick et al., 2013); and CD49b, a marker for IL-10 producing Tr1 Treg cells, is lower in those who respond to viral therapy, thus suggesting a regulatory role for Tr1 Tregs too (Fabien et al., 2014). Indoleamine 2,3-dioxygenase (IDO) production by stimulated monocyte derived DCs was higher in HCV patients compared to healthy controls, and these DCs were more able to induce Tregs, suggesting a role for this Treg induction pathway in chronic HCV (Higashitani et al., 2013). Expression of the inhibitory signaling pathway molecule T cell immunoglobulin and mucin-domain-protein-3 (Tim-3) is upregulated on both Teff and $CD4^+CD25^+FOXP3^+$ Tregs in chronic HCV, correlating with increased Treg and decreased Teff proliferation, suggesting that the Tim-3 pathway controls the Treg/Teff balance in chronic HCV (Moorman et al., 2012). Viral persistence following acute HCV infection is accompanied by increased plasma Galectin-9 (Gal-9) which is the ligand for Tim-3, alongside expanded Gal-9 expressing Tregs and increased expression of Tim-3 and CTLA-4 on HCV-specific $CD8^+$ T cells (Kared et al., 2013). Thus high levels of Tregs likely contribute to viral persistence in HCV infection, and both $FOXP3^+$ and Tr1 Tregs have been implicated. Mechanisms of Treg induction in HCV may be multifactorial but include HCV antigen driven induction, IL-10, IDO, Gal-9/Tim-3, and CTLA-4.

ROLE OF Tregs IN CONTROLLING VACCINE IMMUNOGENICITY

The role that Tregs play in controlling or limiting vaccine immunogenicity remains to be fully determined. Given that Tregs are induced by natural infections to regulate the inflammatory response, it makes sense that Tregs would be induced as part of the immune response to vaccination, particularly for live attenuated vaccines. One might predict that their induction would play a beneficial immunoregulatory role by preventing an overexuberant immune response to the vaccine. However, most studies suggest that Tregs can interfere with the generation of vaccine-induced immunity. Thus, depletion of Tregs pre-vaccination in murine models has been shown to enhance immune responses to some vaccines. In a DEREG mouse model, which allows for *in vivo* depletion of $Foxp3^+$ Treg cells at any point during an immune response using diphtheria toxin (Lahl and Sparwasser,

2011), Treg depletion led to an enhanced anti-tumor response to vaccination against an established melanoma (Klages et al., 2010). A more recent study showed that the short term depletion of Tregs in DEREG mice greatly enhanced vaccine-induced immunity against a solid tumor; increasing NK cells and CD8 T cell activation and IFN-γ production (Mattarollo et al., 2013). Administration of vaccines with anti-CD25 monoclonal Ab has been shown to induce more durable immunity in mice compared to when the vaccine is administered alone, for both BCG and hepatitis B vaccines, which has been attributed to a depletion of $CD25^+$ Tregs (Moore et al., 2005). Ho et al. (2010) showed that antigen-specific Tregs induced by environmental mycobacteria suppress Th1 immune responses, thus compromising the response to BCG vaccination in mice. They also showed a correlation between the pre-existing Tregs and the subsequent vaccine response. Murine studies of Parkinson's disease have shown that Tregs are induced by BCG vaccination (Lacan et al., 2013).

It is difficult to translate these studies into primates and humans since murine Tregs are not phenotypically identical, and *in vivo* depletion of $FOXP3^+$ Tregs in healthy humans presents logistic and ethical challenges. An oral vaccine against simian immunodeficiency virus (SIV) based on a *Lactobacillus* commensal that favors immune tolerance induction was used to induce T cell tolerance to SIV antigens in macaques (Lu et al., 2012). The vaccine-induced $CD8^+$ Tregs that suppressed $CD4^+$ T cell activation and *ex vivo* SIV replication, and provided sterile protection against an intrarectal SIV challenge in 15 of 16 vaccinated macaques. This strategy is thought to work because $CD4^+$ T cell activation drives the initial phase of viral replication, and provides the proof-of-concept that an oral Treg inducing vaccine could prevent the establishment of HIV infection.

Using a DC-based vaccine in HIV patients undergoing cART, it was shown that depletion of the Tregs *in vitro* significantly enhanced the vaccine-induced anti-HIV-1-specific polyfunctional T cell response, suggesting that Tregs can dampen vaccine-induced immunity (Macatangay et al., 2010). This study also showed a marked increase in the $CD4^+CD25^{hi}FOXP3^+$ Treg numbers following vaccination, however, this increase did not correlate with the effector $CD8^+$ T cell vaccine-induced response. Increased FOXP3 mRNA expression has been demonstrated in malaria vaccinated adults; however, the authors concluded that this might be attributed to the participants being naturally exposed to the malaria parasite rather than as a result of vaccination *per se* (Mwacharo et al., 2009).

Very few studies have looked at the role that Tregs play in controlling vaccine immunogenicity in infants. Our group found no correlation between PPD-specific $CD4^+CD25^+FOXP3^+$ Tregs or $CD4^+IL\text{-}10^+$ Tregs, or PPD stimulated total IL-10 production on the day of BCG vaccination of Gambian infants, and subsequent IFN-γ responses to PPD (Burl et al., 2010). No functional Treg assays were conducted in this study. In another study we found that placental associated malaria (PAM) infection is associated with increased malaria-specific $CD4^+CD25^+FOXP3^+$ Tregs (Flanagan et al., 2010) and that PAM also correlates with decreased immunogenicity of BCG vaccination as evidenced by poorer PPD reactivity persisting to 1 year of age compared to PAM negative children (Walther et al., 2012). Whether the Tregs are the cause of this attenuation of BCG responses is not known.

TARGETING Tregs *IN VIVO* TO ENHANCE VACCINE IMMUNOGENICITY

The cancer research field has made considerable advances in dissecting the role that Tregs play in cancer progression and their role in suppressing responses to cancer vaccines. Moreover, trials conducted in animal models and humans have demonstrated that certain drugs and immunotherapies can transiently decrease Treg frequencies *in vivo* leading to improved anti-tumor Teff functions, and in some cases reduced tumor load. Since Treg depletion can enhance inflammation and autoimmunity then such transient depletion, as opposed to long term effects, is desirable. In low doses, the agent cyclophosphamide transiently decreases Treg frequencies while Teff functions are preserved, leading to enhanced responses to vaccine antigens and improved vaccine immunogenicity in mouse and human cancer vaccine trials (Barbon et al., 2010; Le and Jaffee, 2012). Anti-CD25 monoclonal antibodies, which deplete Tregs *in vivo*, enhanced vaccine efficacy in mouse models of pancreatic carcinoma (Keenan et al., 2014) and melanoma (Tan et al., 2013). Basiliximab and Daclizumab are anti-human CD25 MAbs that cause both decreased number and decreased function of Tregs by blocking IL-2 signaling (Goebel et al., 2000; Kohm et al., 2006; Mitchell et al., 2011). Daclizumab has been used in several human breast cancer vaccine trials where it depleted Tregs and improved effector responses, and furthermore may reprogram naïve Tregs to become IFN-γ producers (Rech and Vonderheide, 2009; Rech et al., 2012). The human monoclonal antibody, Ipilimumab, inhibits Tregs by blocking CTLA-4; and was approved by the FDA in 2011 for use in melanoma patients (Peggs et al., 2009).

Certain innate agonists that are being used as vaccine adjuvants preferentially expand Teff over Tregs, e.g., the TLR3 agonist Poly(I:C) and the TLR9 agonist CpG-ODN; whereas others favor Treg expansion, e.g., the TLR7 agonist imiquimod (Perret et al., 2013). OX40 is part of the TNFR superfamily expressed by Teff and Tregs, and the monoclonal antibody increases Teff function while blocking Treg function. OX40 clones have been humanized as potential agents to enhance the immunogenicity of vaccines against infectious diseases (Voo et al., 2013).

An interesting approach is that of local depletion of Tregs at the site of injection of a vaccine. Chemokine receptor 4 (CCR4) antagonists can be used as vaccine adjuvants to target and decrease local recruitment of $CCR4^+$ Tregs in order to amplify vaccine responses at the site of immunization (Bayry, 2014).

Therefore a number of agents that target Tregs are being used experimentally in humans in order to enhance vaccine efficacy. Some of these are non-toxic and safe for use in humans, offering the future prospect of using this approach to enhance immunogenicity of vaccines against infectious diseases including malaria, HIV, and HCV.

TRIALS OF NOVEL VACCINES AGAINST MALARIA, HIV, AND HCV IN INFANTS

Despite the multiple obstacles to successful infant vaccination discussed above, many vaccines are currently delivered in infancy

with good immunogenicity, including the live BCG, measles and yellow fever vaccines; and the inactivated diphtheria, tetanus, pertussis and hepatitis B vaccines, *H. influenzae b*, and pneumococcal conjugate vaccine. The RTS,S/AS01 malaria vaccine is the most advanced malaria vaccine in human clinical trials, having reached phase III testing in children and infants, with potential licensure in 2015. It reduced clinical and severe malaria by 56 and 47.3% respectively in children aged 5–17 months (Bejon et al., 2008; Olotu et al., 2011); but only 31.3 and 36.6% when administered in three doses with routine Expanded Program on Immunization (EPI) vaccines in the 6–12 week old age group (Rts et al., 2012). Follow up of the 5–17 month old vaccinated group over a 4 year period found protection waned to 16.8%; with waning greater among those with higher malaria exposure suggesting that natural immunity to malaria contributes to the waning (Olotu et al., 2013). Whether exposure induced Tregs play a role in this waning has not been investigated.

Fowlpox and modified vaccinia Ankara (MVA) based malaria vaccines have been tested in 1–6 year olds with no evidence of protective efficacy (Bejon et al., 2007). A blood stage vaccine FMP2.1/AS02A has been tested in Malian children aged 1–6 years with an efficacy of <10% (Laurens et al., 2013). The blood stage alum adjuvanted GMZ2 malaria vaccine elicited good inhibitory antibody levels in pre-school children (Jepsen et al., 2013). Prime-boost strategies based on chimp adenovirus vector priming followed by MVA boosting are being tested in children and infants in Africa, and while results of these trials are not yet available the adult studies have shown unprecedented immunogenicity for malaria exposed populations (Ogwang et al., 2013) and should stimulate good immunity in infants.

Only a few human HIV vaccine trials have been conducted in healthy uninfected and HIV-exposed infants. Immunogenicity was limited among healthy Gambian infants given a single dose of MVA.HIVA, but this was not surprising given that MVA alone is known to be poorly immunogenic (Afolabi et al., 2013). In a Ugandan trial, infants were vaccinated at birth, 4, 8, and 12 weeks of age with ALVAC-HIV vCP1521 (ALVAC) candidate HIV vaccine which induced low level CD4 and CD8 T cell responses at 24 months (Kaleebu et al., 2014).

The target population for HCV vaccines include intravenous drug abusers and health care professionals. However, HCV infection is common throughout the world and mother-to-child transmission is well described (Yeung et al., 2001). Thus an infant HCV vaccine would have its place, particularly in resource poor settings where the anti-viral therapies available are currently not affordable. Both therapeutic and prophylactic vaccines are being developed, and several have now entered phase I/II human clinical trials, mostly of therapeutic vaccines in chronically infected cohorts. These include recombinant protein, peptide, DNA, and vector-based vaccines aimed at producing robust anti-T cell responses (Halliday et al., 2011). As far as we are aware no HCV vaccine trials have been conducted in children.

FUTURE PROSPECTS

There is very little in the literature regarding the role of Tregs in infants in general, and even less in respect to vaccine immunogenicity. The fact that functional Tregs are present in high numbers in infancy and have potent suppressive activity, coupled with poor immunological responses to some vaccines in this vulnerable age group, supports a need to better understand the role they play in controlling the response to childhood vaccines in particular. The data available suggest that Tregs suppress immunity to vaccines, and that they can also be induced by vaccination. We have shown that malaria, HIV, and HCV all use Tregs to evade host immune responses, therefore vaccine-induced Tregs would be predicted to reduce the protective efficacy of vaccines against these infections. A better understanding of the role that Tregs and other immunoregulatory factors play in contributing to poor vaccine immunogenicity in childhood would help with the design of better vaccines. Studies in cancer patients have shown that transient Treg inactivation or depletion is a viable approach to enhancing vaccine efficacy. A number of Treg modifying agents are available for use in humans, therefore this approach is a very real prospect for the future and may be particularly applicable to neonates and infants.

REFERENCES

Abbas, A. K., Benoist, C., Bluestone, J. A., Campbell, D. J., Ghosh, S., Hori, S., et al. (2013). Regulatory T cells: recommendations to simplify the nomenclature. *Nat. Immunol.* 14, 307–308. doi: 10.1038/ni.2554

Abel, S., Luckheide, N., Westendorf, A. M., Geffers, R., Roers, A., Muller, W., et al. (2012). Strong impact of $CD4^+$ $Foxp3^+$ regulatory T cells and limited effect of T cell-derived IL-10 on pathogen clearance during *Plasmodium yoelii* infection. *J. Immunol.* 188, 5467–5477. doi: 10.4049/jimmunol.1102223

Adkins, B., Leclerc, C., and Marshall-Clarke, S. (2004). Neonatal adaptive immunity comes of age. *Nat. Rev. Immunol.* 4, 553–564. doi: 10.1038/nri1394

Afolabi, M. O., Ndure, J., Drammeh, A., Darboe, F., Mehedi, S. R., Rowland-Jones, S. L., et al. (2013). A phase I randomized clinical trial of candidate human immunodeficiency virus type 1 vaccine MVA.HIVA administered to Gambian infants. *PLoS ONE* 8:e78289. doi: 10.1371/journal.pone.0078289

Aksoy, E., Albarani, V., Nguyen, M., Laes, J. F., Ruelle, J. L., De Wit, D., et al. (2007). Interferon regulatory factor 3-dependent responses to lipopolysaccharide are selectively blunted in cord blood cells. *Blood* 109, 2887–2893. doi: 10.1182/blood-2006-06-027862

Albrecht, P., Ennis, F. A., Saltzman, E. J., and Krugman, S. (1977). Persistence of maternal antibody in infants beyond 12 months: mechanism of measles vaccine failure. *J. Pediatr.* 91, 715–718. doi: 10.1016/S0022-3476(77)81021-4

Andersson, P. O., Olsson, A., and Wadenvik, H. (2002). Reduced transforming growth factor-beta1 production by mononuclear cells from patients with active chronic idiopathic thrombocytopenic purpura. *Br. J. Haematol.* 116, 862–867. doi: 10.1046/j.0007-1048.2002.03345.x

Banchereau, J., Pascual, V., and O'Garra, A. (2012). From IL-2 to IL-37: the expanding spectrum of anti-inflammatory cytokines. *Nat. Immunol.* 13, 925–931. doi: 10.1038/ni.2406

Barbon, C. M., Yang, M., Wands, G. D., Ramesh, R., Slusher, B. S., Hedley, M. L., et al. (2010). Consecutive low doses of cyclophosphamide preferentially target Tregs and potentiate T cell responses induced by DNA PLG microparticle immunization. *Cell. Immunol.* 262, 150–161. doi: 10.1016/j.cellimm.2010.02.007

Bayry, J. (2014). Regulatory T cells as adjuvant target for enhancing the viral disease vaccine efficacy. *Virusdisease* 25, 18–25. doi: 10.1007/s13337-013-0187-3

Bejon, P., Lusingu, J., Olotu, A., Leach, A., Lievens, M., Vekemans, J., et al. (2008). Efficacy of RTS,S/AS01E vaccine against malaria in children 5 to 17 months of age. *N. Engl. J. Med.* 359, 2521–2532. doi: 10.1056/NEJMoa0807381

Bejon, P., Ogada, E., Mwangi, T., Milligan, P., Lang, T., Fegan, G., et al. (2007). Extended follow-up following a phase 2b randomized trial of the candidate malaria vaccines FP9 ME-TRAP and MVA ME-TRAP among children in Kenya. *PLoS ONE* 2:e707. doi: 10.1371/journal.pone.0000707

Belderbos, M. E., Van Bleek, G. M., Levy, O., Blanken, M. O., Houben, M. L., Schuijff, L., et al. (2009). Skewed pattern of Toll-like receptor 4-mediated cytokine production in human neonatal blood: low LPS-induced IL-12p70 and high IL-10 persist throughout the first month of life. *Clin. Immunol.* 133, 228–237. doi: 10.1016/j.clim.2009.07.003

Belkaid, Y. (2007). Regulatory T cells and infection: a dangerous necessity. *Nat. Rev. Immunol.* 7, 875–888. doi: 10.1038/nri2189

Belkaid, Y. (2008). Paradoxical roles of Foxp3$^+$ T cells during infection: from regulators to regulators. *Cell Host Microbe* 3, 341–343. doi: 10.1016/j.chom.2008.05.011

Belkaid, Y., Piccirillo, C. A., Mendez, S., Shevach, E. M., and Sacks, D. L. (2002). CD4$^+$CD25$^+$ regulatory T cells control *Leishmania* major persistence and immunity. *Nature* 420, 502–507. doi: 10.1038/nature01152

Belkaid, Y., and Tarbell, K. (2009). Regulatory T cells in the control of host-microorganism interactions (*). *Annu. Rev. Immunol.* 27, 551–589. doi: 10.1146/annurev.immunol.021908.132723

Bennett, C. L., and Ochs, H. D. (2001). IPEX is a unique X-linked syndrome characterized by immune dysfunction, polyendocrinopathy, enteropathy, and a variety of autoimmune phenomena. *Curr. Opin. Pediatr.* 13, 533–538. doi: 10.1097/00008480-200112000-00007

Bjorkholm, B., Granstrom, M., Taranger, J., Wahl, M., and Hagberg, L. (1995). Influence of high titers of maternal antibody on the serologic response of infants to diphtheria vaccination at three, five and twelve months of age. *Pediatr. Infect. Dis. J.* 14, 846–850. doi: 10.1097/00006454-199510000-00005

Burl, S., Adetifa, U. J., Cox, M., Touray, E., Ota, M. O., Marchant, A., et al. (2010). Delaying *Bacillus* Calmette-Guerin vaccination from birth to 4 1/2 months of age reduces postvaccination Th1 and IL-17 responses but leads to comparable mycobacterial responses at 9 months of age. *J. Immunol.* 185, 2620–2628. doi: 10.4049/jimmunol.1000552

Burl, S., Townend, J., Njie-Jobe, J., Cox, M., Adetifa, U. J., Touray, E., et al. (2011). Age-dependent maturation of Toll-like receptor-mediated cytokine responses in Gambian infants. *PLoS ONE* 6:e18185. doi: 10.1371/journal.pone.0018185

Burstyn, D. G., Baraff, L. J., Peppler, M. S., Leake, R. D., St Geme, J. Jr., and Manclark, C. R. (1983). Serological response to filamentous hemagglutinin and lymphocytosis-promoting toxin of *Bordetella pertussis*. *Infect. Immun.* 41, 1150–1156.

Carr, R. (2000). Neutrophil production and function in newborn infants. *Br. J. Haematol.* 110, 18–28. doi: 10.1046/j.1365-2141.2000.01992.x

Chang, K. M. (2007). Regulatory T cells in hepatitis C virus infection. *Hepatol. Res.* 37(Suppl. 3), S327–S330. doi: 10.1111/j.1872-034X.2007.00220.x

Chen, Y., Kuchroo, V. K., Inobe, J., Hafler, D. A., and Weiner, H. L. (1994). Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science* 265, 1237–1240. doi: 10.1126/science.7520605

Ciubotariu, R., Colovai, A. I., Pennesi, G., Liu, Z., Smith, D., Berlocco, P., et al. (1998). Specific suppression of human CD4$^+$ Th cell responses to pig MHC antigens by CD8$^+$CD28$^-$ regulatory T cells. *J. Immunol.* 161, 5193–5202.

Claesson, B. A., Schneerson, R., Robbins, J. B., Johansson, J., Lagergard, T., Taranger, J., et al. (1989). Protective levels of serum antibodies stimulated in infants by two injections of *Haemophilus influenzae* type b capsular polysaccharide-tetanus toxoid conjugate. *J. Pediatr.* 114, 97–100. doi: 10.1016/S0022-3476(89)80611-0

Clemente, A., Caporale, R., Sannella, A. R., Majori, G., Severini, C., Fadigati, G., et al. (2011). *Plasmodium falciparum* soluble extracts potentiate the suppressive function of polyclonal T regulatory cells through activation of TGFbeta-mediated signals. *Cell. Microbiol.* 13, 1328–1338. doi: 10.1111/j.1462-5822.2011.01622.x

Collison, L. W., Workman, C. J., Kuo, T. T., Boyd, K., Wang, Y., Vignali, K. M., et al. (2007). The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature* 450, 566–569. doi: 10.1038/nature06306

Cusick, M. F., Libbey, J. E., Cox Gill, J., Fujinami, R. S., and Eckels, D. D. (2013). CD4$^+$ T-cell engagement by both wild-type and variant HCV peptides modulates the conversion of viral clearing helper T cells to Tregs. *Future Virol.* 8. doi: 10.2217/fvl.13.49

Cusick, M. F., Schiller, J. J., Gill, J. C., and Eckels, D. D. (2011). Hepatitis C virus induces regulatory T cells by naturally occurring viral variants to suppress T cell responses. *Clin. Dev. Immunol.* 2011:806061. doi: 10.1155/2011/806061

Danis, B., George, T. C., Goriely, S., Dutta, B., Renneson, J., Gatto, L., et al. (2008). Interferon regulatory factor 7-mediated responses are defective in cord blood plasmacytoid dendritic cells. *Eur. J. Immunol.* 38, 507–517. doi: 10.1002/eji.200737760

Daum, R. S., Siber, G. R., Ballanco, G. A., and Sood, S. K. (1991). Serum anticapsular antibody response in the first week after immunization of adults and infants with the *Haemophilus influenzae* type b-*Neisseria meningitidis* outer membrane protein complex conjugate vaccine. *J. Infect. Dis.* 164, 1154–1159. doi: 10.1093/infdis/164.6.1154

De La Rosa, M., Rutz, S., Dorninger, H., and Scheffold, A. (2004). Interleukin-2 is essential for CD4$^+$CD25$^+$ regulatory T cell function. *Eur. J. Immunol.* 34, 2480–2488. doi: 10.1002/eji.200425274

De Wit, D., Olislagers, V., Goriely, S., Vermeulen, F., Wagner, H., Goldman, M., et al. (2004). Blood plasmacytoid dendritic cell responses to CpG oligodeoxynucleotides are impaired in human newborns. *Blood* 103, 1030–1032. doi: 10.1182/blood-2003-04-1216

Dietze, K. K., Zelinskyy, G., Liu, J., Kretzmer, F., Schimmer, S., and Dittmer, U. (2013). Combining regulatory T cell depletion and inhibitory receptor blockade improves reactivation of exhausted virus-specific CD8$^+$ T cells and efficiently reduces chronic retroviral loads. *PLoS Pathog.* 9:e1003798. doi: 10.1371/journal.ppat.1003798

Dowling, D. J., Tan, Z., Prokopowicz, Z. M., Palmer, C. D., Matthews, M. A., Dietsch, G. N., et al. (2013). The ultra-potent and selective TLR8 agonist VTX-294 activates human newborn and adult leukocytes. *PLoS ONE* 8:e58164. doi: 10.1371/journal.pone.0058164

Duhen, T., Duhen, R., Lanzavecchia, A., Sallusto, F., and Campbell, D. J. (2012). Functionally distinct subsets of human FOXP3$^+$ Treg cells that phenotypically mirror effector Th cells. *Blood* 119, 4430–4440. doi: 10.1182/blood-2011-11-392324

Englund, J. A., Anderson, E. L., Reed, G. F., Decker, M. D., Edwards, K. M., Pichichero, M. E., et al. (1995). The effect of maternal antibody on the serologic response and the incidence of adverse reactions after primary immunization with acellular and whole-cell pertussis vaccines combined with diphtheria and tetanus toxoids. *Pediatrics* 96, 580–584.

Fabien, S., Olivier, M., Khaldoun, G., Vivian, V., Lynda, A., Laurissa, O., et al. (2014). CD49b, a major marker of regulatory T-cells type 1, predicts the response to antiviral therapy of recurrent hepatitis C after liver transplantation. *Biomed Res. Int.* 2014:290878. doi: 10.1155/2014/290878

Fan, H., Yang, J., Hao, J., Ren, Y., Chen, L., Li, G., et al. (2012). Comparative study of regulatory T cells expanded ex vivo from cord blood and adult peripheral blood. *Immunology* 136, 218–230. doi: 10.1111/j.1365-2567.2012.03573.x

Filaci, G., Fravega, M., Negrini, S., Procopio, F., Fenoglio, D., Rizzi, M., et al. (2004). Nonantigen specific CD8$^+$ T suppressor lymphocytes originate from CD8$^+$CD28$^-$ T cells and inhibit both T-cell proliferation and CTL function. *Hum. Immunol.* 65, 142–156. doi: 10.1016/j.humimm.2003.12.001

Flanagan, K. L., Halliday, A., Burl, S., Landgraf, K., Jagne, Y. J., Noho-Konteh, F., et al. (2010). The effect of placental malaria infection on cord blood and maternal immunoregulatory responses at birth. *Eur. J. Immunol.* 40, 1062–1072. doi: 10.1002/eji.200939638

Fontenot, J. D., Gavin, M. A., and Rudensky, A. Y. (2003). Foxp3 programs the development and function of CD4$^+$CD25$^+$ regulatory T cells. *Nat. Immunol.* 4, 330–336. doi: 10.1038/ni904

Fujimaki, W., Takahashi, N., Ohnuma, K., Nagatsu, M., Kurosawa, H., Yoshida, S., et al. (2008). Comparative study of regulatory T cell function of human CD25CD4 T cells from thymocytes, cord blood, and adult peripheral blood. *Clin. Dev. Immunol.* 2008:305859. doi: 10.1155/2008/305859

Gaardbo, J. C., Hartling, H. J., Ronit, A., Springborg, K., Gjerdrum, L. M., Ralfkiaer, E., et al. (2014). Regulatory T cells in HIV-infected immunological non-responders are increased in blood but depleted in lymphoid tissue and predict immunological reconstitution. *J. Acquir. Immune Defic. Syndr.* 66, 349–357. doi: 10.1097/QAI.0000000000000173

Gagliani, N., Magnani, C. F., Huber, S., Gianolini, M. E., Pala, M., Licona-Limon, P., et al. (2013). Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. *Nat. Med.* 19, 739–746. doi: 10.1038/nm.3179

Gambineri, E., Torgerson, T. R., and Ochs, H. D. (2003). Immune dysregulation, polyendocrinopathy, enteropathy, and X-linked inheritance (IPEX), a syndrome of systemic autoimmunity caused by mutations of FOXP3, a critical regulator of T-cell homeostasis. *Curr. Opin. Rheumatol.* 15, 430–435. doi: 10.1097/00002281-200307000-00010

Gans, H. A., Arvin, A. M., Galinus, J., Logan, L., Dehovitz, R., and Maldonado, Y. (1998). Deficiency of the humoral immune response to measles vaccine in infants immunized at age 6 months. *JAMA* 280, 527–532. doi: 10.1001/jama.280.6.527

Ghazal, P., Dickinson, P., and Smith, C. L. (2013). Early life response to infection. *Curr. Opin. Infect. Dis.* 26, 213–218. doi: 10.1097/QCO.0b013e32835fb8bf

Godfrey, W. R., Spoden, D. J., Ge, Y. G., Baker, S. R., Liu, B., Levine, B. L., et al. (2005). Cord blood CD4(+)CD25(+)-derived T regulatory cell lines express

FoxP3 protein and manifest potent suppressor function. *Blood* 105, 750–758. doi: 10.1182/blood-2004-06-2467

Goebel, J., Stevens, E., Forrest, K., and Roszman, T. L. (2000). Daclizumab (Zenapax) inhibits early interleukin-2 receptor signal transduction events. *Transpl. Immunol.* 8, 153–159. doi: 10.1016/S0966-3274(00)00021-6

Goriely, S., Van Lint, C., Dadkhah, R., Libin, M., De Wit, D., Demonte, D., et al. (2004). A defect in nucleosome remodeling prevents IL-12(p35) gene transcription in neonatal dendritic cells. *J. Exp. Med.* 199, 1011–1016. doi: 10.1084/jem.20031272

Gregori, S., Goudy, K. S., and Roncarolo, M. G. (2012). The cellular and molecular mechanisms of immuno-suppression by human type 1 regulatory T cells. *Front. Immunol.* 3:30. doi: 10.3389/fimmu.2012.00030

Gregori, S., Tomasoni, D., Pacciani, V., Scirpoli, M., Battaglia, M., Magnani, C. F., et al. (2010). Differentiation of type 1 T regulatory cells (Tr1) by tolerogenic DC-10 requires the IL-10-dependent ILT4/HLA-G pathway. *Blood* 116, 935–944. doi: 10.1182/blood-2009-07-234872

Griffioen, A. W., Franklin, S. W., Zegers, B. J., and Rijkers, G. T. (1993). Expression and functional characteristics of the complement receptor type 2 on adult and neonatal B lymphocytes. *Clin. Immunol. Immunopathol.* 69, 1–8. doi: 10.1006/clin.1993.1142

Groux, H., O'Garra, A., Bigler, M., Rouleau, M., Antonenko, S., De Vries, J. E., et al. (1997). A $CD4^+$ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389, 737–742. doi: 10.1038/39614

Guilmot, A., Hermann, E., Braud, V. M., Carlier, Y., and Truyens, C. (2011). Natural killer cell responses to infections in early life. *J. Innate Immun.* 3, 280–288. doi: 10.1159/000323934

Halliday, J., Klenerman, P., and Barnes, E. (2011). Vaccination for hepatitis C virus: closing in on an evasive target. *Expert Rev. Vaccines* 10, 659–672. doi: 10.1586/erv.11.55

Hatakeyama, M., Tsudo, M., Minamoto, S., Kono, T., Doi, T., Miyata, T., et al. (1989). Interleukin-2 receptor beta chain gene: generation of three receptor forms by cloned human alpha and beta chain cDNA's. *Science* 244, 551–556. doi: 10.1126/science.2785715

Higashitani, K., Kanto, T., Kuroda, S., Yoshio, S., Matsubara, T., Kakita, N., et al. (2013). Association of enhanced activity of indoleamine 2,3-dioxygenase in dendritic cells with the induction of regulatory T cells in chronic hepatitis C infection. *J. Gastroenterol.* 48, 660–670. doi: 10.1007/s00535-012-0667-z

Ho, P., Wei, X., and Seah, G. T. (2010). Regulatory T cells induced by *Mycobacterium chelonae* sensitization influence murine responses to bacille Calmette-Guerin. *J. Leukoc. Biol.* 88, 1073–1080. doi: 10.1189/jlb.0809582

Horenstein, A. L., Chillemi, A., Zaccarello, G., Bruzzone, S., Quarona, V., Zito, A., et al. (2013). A CD38/CD203a/CD73 ectoenzymatic pathway independent of CD39 drives a novel adenosinergic loop in human T lymphocytes. *Oncoimmunology* 2:e26246. doi: 10.4161/onci.26246

Hori, S., Nomura, T., and Sakaguchi, S. (2003). Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299, 1057–1061. doi: 10.1126/science.1079490

Hu, D., Ikizawa, K., Lu, L., Sanchirico, M. E., Shinohara, M. L., and Cantor, H. (2004). Analysis of regulatory CD8 T cells in Qa-1-deficient mice. *Nat. Immunol.* 5, 516–523. doi: 10.1038/ni1063

Jepsen, M. P., Jogdand, P. S., Singh, S. K., Esen, M., Christiansen, M., Issifou, S., et al. (2013). The malaria vaccine candidate GMZ2 elicits functional antibodies in individuals from malaria endemic and non-endemic areas. *J. Infect. Dis.* 208, 479–488. doi: 10.1093/infdis/jit185

Jones, C., Pollock, L., Barnett, S. M., Battersby, A., and Kampmann, B. (2014). The relationship between concentration of specific antibody at birth and subsequent response to primary immunization. *Vaccine* 32, 996–1002. doi: 10.1016/j.vaccine.2013.11.104

Kaleebu, P., Njai, H. F., Wang, L., Jones, N., Ssewanyana, I., Richardson, P., et al. (2014). Immunogenicity of ALVAC-HIV vCP1521 in infants of HIV-1-infected women in Uganda (HPTN 027): the first pediatric HIV vaccine trial in Africa. *J. Acquir. Immune Defic. Syndr.* 65, 268–277. doi: 10.1097/01.qai.0000435600.65845.31

Kanegane, H., Miyawaki, T., Kato, K., Yokoi, T., Uehara, T., Yachie, A., et al. (1991). A novel subpopulation of $CD45RA^+$ $CD4^+$ T cells expressing IL-2 receptor alpha-chain (CD25) and having a functionally transitional nature into memory cells. *Int. Immunol.* 3, 1349–1356. doi: 10.1093/intimm/3.12.1349

Kanra, G., Yalcin, S. S., Ceyhan, M., and Yurdakok, K. (2000). Clinical trial to evaluate immunogenicity and safety of inactivated hepatitis A vaccination starting at 2-month-old children. *Turk. J. Pediatr.* 42, 105–108.

Kao, J. Y., Zhang, M., Miller, M. J., Mills, J. C., Wang, B., Liu, M., et al. (2010). *Helicobacter pylori* immune escape is mediated by dendritic cell-induced Treg skewing and Th17 suppression in mice. *Gastroenterology* 138, 1046–1054. doi: 10.1053/j.gastro.2009.11.043

Kared, H., Fabre, T., Bedard, N., Bruneau, J., and Shoukry, N. H. (2013). Galectin-9 and IL-21 mediate cross-regulation between Th17 and Treg cells during acute hepatitis C. *PLoS Pathog.* 9:e1003422. doi: 10.1371/journal.ppat.1003422

Keenan, B. P., Saenger, Y., Kafrouni, M. I., Leubner, A., Lauer, P., Maitra, A., et al. (2014). A *Listeria* vaccine and depletion of T-regulatory cells activate immunity against early stage pancreatic intraepithelial neoplasms and prolong survival of mice. *Gastroenterology* 146, 1784.e6–1794.e6. doi: 10.1053/j.gastro.2014.02.055

Klages, K., Mayer, C. T., Lahl, K., Loddenkemper, C., Teng, M. W., Ngiow, S. F., et al. (2010). Selective depletion of $Foxp3^+$ regulatory T cells improves effective therapeutic vaccination against established melanoma. *Cancer Res.* 70, 7788–7799. doi: 10.1158/0008-5472.CAN-10-1736

Kohm, A. P., Mcmahon, J. S., Podojil, J. R., Begolka, W. S., Degutes, M., Kasprowicz, D. J., et al. (2006). Cutting Edge: Anti-CD25 monoclonal antibody injection results in the functional inactivation, not depletion, of $CD4^+CD25^+$ T regulatory cells. *J. Immunol.* 176, 3301–3305. doi: 10.4049/jimmunol.176.6.3301

Kollmann, T. R., Crabtree, J., Rein-Weston, A., Blimkie, D., Thommai, F., Wang, X. Y., et al. (2009). Neonatal innate TLR-mediated responses are distinct from those of adults. *J. Immunol.* 183, 7150–7160. doi: 10.4049/jimmunol.0901481

Kollmann, T. R., Levy, O., Montgomery, R. R., and Goriely, S. (2012). Innate immune function by Toll-like receptors: distinct responses in newborns and the elderly. *Immunity* 37, 771–783. doi: 10.1016/j.immuni.2012.10.014

Koukouikila-Koussounda, F., Ntoumi, F., Ndounga, M., Tong, H. V., Abena, A. A., and Velavan, T. P. (2013). Genetic evidence of regulatory gene variants of the STAT6, IL10R and FOXP3 locus as a susceptibility factor in uncomplicated malaria and parasitaemia in Congolese children. *Malar. J.* 12:9. doi: 10.1186/1475-2875-12-9

Kruetzmann, S., Rosado, M. M., Weber, H., Germing, U., Tournilhac, O., Peter, H. H., et al. (2003). Human immunoglobulin M memory B cells controlling *Streptococcus pneumoniae* infections are generated in the spleen. *J. Exp. Med.* 197, 939–945. doi: 10.1084/jem.20022020

Lacan, G., Dang, H., Middleton, B., Horwitz, M. A., Tian, J., Melega, W. P., et al. (2013). *Bacillus* Calmette-Guerin vaccine-mediated neuroprotection is associated with regulatory T-cell induction in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease. *J. Neurosci. Res.* 91, 1292–1302. doi: 10.1002/jnr.23253

Lahl, K., and Sparwasser, T. (2011). In vivo depletion of $FoxP3^+$ Tregs using the DEREG mouse model. *Methods Mol. Biol.* 707, 157–172. doi: 10.1007/978-1-61737-979-6_10

Langrish, C. L., Buddle, J. C., Thrasher, A. J., and Goldblatt, D. (2002). Neonatal dendritic cells are intrinsically biased against Th-1 immune responses. *Clin. Exp. Immunol.* 128, 118–123. doi: 10.1046/j.1365-2249.2002.01817.x

Laurens, M. B., Thera, M. A., Coulibaly, D., Ouattara, A., Kone, A. K., Guindo, A. B., et al. (2013). Extended safety, immunogenicity and efficacy of a blood-stage malaria vaccine in Malian children: 24-month follow-up of a randomized, double-blinded phase 2 trial. *PLoS ONE* 8:e79323. doi: 10.1371/journal.pone.0079323

Le, D. T., and Jaffee, E. M. (2012). Regulatory T-cell modulation using cyclophosphamide in vaccine approaches: a current perspective. *Cancer Res.* 72, 3439–3444. doi: 10.1158/0008-5472.CAN-11-3912

Levings, M. K., and Roncarolo, M. G. (2000). T-regulatory 1 cells: a novel subset of CD4 T cells with immunoregulatory properties. *J. Allergy Clin. Immunol.* 106, S109–S112. doi: 10.1067/mai.2000.106635

Levy, O. (2007). Innate immunity of the newborn: basic mechanisms and clinical correlates. *Nat. Rev. Immunol.* 7, 379–390. doi: 10.1038/nri2075

Levy, O., Coughlin, M., Cronstein, B. N., Roy, R. M., Desai, A., and Wessels, M. R. (2006). The adenosine system selectively inhibits TLR-mediated TNF-alpha production in the human newborn. *J. Immunol.* 177, 1956–1966. doi: 10.4049/jimmunol.177.3.1956

Levy, O., and Wynn, J. L. (2014). A prime time for trained immunity: innate immune memory in newborns and infants. *Neonatology* 105, 136–141. doi: 10.1159/000356035

Lin, S. J., Lu, C. H., Yan, D. C., Lee, P. T., Hsiao, H. S., and Kuo, M. L. (2014). Expansion of regulatory T cells from umbilical cord blood and adult peripheral blood CD4CD25 T cells. *Immunol. Res.* doi: 10.1007/s12026-014-8488-1 [Epub ahead of print].

Liu, W., Putnam, A. L., Xu-Yu, Z., Szot, G. L., Lee, M. R., Zhu, S., et al. (2006). CD127 expression inversely correlates with FoxP3 and suppressive function of human $CD4^+$ T reg cells. *J. Exp. Med.* 203, 1701–1711. doi: 10.1084/jem.20060772

Liu, Y., Lan, Q., Lu, L., Chen, M., Xia, Z., Ma, J., et al. (2014). Phenotypic and functional characteristic of a newly identified $CD8^+$ $Foxp3^-$ $CD103^+$ regulatory T cells. *J. Mol. Cell Biol.* 6, 81–92. doi: 10.1093/jmcb/mjt026

Lu, L., Kim, H. J., Werneck, M. B., and Cantor, H. (2008). Regulation of $CD8^+$ regulatory T cells: Interruption of the NKG2A-Qa-1 interaction allows robust suppressive activity and resolution of autoimmune disease. *Proc. Natl. Acad. Sci. U.S.A.* 105, 19420–19425. doi: 10.1073/pnas.0810383105

Lu, W., Chen, S., Lai, C., Guo, W., Fu, L., and Andrieu, J. M. (2012). Induction of $CD8^+$ regulatory T cells protects macaques against SIV challenge. *Cell Rep.* 2, 1736–1746. doi: 10.1016/j.celrep.2012.11.016

Luciano, A. A., Arbona-Ramirez, I. M., Ruiz, R., Llorens-Bonilla, B. J., Martinez-Lopez, D. G., Funderburg, N., et al. (2014). Alterations in regulatory T cell subpopulations seen in preterm infants. *PLoS ONE* 9:e95867. doi: 10.1371/journal.pone.0095867

Ly, N. P., Ruiz-Perez, B., Mcloughlin, R. M., Visness, C. M., Wallace, P. K., Cruikshank, W. W., et al. (2009). Characterization of regulatory T cells in urban newborns. *Clin. Mol. Allergy* 7:8. doi: 10.1186/1476-7961-7-8

Macatangay, B. J., Szajnik, M. E., Whiteside, T. L., Riddler, S. A., and Rinaldo, C. R. (2010). Regulatory T cell suppression of Gag-specific CD8 T cell polyfunctional response after therapeutic vaccination of HIV-1-infected patients on ART. *PLoS ONE* 5:e9852. doi: 10.1371/journal.pone.0009852

Macdonald, A. J., Duffy, M., Brady, M. T., Mckiernan, S., Hall, W., Hegarty, J., et al. (2002). CD4 T helper type 1 and regulatory T cells induced against the same epitopes on the core protein in hepatitis C virus-infected persons. *J. Infect. Dis.* 185, 720–727. doi: 10.1086/339340

Mandapathil, M., Hilldorfer, B., Szczepanski, M. J., Czystowska, M., Szajnik, M., Ren, J., et al. (2010). Generation and accumulation of immunosuppressive adenosine by human $CD4^+CD25highFOXP3^+$ regulatory T cells. *J. Biol. Chem.* 285, 7176–7186. doi: 10.1074/jbc.M109.047423

Marchant, A., Goetghebuer, T., Ota, M. O., Wolfe, I., Ceesay, S. J., De Groote, D., et al. (1999). Newborns develop a Th1-type immune response to *Mycobacterium bovis Bacillus* Calmette-Guerin vaccination. *J. Immunol.* 163, 2249–2255.

Mattarollo, S. R., Steegh, K., Li, M., Duret, H., Foong Ngiow, S., and Smyth, M. J. (2013). Transient Foxp3(+) regulatory T-cell depletion enhances therapeutic anticancer vaccination targeting the immune-stimulatory properties of NKT cells. *Immunol. Cell Biol.* 91, 105–114. doi: 10.1038/icb.2012.58

Mayer, C. T., Floess, S., Baru, A. M., Lahl, K., Huehn, J., and Sparwasser, T. (2011). $CD8^+$ $Foxp3^+$ T cells share developmental and phenotypic features with classical $CD4^+$ $Foxp3^+$ regulatory T cells but lack potent suppressive activity. *Eur. J. Immunol.* 41, 716–725. doi: 10.1002/eji.201040913

Mayer, E., Bannert, C., Gruber, S., Klunker, S., Spittler, A., Akdis, C. A., et al. (2012). Cord blood derived $CD4^+$ CD25(high) T cells become functional regulatory T cells upon antigen encounter. *PLoS ONE* 7:e29355. doi: 10.1371/journal.pone.0029355

Mills, K. H., and Mcguirk, P. (2004). Antigen-specific regulatory T cells – their induction and role in infection. *Semin. Immunol.* 16, 107–117. doi: 10.1016/j.smim.2003.12.006

Minigo, G., Woodberry, T., Piera, K. A., Salwati, E., Tjitra, E., Kenangalem, E., et al. (2009). Parasite-dependent expansion of TNF receptor II-positive regulatory T cells with enhanced suppressive activity in adults with severe malaria. *PLoS Pathog.* 5:e1000402. doi: 10.1371/journal.ppat.1000402

Mitchell, D. A., Cui, X., Schmittling, R. J., Sanchez-Perez, L., Snyder, D. J., Congdon, K. L., et al. (2011). Monoclonal antibody blockade of IL-2 receptor alpha during lymphopenia selectively depletes regulatory T cells in mice and humans. *Blood* 118, 3003–3012. doi: 10.1182/blood-2011-02-334565

Miyara, M., Yoshioka, Y., Kitoh, A., Shima, T., Wing, K., Niwa, A., et al. (2009). Functional delineation and differentiation dynamics of human $CD4^+$ T cells expressing the FoxP3 transcription factor. *Immunity* 30, 899–911. doi: 10.1016/j.immuni.2009.03.019

Moore, A. C., Gallimore, A., Draper, S. J., Watkins, K. R., Gilbert, S. C., and Hill, A. V. (2005). Anti-CD25 antibody enhancement of vaccine-induced immunogenicity: increased durable cellular immunity with reduced immunodominance. *J. Immunol.* 175, 7264–7273. doi: 10.4049/jimmunol.175.11.7264

Moorman, J. P., Wang, J. M., Zhang, Y., Ji, X. J., Ma, C. J., Wu, X. Y., et al. (2012). Tim-3 pathway controls regulatory and effector T cell balance during hepatitis C virus infection. *J. Immunol.* 189, 755–766. doi: 10.4049/jimmunol.1200162

Mwacharo, J., Dunachie, S. J., Kai, O., Hill, A. V., Bejon, P., and Fletcher, H. A. (2009). Quantitative PCR evaluation of cellular immune responses in Kenyan children vaccinated with a candidate malaria vaccine. *PLoS ONE* 4:e8434. doi: 10.1371/journal.pone.0008434

Nettenstrom, L., Alderson, K., Raschke, E. E., Evans, M. D., Sondel, P. M., Olek, S., et al. (2013). An optimized multi-parameter flow cytometry protocol for human T regulatory cell analysis on fresh and viably frozen cells, correlation with epigenetic analysis, and comparison of cord and adult blood. *J. Immunol. Methods* 387, 81–88. doi: 10.1016/j.jim.2012.09.014

Ngamphaiboon, J., Theamboonlert, A., and Poovorawan, Y. (1998). Serum IgG subclass levels in a group of healthy Thai children. *Asian Pac. J. Allergy Immunol.* 16, 49–55.

Nguyen, M., Leuridan, E., Zhang, T., De Wit, D., Willems, F., Van Damme, P., et al. (2010). Acquisition of adult-like TLR4 and TLR9 responses during the first year of life. *PLoS ONE* 5:e10407. doi: 10.1371/journal.pone.0010407

Ogwang, C., Afolabi, M., Kimani, D., Jagne, Y. J., Sheehy, S. H., Bliss, C. M., et al. (2013). Safety and immunogenicity of heterologous prime-boost immunisation with *Plasmodium falciparum* malaria candidate vaccines, ChAd63 ME-TRAP and MVA ME-TRAP, in healthy Gambian and Kenyan adults. *PLoS ONE* 8:e57726. doi: 10.1371/journal.pone.0057726

Olotu, A., Fegan, G., Wambua, J., Nyangweso, G., Awuondo, K. O., Leach, A., et al. (2013). Four-year efficacy of RTS,S/AS01E and its interaction with malaria exposure. *N. Engl. J. Med.* 368, 1111–1120. doi: 10.1056/NEJMoa1207564

Olotu, A., Lusingu, J., Leach, A., Lievens, M., Vekemans, J., Msham, S., et al. (2011). Efficacy of RTS,S/AS01E malaria vaccine and exploratory analysis on anti-circumsporozoite antibody titres and protection in children aged 5-17 months in Kenya and Tanzania: a randomised controlled trial. *Lancet Infect. Dis.* 11, 102–109. doi: 10.1016/S1473-3099(10)70262-0

Patton, D. T., Wilson, M. D., Rowan, W. C., Soond, D. R., and Okkenhaug, K. (2011). The PI3K p110delta regulates expression of CD38 on regulatory T cells. *PLoS ONE* 6:e17359. doi: 10.1371/journal.pone.0017359

Peggs, K. S., Quezada, S. A., Chambers, C. A., Korman, A. J., and Allison, J. P. (2009). Blockade of CTLA-4 on both effector and regulatory T cell compartments contributes to the antitumor activity of anti-CTLA-4 antibodies. *J. Exp. Med.* 206, 1717–1725. doi: 10.1084/jem.20082492

Perez-Machado, M. A., Ashwood, P., Thomson, M. A., Latcham, F., Sim, R., Walker-Smith, J. A., et al. (2003). Reduced transforming growth factor-beta1-producing T cells in the duodenal mucosa of children with food allergy. *Eur. J. Immunol.* 33, 2307–2315. doi: 10.1002/eji.200323308

Perret, R., Sierro, S. R., Botelho, N. K., Corgnac, S., Donda, A., and Romero, P. (2013). Adjuvants that improve the ratio of antigen-specific effector to regulatory T cells enhance tumor immunity. *Cancer Res.* 73, 6597–6608. doi: 10.1158/0008-5472.CAN-13-0875

Philbin, V. J., Dowling, D. J., Gallington, L. C., Cortes, G., Tan, Z., Suter, E. E., et al. (2012). Imidazoquinoline Toll-like receptor 8 agonists activate human newborn monocytes and dendritic cells through adenosine-refractory and caspase-1-dependent pathways. *J. Allergy Clin. Immunol.* 130, 195.e9–204.e9. doi: 10.1016/j.jaci.2012.02.042

Philbin, V. J., and Levy, O. (2009). Developmental biology of the innate immune response: implications for neonatal and infant vaccine development. *Pediatr. Res.* 65, 98R–105R. doi: 10.1203/PDR.0b013e31819f195d

Power Coombs, M. R., Belderbos, M. E., Gallington, L. C., Bont, L., and Levy, O. (2011). Adenosine modulates Toll-like receptor function: basic mechanisms and translational opportunities. *Expert Rev. Anti Infect. Ther.* 9, 261–269. doi: 10.1586/eri.10.158

Raskovalova, T., Huang, X., Sitkovsky, M., Zacharia, L. C., Jackson, E. K., and Gorelik, E. (2005). Gs protein-coupled adenosine receptor signaling

and lytic function of activated NK cells. *J. Immunol.* 175, 4383–4391. doi: 10.4049/jimmunol.175.7.4383

Rech, A. J., Mick, R., Martin, S., Recio, A., Aqui, N. A., Powell, D. J., et al. (2012). CD25 blockade depletes and selectively reprograms regulatory T cells in concert with immunotherapy in cancer patients. *Sci. Transl. Med.* 4:134ra62.

Rech, A. J., and Vonderheide, R. H. (2009). Clinical use of anti-CD25 antibody daclizumab to enhance immune responses to tumor antigen vaccination by targeting regulatory T cells. *Ann. N. Y. Acad. Sci.* 1174, 99–106. doi: 10.1111/j.1749-6632.2009.04939.x

Roncarolo, M. G., Gregori, S., Battaglia, M., Bacchetta, R., Fleischhauer, K., and Levings, M. K. (2006). Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunol. Rev.* 212, 28–50. doi: 10.1111/j.0105-2896.2006.00420.x

Rts, S. C. T. P., Agnandji, S. T., Lell, B., Fernandes, J. F., Abossolo, B. P., 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

Saison, J., Ferry, T., Demaret, J., Maucort Boulch, D., Venet, F., Perpoint, T., et al. (2014). Association between discordant immunological response to highly active anti-retroviral therapy, regulatory T cell percentage, immune cell activation and very low-level viraemia in HIV-infected patients. *Clin. Exp. Immunol.* 176, 401–409. doi: 10.1111/cei.12278

Sakaguchi, S. (2000). Regulatory T cells: key controllers of immunologic self-tolerance. *Cell* 101, 455–458. doi: 10.1016/S0092-8674(00)80856-9

Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M., and Toda, M. (1995). Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* 155, 1151–1164.

Sallusto, F., Lenig, D., Forster, R., Lipp, M., and Lanzavecchia, A. (1999). Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401, 708–712. doi: 10.1038/44385

Sansom, D. M., and Walker, L. S. (2006). The role of CD28 and cytotoxic T-lymphocyte antigen-4 (CTLA-4) in regulatory T-cell biology. *Immunol. Rev.* 212, 131–148. doi: 10.1111/j.0105-2896.2006.00419.x

Santner-Nanan, B., Seddiki, N., Zhu, E., Quent, V., Kelleher, A., Fazekas De St Groth, B., et al. (2008). Accelerated age-dependent transition of human regulatory T cells to effector memory phenotype. *Int. Immunol.* 20, 375–383. doi: 10.1093/intimm/dxm151

Scalzo-Inguanti, K., and Plebanski, M. (2011). CD38 identifies a hypo-proliferative IL-13-secreting $CD4^+$ T-cell subset that does not fit into existing naive and memory phenotype paradigms. *Eur. J. Immunol.* 41, 1298–1308. doi: 10.1002/eji.201040726

Schmetterer, K. G., Neunkirchner, A., and Pickl, W. F. (2012). Naturally occurring regulatory T cells: markers, mechanisms, and manipulation. *FASEB J.* 26, 2253–2276. doi: 10.1096/fj.11-193672

Scholzen, A., Minigo, G., and Plebanski, M. (2010). Heroes or villains? T regulatory cells in malaria infection. *Trends Parasitol.* 26, 16–25. doi: 10.1016/j.pt.2009.10.004

Scholzen, A., Mittag, D., Rogerson, S. J., Cooke, B. M., and Plebanski, M. (2009). *Plasmodium falciparum*-mediated induction of human CD25Foxp3 CD4 T cells is independent of direct TCR stimulation and requires IL-2, IL-10 and TGFbeta. *PLoS Pathog.* 5:e1000543. doi: 10.1371/journal.ppat.1000543

Seddiki, N., Santner-Nanan, B., Martinson, J., Zaunders, J., Sasson, S., Landay, A., et al. (2006). Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J. Exp. Med.* 203, 1693–1700. doi: 10.1084/jem.20060468

Serana, F., Chiarini, M., Quiros-Roldan, E., Gotti, D., Zanotti, C., Sottini, A., et al. (2014). Modulation of regulatory T-cell subsets in very long-term treated aviremic HIV(+) patients and untreated viremic patients. *Open AIDS J.* 8, 1–6. doi: 10.2174/1874613601408010001

Siegrist, C. A. (2003). Mechanisms by which maternal antibodies influence infant vaccine responses: review of hypotheses and definition of main determinants. *Vaccine* 21, 3406–3412. doi: 10.1016/S0264-410X(03)00342-6

Siegrist, C. A., Barrios, C., Martinez, X., Brandt, C., Berney, M., Cordova, M., et al. (1998). Influence of maternal antibodies on vaccine responses: inhibition of antibody but not T cell responses allows successful early prime-boost strategies in mice. *Eur. J. Immunol.* 28, 4138–4148. doi: 10.1002/(SICI)1521-4141(199812)28:12<4138::AID-IMMU4138>3.0.CO;2-L

Simone, R., Zicca, A., and Saverino, D. (2008). The frequency of regulatory $CD3^+CD8^+CD28^-$ $CD25^+$ T lymphocytes in human peripheral blood increases with age. *J. Leukoc. Biol.* 84, 1454–1461. doi: 10.1189/jlb.0907627

Sitkovsky, M. V., and Ohta, A. (2005). The 'danger' sensors that STOP the immune response: the A2 adenosine receptors? *Trends Immunol.* 26, 299–304. doi: 10.1016/j.it.2005.04.004

Smith, T. R., and Kumar, V. (2008). Revival of $CD8^+$ Treg-mediated suppression. *Trends Immunol.* 29, 337–342. doi: 10.1016/j.it.2008.04.002

Suzuki, M., Jagger, A. L., Konya, C., Shimojima, Y., Pryshchep, S., Goronzy, J. J., et al. (2012). $CD8^+CD45RA^+CCR7^+FOXP3^+$ T cells with immunosuppressive properties: a novel subset of inducible human regulatory T cells. *J. Immunol.* 189, 2118–2130. doi: 10.4049/jimmunol.1200122

Suzuki, M., Konya, C., Goronzy, J. J., and Weyand, C. M. (2008). Inhibitory $CD8^+$ T cells in autoimmune disease. *Hum. Immunol.* 69, 781–789. doi: 10.1016/j.humimm.2008.08.283

Takahata, Y., Nomura, A., Takada, H., Ohga, S., Furuno, K., Hikino, S., et al. (2004). $CD25^+CD4^+$ T cells in human cord blood: an immunoregulatory subset with naive phenotype and specific expression of forkhead box p3 (Foxp3) gene. *Exp. Hematol.* 32, 622–629. doi: 10.1016/j.exphem.2004.03.012

Tan, C., Reddy, V., Dannull, J., Ding, E., Nair, S. K., Tyler, D. S., et al. (2013). Impact of anti-CD25 monoclonal antibody on dendritic cell-tumor fusion vaccine efficacy in a murine melanoma model. *J. Transl. Med.* 11:148. doi: 10.1186/1479-5876-11-148

Todryk, S. M., Bejon, P., Mwangi, T., Plebanski, M., Urban, B., Marsh, K., et al. (2008). Correlation of memory T cell responses against TRAP with protection from clinical malaria, and CD4 CD25 high T cells with susceptibility in Kenyans. *PLoS ONE* 3:e2027. doi: 10.1371/journal.pone.0002027

Tosello, V., Odunsi, K., Souleimanian, N. E., Lele, S., Shrikant, P., Old, L. J., et al. (2008). Differential expression of CCR7 defines two distinct subsets of human memory $CD4^+CD25^+$ Tregs. *Clin. Immunol.* 126, 291–302. doi: 10.1016/j.clim.2007.11.008

Uss, E., Rowshani, A. T., Hooibrink, B., Lardy, N. M., Van Lier, R. A., and Ten Berge, I. J. (2006). CD103 is a marker for alloantigen-induced regulatory $CD8^+$ T cells. *J. Immunol.* 177, 2775–2783. doi: 10.4049/jimmunol.177.5.2775

Vieira, P. L., Christensen, J. R., Minaee, S., O'Neill, E. J., Barrat, F. J., Boonstra, A., et al. (2004). IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring $CD4^+CD25^+$ regulatory T cells. *J. Immunol.* 172, 5986–5993. doi: 10.4049/jimmunol.172.10.5986

Voo, K. S., Bover, L., Harline, M. L., Vien, L. T., Facchinetti, V., Arima, K., et al. (2013). Antibodies targeting human OX40 expand effector T cells and block inducible and natural regulatory T cell function. *J. Immunol.* 191, 3641–3650. doi: 10.4049/jimmunol.1202752

Walker, L. S. (2013). Treg and CTLA-4: two intertwining pathways to immune tolerance. *J. Autoimmun.* 45, 49–57. doi: 10.1016/j.jaut.2013.06.006

Walther, B., Miles, D. J., Waight, P., Palmero, M. S., Ojuola, O., Touray, E. S., et al. (2012). Placental malaria is associated with attenuated CD4 T-cell responses to tuberculin PPD 12 months after BCG vaccination. *BMC Infect. Dis.* 12:6. doi: 10.1186/1471-2334-12-6

Walther, M., Jeffries, D., Finney, O. C., Njie, M., Ebonyi, A., Deininger, S., et al. (2009). Distinct roles for FOXP3 and FOXP3 CD4 T cells in regulating cellular immunity to uncomplicated and severe *Plasmodium falciparum* malaria. *PLoS Pathog.* 5:e1000364. doi: 10.1371/journal.ppat.1000364

Walther, M., Tongren, J. E., Andrews, L., Korbel, D., King, E., Fletcher, H., et al. (2005). Upregulation of TGF-beta, FOXP3, and $CD4^+CD25^+$ regulatory T cells correlates with more rapid parasite growth in human malaria infection. *Immunity* 23, 287–296. doi: 10.1016/j.immuni.2005.08.006

Wammes, L. J., Wiria, A. E., Toenhake, C. G., Hamid, F., Liu, K. Y., Suryani, H., et al. (2013). Asymptomatic plasmodial infection is associated with increased tumor necrosis factor receptor II-expressing regulatory T cells and suppressed type 2 immune responses. *J. Infect. Dis.* 207, 1590–1599. doi: 10.1093/infdis/jit058

Wang, W. H., Ming, L., Wang, Y., Kan, Q. C., and Zhang, X. Y. (2013). High frequency of regulatory T cells among HIV type 1-infected men who have sex with men correlates with disease progression. *Chin. Med. J.* (*Engl.*) 126, 2054–2061.

Wing, J. B., and Sakaguchi, S. (2012). Multiple Treg suppressive modules and their adaptability. *Front. Immunol.* 3:178. doi: 10.3389/fimmu.2012.00178

Wing, K., Ekmark, A., Karlsson, H., Rudin, A., and Suri-Payer, E. (2002). Characterization of human $CD25^+$ $CD4^+$ T cells in thymus, cord and adult blood. *Immunology* 106, 190–199. doi: 10.1046/j.1365-2567.2002.01412.x

Wing, K., Larsson, P., Sandstrom, K., Lundin, S. B., Suri-Payer, E., and Rudin, A. (2005). $CD4^+$ $CD25^+$ $FOXP3^+$ regulatory T cells from human thymus and cord blood suppress antigen-specific T cell responses. *Immunology* 115, 516–525. doi: 10.1111/j.1365-2567.2005.02186.x

Wing, K., Yamaguchi, T., and Sakaguchi, S. (2011). Cell-autonomous and -non-autonomous roles of CTLA-4 in immune regulation. *Trends Immunol.* 32, 428–433. doi: 10.1016/j.it.2011.06.002

Wu, J. J., Chen, G., Liu, J., Wang, T., Zheng, W., and Cao, Y. M. (2010). Natural regulatory T cells mediate the development of cerebral malaria by modifying the pro-inflammatory response. *Parasitol. Int.* 59, 232–241. doi: 10.1016/j.parint.2010.02.007

Yeung, L. T., King, S. M., and Roberts, E. A. (2001). Mother-to-infant transmission of hepatitis C virus. *Hepatology* 34, 223–229. doi: 10.1053/jhep.2001.25885

Zandvoort, A., and Timens, W. (2002). The dual function of the splenic marginal zone: essential for initiation of anti-TI-2 responses but also vital in the general first-line defense against blood-borne antigens. *Clin. Exp. Immunol.* 130, 4–11. doi: 10.1046/j.1365-2249.2002.01953.x

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.

Received: 06 June 2014; accepted: 25 August 2014; published online: 11 September 2014.

Citation: Ndure J and Flanagan KL (2014) Targeting regulatory T cells to improve vaccine immunogenicity in early life. Front. Microbiol. ***5****:477. doi: 10.3389/fmicb.2014.00477*

This article was submitted to Microbial Immunology, a section of the journal Frontiers in Microbiology.

Copyright © 2014 Ndure and Flanagan. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in
MICROBIOLOGY

MINI REVIEW ARTICLE
published: 13 October 2014
doi: 10.3389/fmicb.2014.00514

Influence of HIV and HCV on T cell antigen presentation and challenges in the development of vaccines

***Mina John*[1,2] *and Silvana Gaudieri*[1,3] ***

[1] *Institute for Immunology and Infectious Diseases, Murdoch University, Murdoch, WA, Australia*
[2] *Department of Clinical Immunology, PathWest Laboratory Medicine WA, Royal Perth Hospital, Perth, WA, Australia*
[3] *School of Anatomy, Physiology and Human Biology, University of Western Australia, Crawley, WA, Australia*

Edited by:
Eric J. Gowans, The University of Adelaide, Australia

Reviewed by:
Joseph Torresi, University of Melbourne, Australia
Andrew R. Lloyd, University of New South Wales, Australia

****Correspondence:***
Silvana Gaudieri, School of Anatomy, Physiology and Human Biology, University of Western Australia, Stirling Highway, Crawley, WA 6009, Australia
e-mail: silvana.gaudieri@uwa.edu.au

Some of the central challenges for developing effective vaccines against HIV and hepatitis C virus (HCV) are similar. Both infections are caused by small, highly mutable, rapidly replicating RNA viruses with the ability to establish long-term chronic pathogenic infection in human hosts. HIV has caused 60 million infections globally and HCV 180 million and both viruses may co-exist among certain populations by virtue of common blood-borne, sexual, or vertical transmission. Persistence of both pathogens is achieved by evasion of intrinsic, innate, and adaptive immune defenses but with some distinct mechanisms reflecting their differences in evolutionary history, replication characteristics, cell tropism, and visibility to mucosal versus systemic and hepatic immune responses. A potent and durable antibody and T cell response is a likely requirement of future HIV and HCV vaccines. Perhaps the single biggest difference between the two vaccine design challenges is that in HCV, a natural model of protective immunity can be found in those who resolve acute infection spontaneously. Such spontaneous resolvers exhibit durable and functional $CD4^+$ and $CD8^+$ T cell responses (Diepolder et al., 1995; Cooper et al., 1999; Thimme et al., 2001; Grakoui et al., 2003; Lauer et al., 2004; Schulze Zur Wiesch et al., 2012). However, frequent re-infection suggests partial or lack of protective immunity against heterologous HCV strains, possibly indicative of the degree of genetic diversity of circulating HCV genotypes and subtypes. There is no natural model of protective immunity in HIV, however, studies of "elite controllers," or individuals who have durably suppressed levels of plasma HIV RNA without antiretroviral therapy, has provided the strongest evidence for $CD8^+$ T cell responses in controlling viremia and limiting reservoir burden in established infection. Here we compare and contrast the specific mechanisms of immune evasion used by HIV and HCV, which subvert adaptive human leukocyte antigen (HLA)-restricted T cell immunity in natural infection, and the challenges these pose for designing effective preventative or therapeutic vaccines.

Keywords: HIV, HCV, viral immune escape, preventative vaccine, anti-viral immune responses

LEADS FROM GENETIC ASSOCIATION STUDIES SUPPORT IMPORTANCE OF IMMUNOLOGICAL MECHANISMS IN VIRAL INFECTION OUTCOME

Genetic determinants of spontaneous HCV infection clearance and HIV viral control using genome-wide association studies (GWAS) and candidate gene studies have added crucial insight into the influence of the host immune response on infection outcome. For HIV the strongest genetic determinant of viral load set-point and $CD4^+$ T cell decline following infection, aside from variants in the CCR5 molecule used by HIV for cell entry, are specific HLA class I alleles (e.g., HLA-B27 and HLA-B57; reviewed in O'Brien et al., 2001) involved in T cell antigen presentation. More recently, a GWAS has shown the association of HLA-C with viral control (International HIVCS et al., 2010). The variation at HLA-C associated with HIV outcome appears to affect cell surface expression of the HLA molecule (Thomas et al., 2009b). The HLA class I molecules also act as ligands for natural killer (NK) cell receptors and this interaction is known to influence the activation threshold for NK cells. Particular combinations of killer immunoglobulin-like receptors (KIR) and HLA class I ligands are strongly associated with HIV infection outcome (Martin et al., 2002).

For HCV, studies that examine host genetic associations with infection outcome clearly indicate that genotypic differences in the interferon pathway such as interferon lambda 3 (IFN-λ3) (Thomas et al., 2009a; Rauch et al., 2010), NK cell cytotoxicity activation threshold (Khakoo et al., 2004) and specific HLA class I and II alleles (McKiernan et al., 2004; Miki et al., 2013) are strongly associated with resolution following HCV infection (reviewed in Rauch et al., 2009a).

For both HIV and HCV, the genetic leads support the involvement of $CD8^+$ T cells and antigen presentation in infection outcome. Further evidence can be obtained from the observed heterozygote advantage at the HLA loci for both viral infections (Carrington et al., 1999; Hraber et al., 2007), likely reflecting the presentation of an increased number of T cell targets.

VIRAL EFFECTS ON ANTIGEN PRESENTATION

To establish chronic infection, viruses such as HIV and HCV must evade the host's T cell response. The host's T cell response is governed by the assembly and presentation of antigen via the polymorphic HLA class I and II molecules. In the case of HLA class I presentation of viral peptides to $CD8^+$ cytotoxic T cells (CTL), the process requires correct folding of the HLA class I molecules with b2-microglobulin in the endoplasmic reticulum. In parallel, the viral peptides that have been processed by the proteasome complex in the cytosol are then loaded onto the HLA class I-β2-microglobulin complex via the transporter associated with antigen presentation (TAP) protein. This tertiary structure is then translocated to the surface of the cell via the golgi apparatus for presentation to a CTL with the appropriate T cell receptor. Both HIV and HCV have evolved several mechanisms to disrupt this pathway including reduction of HLA class I expression and mutational escape from antigen presentation.

EFFECTS ON HLA EXPRESSION

For HCV, the proteins core (Miyamoto et al., 2007) and NS3 (Khu et al., 2004) have been shown to affect the function of the proteasome complex (reviewed in Osna, 2009) and potentially HLA class I presentation. Other evidence from the Huh-7 subgenomic replicon system, suggests that HCV infection reduces HLA class I surface expression via a stress-mediated mechanism that lowers the efficacy of HLA class I folding in the endoplasmic reticulum, although the mechanism does not appear to be specific for HLA class I molecules (Tardif and Siddiqui, 2003). However, another study by Herzer et al. (2003) utilizing liver cell lines and plasmid constructs showed increased HLA class I expression via the action of the HCV core protein on TAP1 (function is dependent on p53). Interestingly, increased HLA class I expression was only seen in HepG2 cells (contain functional p53) and not in Huh-7 cells (exhibit a non-functional p53), not for HLA class II (using a pan HLA-DR antibody) and not for other HCV proteins used in a plasmid construct. However, the change in HLA class I expression on the HepG2 cells did not appear to affect $CD8^+$ T cell recognition and may instead be related to NK cell cytotoxicity.

The ability to differentiate the effect of HCV on the expression of the different HLA class I loci will be critical given the differing functions/interactions of HLA-A, -B, and -C alleles with NK cell receptors and potentially $CD8^+$ T cell antigen presentation. It should be noted that in the studies described above, the pan HLA-class I antibody W6/32 was used and this antibody does not differentiate between the HLA class I loci.

Interactions between HIV and HLA surface expression are well established. HIV Nef in particular down-modulates cell surface expression of HLA-A and -B molecules, rendering them less visible to cytotoxic $CD8^+$ T cells, however HLA-C and -E are not selectively down-modulated, which renders them resistant to NK-mediated lysis (Cohen et al., 1999). More recently, differential expression levels of different HLA-C alleles mediated through micro-RNA regulation were found to be important in influencing HIV-1 control. Increased cell surface expression levels of HLA-C were significantly associated with reduced longitudinal viral load and rate of decline in $CD4^+$ T cell count in a study involving over 5000 individuals with pre-treatment HIV-1 infection (Apps et al., 2013). Furthermore, this effect was independent of all other HLA allele-specific effects and was robust across different ethnic groups with distinct HLA-C allele frequency distributions and linkage relationships with HLA-A and -B alleles. Further, differential HLA-C expression levels correlated with measured CTL responses and frequency of viral escape mutation, signifying a direct modulatory effect on disease outcome mediated through the quality of HLA-C restricted T cell responses. While this is a "peptide-independent" mechanism of control, it points to the importance of providing sufficient epitopes for HLA-C in a vaccine immunogen not liable to escape from responses binding with high or low expressing HLA-C alleles.

Human leukocyte antigen class II presentation by antigen presenting cells (APCs) to $CD4^+$ T cells is important for both HIV and HCV, but less is known about how these viruses affect HLA class II presentation. In general, nascent HLA class II molecules in the endoplasmic reticulum of APCs such as dendritic cells associate with the invariant chain protein, which acts to prevent the binding of endogenous peptides in the HLA class II pocket as well as a chaperone for the HLA class II molecule to the golgi apparatus for transportation to the cell surface. However, cell surface expression of HLA class II molecules is not possible until the invariant chain is degraded by a protease such as cathepsin.

Hepatitis C virus is known to affect dendritic cell function and maturation and has been shown to inhibit HLA class II (HLA-DR) expression on dendritic cells (Siavoshian et al., 2005; Averill et al., 2007; Saito et al., 2008). Subsequent studies have shown that dendritic cells exposed to HCV exhibit decreased expression of Cathepsin S with a corresponding decrease of HLA-DR expression on the cell surface, mainly mediated through the HCV proteins core and NS5A (Kim et al., 2012). Interestingly, hepatocytes may act as APCs in the liver and similar interactions were observed when these cells are transfected with core and NS5A (Kim et al., 2012). More should be examined in this area for HCV as $CD4^+$ T cells are critical in HCV infection outcome based on $CD4^+$ T cell depletion and HLA class II tetramer studies that clearly show lack of $CD4^+$ T cell help and a collapse in HCV-specific $CD4^+$ T cell responses within months of acute HCV infection is strongly associated with persistence (Lucas et al., 2007; Schulze Zur Wiesch et al., 2012).

Although less studied compared to interactions with HLA class I, HIV Nef has been shown to influence HLA class II surface expression through effects on intracellular trafficking (Stumptner-Cuvelette et al., 2001). Notably, slower progression of pediatric HIV disease has been associated with *nef* variants, which induced greater down-modulation of surface HLA class II expression, possibly through reducing $CD4^+$ T cell activation and therefore cell loss (Schindlera et al., 2007).

VIRAL ESCAPE, DIVERSITY AND POPULATION LEVEL ADAPTATION

HIV and HCV have error-prone polymerases, rapid replication cycles and in the case of HIV high intracellular recombination rate, allowing for rapid generation, and selective outgrowth of mutant strains, which escape antigen-specific antiviral responses mediated by T cells and NK cells. There is now an extensive literature documenting the predictable mutational networks, which arise in circulating HIV and HCV strains as a result

of escape from HLA-restricted T cell responses (Moore et al., 2002; Gaudieri et al., 2006; Rauch et al., 2009b). The antigenic diversity, which partly results from this escape mechanism, is extreme compared to other vaccine-preventable virus infections, and therefore requires especially broad-based immunity from vaccines against HIV and HCV. What makes T cell escape particularly notable is that HLA, which mediates the peptide specific targeting of virally infected cells, is the most polymorphic of human gene systems, having become so as a result of myriad microbial selective pressures in human evolution (Prugnolle et al., 2005). To retain or even increase *in vivo* fitness despite mutation in the context of the great diversity of HLA types across a pandemic infection underscores the plasticity of these viruses and the challenge of vaccinating against them at the population level.

In terms of the diversity challenge for vaccines, among the nine phylogenetically distinct HIV-1 group M subtypes, subtypes C and B account for the majority of the global epidemic but have as much as 30–40% inter-subtype diversity at certain segments of the genome. Phylogenetic trees based on HCV sequences indicate the challenge of diversity with HCV, which has an up to 3000-fold higher replication rate than HIV and the absence of any constraint imposed by overlapping open reading frames. HCV genotype 1 is as diverse as all the subtypes of HIV (**Figure 1**). HCV is classified into seven genotypes that differ by about 20–30% at the amino acid level and multiple subtypes for each genotype that differ by 10–15% (Smith et al., 2014). We have previously shown that the polymorphism profile of the different genotypes along sites in the non-structural proteins of HCV vary and supports the observation that there is limited overlap in viral adaptations between genotypes (Rauch et al., 2009b). The limited overlap in the adaptation profile of the genotype 1a and 3a strains likely reflects both different T cell targets as well as different fitness costs associated with variations at specific sites (Salloum et al., 2008; Dazert et al., 2009).

A multi-epitope approach using non-structural proteins has been successful to elicit effective immunity against heterologous HCV strains suggesting potential for effective vaccine development (Folgori et al., 2006; Lang Kuhs et al., 2012). However, a limitation of vaccines developed for HCV is that the use of a small number of T cell epitopes are not sufficient to cover the high variability of HCV observed at the population level (Firbas et al., 2006; Klade et al., 2008). A paucity in the number of known HCV-specific HLA-restricted T cell epitopes is a challenge for a T cell based HCV vaccine.

A further implication of T cell escape is the degree to which escape can accumulate over time in viruses circulating in populations, rendering natural, and vaccine-induced $CD8^+$ T cell responses ineffective against transmitting strains, especially those restricted by common HLA alleles. The frequency of certain HLA-driven escape mutations in HIV are highly correlated to HLA allele frequency across ethnically diverse populations, including for some well-known escape networks associated with "protective" HLA alleles (Kawashima et al., 2009). This is an inherently difficult phenomenon to prove, however, as the more such adaptations might accumulate in a population, the less polymorphism and less statistical power to show a correlation with any host trait as evidence of an adaptive process. Notably early population-based studies of HIV and HCV escape detected significant associations between common population HLA alleles and the presence of population consensus amino acids in autologous viruses, which raised the possibility that these were HLA-driven adaptations that had become fixed at the population level (Moore et al., 2002). This clearly has implications for vaccine immunogens, which may include such "population-adapted" areas.

CURRENT VACCINE DEVELOPMENT

There is recognition of the need to stimulate both arms of the adaptive immune response for an effective preventative HCV vaccine (reviewed in Swadling et al., 2013) and evidence to support

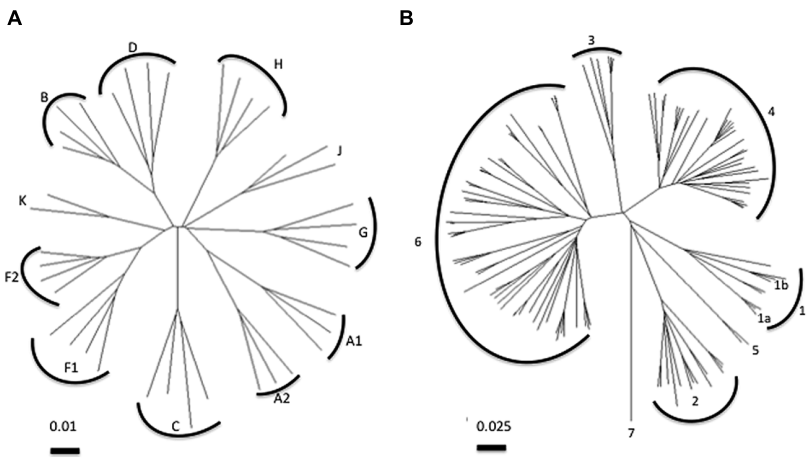

FIGURE 1 | Phylogenetic analysis of (A) HIV pol and (B) HCV NS5B polymerase sequences. Neighbor-joining trees were constructed using the Tamura-Nei model. Note the distance bar for HIV corresponds to 0.01 substitutions per site and for HCV 0.025 substitutions per site. Common HCV subtypes 1a and 1b are indicated on tree. HCV subtype and HIV clade sequences obtained from www.hcv.lanl.gov and www.hiv.lanl.gov, respectively.

the inclusion of both structural and non-structural proteins (reviewed in Torresi et al., 2011). Previous studies have shown evidence of cross-reactive neutralizing antibodies (NAbs), particularly in the chimpanzee model (Choo et al., 1994; Forns et al., 2000; Rollier et al., 2007; Meunier et al., 2011), but limited data on vaccine candidates that elicit both NAbs and HCV-specific T cell responses. Recently, encouraging results have been reported by Martinez-Donato et al. (2014) who utilized a mixture of HCV core, E1, E2, and NS3 in Alum (MixprotHC; containing likely conserved T cell epitopes) from a genotype 1b strain to induce cross-reactive IgG NAbs (to genotype 1a and 2a) and broad HCV-specific $CD4^+$ and $CD8^+$ T cell responses (detected via proliferation and IFN-gamma ELISpot assays) in immunized mice (BALB/c) and African Green Monkeys. Importantly, immunization with MixprotHC also suppresses viremia in a surrogate challenge model in mice. Other vector-based and DNA-based vaccine candidates exist (reviewed in Swadling et al., 2013) and outcomes from Phase II trials should be informative as to their likely efficacy in "at-risk" populations.

In the comparatively much larger and now 30 year old field of HIV vaccine development, the lack of an effective vaccine points to the many remaining barriers to inducing broadly NAbs or effective $CD8^+$ T cells capable of acting and persisting at the site of mucosal HIV entry. Many current vaccine strategies progressing to clinical studies seek to address some of the evasion mechanisms discussed here. For example, there has been testing of various diversity-combating immunogen design approaches, including mosaic vaccines in which inclusion of variant epitopes is optimized, as well as strategies based on conserved immunogens sequences. There are numerous adjuvants, vectors and delivery vehicles designed to improve the efficiency of antigen presentation of vaccine antigens in order to stimulate effective antiviral responses. There are two recent vaccine programs, however, which raise the intriguing possibility that vaccines may need to induce mechanisms of antigen presentation that are highly distinct from those seen in natural infection for their protective effects. A novel "tolerogenic" vaccine consisting of inactivated simian immunodeficiency virus (SIV) mac239 particles with particular bacterial adjuvants has been shown to elicit $CD8^+$ T-regulatory cells in vaccinated macaques. These T cell were not cytolytic but were able to suppress the activation of SIV-positive $CD4^+$ T cells, rendering them less susceptible to SIV infection after challenge. In addition, these $CD8^+$ T cells were found to be uniquely restricted by non-classical MHCIb/E molecules (Andrieu et al., 2014), corresponding to HLA-E in humans. Interestingly, recent data shows HLA-E expression in liver biopsies correlates with HCV viral load in chronic HCV-infected subjects and NK cells lacking the inhibitory receptor for HLA-E (NKG2A) is associated with protection from HCV infection in high-risk exposure subjects (Thoens et al., 2014). To date there has been no examination of non-classical HLA-restricted $CD8^+$ T cells in HCV infection. In contrast to the $CD8^+$ "T-regulatory" type cells described above, a vaccine based on a rhesis CMV vector has produced durable protection or clearance of SIV challenge infections in vaccinated macaques associated with induction of effector memory $CD8^+$ T cell responses. However, these $CD8^+$ T cells have been found to target a diverse array of promiscuous or dominant epitopes restricted by HLA class II alleles, rather than HLA class I (Hansen et al., 2013). In both these examples, properties of the vaccine appear to violate the usual rules of $CD8^+$ T cell priming and both show promising efficacy in the SIV-macaque model, suggesting novel ways in which vaccines may avoid the evolutionary solutions that SIV/HIV may have developed in natural infection.

CONCLUSION

In general, induction of $CD4^+$ and $CD8^+$ T cell responses a key aim of most current vaccine candidates for HIV and HCV, together with innate and humoral immunity as part of a coordinated and long lived immune response. For preventative vaccines, the efficacy of $CD4^+$ and $CD8^+$ T cells will crucially depend on the extent to which the vaccine induced T cells can overcome natural effects of these viruses on HLA expression, antigen presentation and HLA-associated viral diversity.

REFERENCES

Andrieu, J. M., Chen, S., Lai, C., Guo, W., and Lu, W. (2014). Mucosal SIV vaccines comprising inactivated virus particles and bacterial adjuvants induce CD8(+) T-regulatory cells that suppress SIV-positive CD4(+) T-cell activation and prevent SIV infection in the Macaque Model. *Front. Immunol.* 5:297. doi: 10.3389/fimmu.2014.00297

Apps, R., Qi, Y., Carlson, J. M., Chen, H., Gao, X., Thomas, R., et al. (2013). Influence of HLA-C expression level on HIV control. *Science* 340, 87–91. doi: 10.1126/science.1232685

Averill, L., Lee, W. M., and Karandikar, N. J. (2007). Differential dysfunction in dendritic cell subsets during chronic HCV infection. *Clin. Immunol.* 123, 40–49. doi: 10.1016/j.clim.2006.12.001

Carrington, M., Nelson, G. W., Martin, M. P., Kissner, T., Vlahov, D., Goedert, J. J., et al. (1999). HLA and HIV-1: heterozygote advantage and B*35-Cw*04 disadvantage. *Science* 283, 1748–1752. doi: 10.1126/science.283.5408.1748

Choo, Q. L., Kuo, G., Ralston, R., Weiner, A., Chien, D., Van Nest, G., et al. (1994). Vaccination of chimpanzees against infection by the hepatitis C virus. *Proc. Natl. Acad. Sci. U.S.A.* 91, 1294–1298. doi: 10.1073/pnas.91.4.1294

Cohen, G. B., Gandhi, R. T., Davis, D. M., Mandelboim, O., Chen, B. K., Strominger, J. L., et al. (1999). The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. *Immunity* 10, 661–671. doi: 10.1016/S1074-7613(00)80065-5

Cooper, S., Erickson, A. L., Adams, E. J., Kansopon, J., Weiner, A. J., Chien, D. Y., et al. (1999). Analysis of a successful immune response against hepatitis C virus. *Immunity* 10, 439–449. doi: 10.1016/S1074-7613(00)80044-8

Dazert, E., Neumann-Haefelin, C., Bressanelli, S., Fitzmaurice, K., Kort, J., Timm, J., et al. (2009). Loss of viral fitness and cross-recognition by $CD8^+$ T cells limit HCV escape from a protective HLA-B27-restricted human immune response. *J. Clin. Invest.* 119, 376–386. doi: 10.1172/JCI36587

Diepolder, H. M., Zachoval, R., Hoffmann, R. M., Wierenga, E. A., Santantonio, T., Jung, M. C., et al. (1995). Possible mechanism involving T-lymphocyte response to non-structural protein 3 in viral clearance in acute hepatitis C virus infection. *Lancet* 346, 1006–1007. doi: 10.1016/S0140-6736(95)91691-1

Firbas, C., Jilma, B., Tauber, E., Buerger, V., Jelovcan, S., Lingnau, K., et al. (2006). Immunogenicity and safety of a novel therapeutic hepatitis C virus (HCV) peptide vaccine: a randomized, placebo controlled trial for dose optimization in 128 healthy subjects. *Vaccine* 24, 4343–4353. doi: 10.1016/j.vaccine.2006.03.009

Folgori, A., Capone, S., Ruggeri, L., Meola, A., Sporeno, E., Ercole, B. B., et al. (2006). A T-cell HCV vaccine eliciting effective immunity against heterologous virus challenge in chimpanzees. *Nat. Med.* 12, 190–197. doi: 10.1038/nm1353

Forns, X., Payette, P. J., Ma, X., Satterfield, W., Eder, G., Mushahwar, I. K., et al. (2000). Vaccination of chimpanzees with plasmid DNA encoding the hepatitis C virus (HCV) envelope E2 protein modified the infection after challenge with homologous monoclonal HCV. *Hepatology* 32, 618–625. doi: 10.1053/jhep.2000.9877

Gaudieri, S., Rauch, A., Park, L. P., Freitas, E., Herrmann, S., Jeffrey, G., et al. (2006). Evidence of viral adaptation to HLA class I-restricted immune pressure in chronic hepatitis C virus infection. *J. Virol.* 80, 11094–11104. doi: 10.1128/JVI.00912-06

Grakoui, A., Shoukry, N. H., Woollard, D. J., Han, J. H., Hanson, H. L., Ghrayeb, J., et al. (2003). HCV persistence and immune evasion in the absence of memory T cell help. *Science* 302, 659–662. doi: 10.1126/science.1088774

Hansen, S. G., Sacha, J. B., Hughes, C. M., Ford, J. C., Burwitz, B. J., Scholz, I., et al. (2013). Cytomegalovirus vectors violate $CD8^+$ T cell epitope recognition paradigms. *Science* 340, 1237874. doi: 10.1126/science.1237874

Herzer, K., Falk, C. S., Encke, J., Eichhorst, S. T., Ulsenheimer, A., Seliger, B., et al. (2003). Upregulation of major histocompatibility complex class I on liver cells by hepatitis C virus core protein via p53 and TAP1 impairs natural killer cell cytotoxicity. *J. Virol.* 77, 8299–8309. doi: 10.1128/JVI.77.15.8299-8309.2003

Hraber, P., Kuiken, C., and Yusim, K. (2007). Evidence for human leukocyte antigen heterozygote advantage against hepatitis C virus infection. *Hepatology* 46, 1713–1721. doi: 10.1002/hep.21889

International HIVCS, Pereyra, F., Jia, X., McLaren, P. J., Telenti, A., de Bakker, P. I., et al. (2010). The major genetic determinants of HIV-1 control affect HLA class I peptide presentation. *Science* 330, 1551–1557. doi: 10.1126/science.1195271

Kawashima, Y., Pfafferott, K., Frater, J., Matthews, P., Payne, R., Addo, M., et al. (2009). Adaptation of HIV-1 to human leukocyte antigen class I. *Nature* 458, 641–645. doi: 10.1038/nature07746

Khakoo, S. I., Thio, C. L., Martin, M. P., Brooks, C. R., Gao, X., Astemborski, J., et al. (2004). HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. *Science* 305, 872–874. doi: 10.1126/science.1097670

Khu, Y. L., Tan, Y. J., Lim, S. G., Hong, W., and Goh, P. Y. (2004). Hepatitis C virus non-structural protein NS3 interacts with LMP7, a component of the immunoproteasome, and affects its proteasome activity. *Biochem. J.* 384(Pt 2), 401–409. doi: 10.1042/BJ20040858

Kim, H., Mazumdar, B., Bose, S. K., Meyer, K., Di Bisceglie, A. M., Hoft, D. F., et al. (2012). Hepatitis C virus-mediated inhibition of cathepsin S increases invariant-chain expression on hepatocyte surface. *J. Virol.* 86, 9919–9928. doi: 10.1128/JVI.00388-12

Klade, C. S., Wedemeyer, H., Berg, T., Hinrichsen, H., Cholewinska, G., Zeuzem, S., et al. (2008). Therapeutic vaccination of chronic hepatitis C nonresponder patients with the peptide vaccine IC41. *Gastroenterology* 134, 1385–1395. doi: 10.1053/j.gastro.2008.02.058

Lang Kuhs, K. A., Ginsberg, A. A., Yan, J., Wiseman, R. W., Khan, A. S., Sardesai, N. Y., et al. (2012). Hepatitis C virus NS3/NS4A DNA vaccine induces multiepitope T cell responses in rhesus macaques mimicking human immune responses [corrected]. *Mol. Ther.* 20, 669–678. doi: 10.1038/mt.2011.188

Lauer, G. M., Barnes, E., Lucas, M., Timm, J., Ouchi, K., Kim, A. Y., et al. (2004). High resolution analysis of cellular immune responses in resolved and persistent hepatitis C virus infection. *Gastroenterology* 127, 924–936. doi: 10.1053/j.gastro.2004.06.015

Lucas, M., Ulsenheimer, A., Pfafferot, K., Heeg, M. H., Gaudieri, S., Gruner, N., et al. (2007). Tracking virus-specific $CD4^+$ T cells during and after acute hepatitis C virus infection. *PLoS ONE.* 2:e649. doi: 10.1371/journal.pone.0000649

Martin, M. P., Gao, X., Lee, J. H., Nelson, G. W., Detels, R., Goedert, J. J., et al. (2002). Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. *Nat. Genet.* 31, 429–434. doi: 10.1038/ng934

Martinez-Donato, G., Amador-Canizares, Y., Alvarez-Lajonchere, L., Guerra, I., Perez, A., Dubuisson, J., et al. (2014). Neutralizing antibodies and broad, functional T cell immune response following immunization with hepatitis C virus proteins-based vaccine formulation. *Vaccine* 32, 1720–1726. doi: 10.1016/j.vaccine.2014.01.046

McKiernan, S. M., Hagan, R., Curry, M., McDonald, G. S., Kelly, A., Nolan, N., et al. (2004). Distinct MHC class I and II alleles are associated with hepatitis C viral clearance, originating from a single source. *Hepatology* 40, 108–114. doi: 10.1002/hep.20261

Meunier, J. C., Gottwein, J. M., Houghton, M., Russell, R. S., Emerson, S. U., Bukh, J., et al. (2011). Vaccine-induced cross-genotype reactive neutralizing antibodies against hepatitis C virus. *J. Infect. Dis.* 204, 1186–1190. doi: 10.1093/infdis/jir511

Miki, D., Ochi, H., Takahashi, A., Hayes, C. N., Urabe, Y., Abe, H., et al. (2013). HLA-DQB1*03 confers susceptibility to chronic hepatitis C in Japanese: a genome-wide association study. *PLoS ONE* 8:e84226. doi: 10.1371/journal.pone.0084226

Miyamoto, H., Moriishi, K., Moriya, K., Murata, S., Tanaka, K., Suzuki, T., et al. (2007). Involvement of the PA28gamma-dependent pathway in insulin resistance induced by hepatitis C virus core protein. *J. Virol.* 81, 1727–1735. doi: 10.1128/JVI.01683-06

Moore, C. B., John, M., James, I. R., Christiansen, F. T., Witt, C. S., and Mallal, S. A. (2002). Evidence of HIV-1 adaptation to HLA-restricted immune responses at a population level. *Science* 296, 1439–1443. doi: 10.1126/science.1069660

O'Brien, S. J., Gao, X., and Carrington, M. (2001). HLA and AIDS: a cautionary tale. *Trends Mol. Med.* 7, 379–381. doi: 10.1016/S1471-4914(01)02131-1

Osna, N. A. (2009). Hepatitis C virus and ethanol alter antigen presentation in liver cells. *World J. Gastroenterol.* 15, 1201–1208. doi: 10.3748/wjg.15.1201

Prugnolle, F., Manica, A., Charpentier, M., Guegan, J. F., Guernier, V., and Balloux, F. (2005). Pathogen-driven selection and worldwide HLA class I diversity. *Curr. Biol.* 15, 1022–1027. doi: 10.1016/j.cub.2005.04.050

Rauch, A., Gaudieri, S., Thio, C., and Bochud, P. Y. (2009a). Host genetic determinants of spontaneous hepatitis C clearance. *Pharmacogenomics* 10, 1819–1837. doi: 10.2217/pgs.09.121

Rauch, A., James, I., Pfafferott, K., Nolan, D., Klenerman, P., Cheng, W., et al. (2009b). Divergent adaptation of hepatitis C virus genotypes 1 and 3 to human leukocyte antigen-restricted immune pressure. *Hepatology* 50, 1017–1029. doi: 10.1002/hep.23101

Rauch, A., Kutalik, Z., Descombes, P., Cai, T., Di Iulio, J., Mueller, T., et al. (2010). Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure: a genome-wide association study. *Gastroenterology* 138, 1338–1345, 1345.e1–1345.e7. doi: 10.1053/j.gastro.2009.12.056

Rollier, C. S., Paranhos-Baccala, G., Verschoor, E. J., Verstrepen, B. E., Drexhage, J. A., Fagrouch, Z., et al. (2007). Vaccine-induced early control of hepatitis C virus infection in chimpanzees fails to impact on hepatic PD-1 and chronicity. *Hepatology* 45, 602–613. doi: 10.1002/hep.21573

Saito, K., Ait-Goughoulte, M., Truscott, S. M., Meyer, K., Blazevic, A., Abate, G., et al. (2008). Hepatitis C virus inhibits cell surface expression of HLA-DR, prevents dendritic cell maturation, and induces interleukin-10 production. *J. Virol.* 82, 3320–3328. doi: 10.1128/JVI.02547-07

Salloum, S., Oniangue-Ndza, C., Neumann-Haefelin, C., Hudson, L., Giugliano, S., aus dem Siepen, M., et al. (2008). Escape from HLA-B*08-restricted CD8 T cells by hepatitis C virus is associated with fitness costs. *J. Virol.* 82, 11803–11812. doi: 10.1128/JVI.00997-08

Schindlera, M., Wilduma, S., Casartellib, N., Doriab, M., and Kirchhoff, F. (2007). Nef alleles from children with non-progressive HIV-1 infection modulate MHC-II expression more efficiently than those from rapid progressors. *AIDS* 21, 1103–1107. doi: 10.1097/QAD.0b013e32816aa37c

Schulze Zur Wiesch, J., Ciuffreda, D., Lewis-Ximenez, L., Kasprowicz, V., Nolan, B. E., Streeck, H., et al. (2012). Broadly directed virus-specific $CD4^+$ T cell responses are primed during acute hepatitis C infection, but rapidly disappear from human blood with viral persistence. *J. Exp. Med.* 209, 61–75. doi: 10.1084/jem.20100388

Siavoshian, S., Abraham, J. D., Thumann, C., Kieny, M. P., and Schuster, C. (2005). Hepatitis C virus core, NS3, NS5A, NS5B proteins induce apoptosis in mature dendritic cells. *J. Med. Virol.* 75, 402–411. doi: 10.1002/jmv.20283

Smith, D. B., Bukh, J., Kuiken, C., Muerhoff, A. S., Rice, C. M., Stapleton, J. T., et al. (2014). Expanded classification of hepatitis C virus into 7 genotypes and 67 subtypes: updated criteria and genotype assignment web resource. *Hepatology* 59, 318–327. doi: 10.1002/hep.26744

Stumptner-Cuvelette, P., Morchoisne, S., Dugast, M., Le Gall, S., Raposo, G., Schwartz, O., et al. (2001). HIV-1 Nef impairs MHC class II antigen presentation and surface expression. *Proc. Natl. Acad. Sci. U.S.A.* 98, 12144–12149. doi: 10.1073/pnas.221256498

Swadling, L., Klenerman, P., and Barnes, E. (2013). Ever closer to a prophylactic vaccine for HCV. *Expert Opin. Biol. Ther.* 13, 1109–1124. doi: 10.1517/14712598.2013.791277

Tardif, K. D., and Siddiqui, A. (2003). Cell surface expression of major histocompatibility complex class I molecules is reduced in hepatitis C virus subgenomic replicon-expressing cells. *J. Virol.* 77, 11644–11650. doi: 10.1128/JVI.77.21.11644-11650.2003

Thimme, R., Oldach, D., Chang, K. M., Steiger, C., Ray, S. C., and Chisari, F. V. (2001). Determinants of viral clearance and persistence during acute hepatitis C virus infection. *J. Exp. Med.* 194, 1395–1406. doi: 10.1084/jem.194.10.1395

Thoens, C., Berger, C., Trippler, M., Siemann, H., Lutterbeck, M., Broering, R., et al. (2014). KIR2DL3+NKG2A- natural killer cells are associated with protection from productive hepatitis C virus infection in people who inject drugs. *J. Hepatol.* 61, 475–481. doi: 10.1016/j.jhep.2014.04.020

Thomas, D. L., Thio, C. L., Martin, M. P., Qi, Y., Ge, D., O'Huigin, C., et al. (2009a). Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature* 461, 798–801. doi: 10.1038/nature08463

Thomas, R., Apps, R., Qi, Y., Gao, X., Male, V., O'HUigin, C., et al. (2009b). HLA-C cell surface expression and control of HIV/AIDS correlate with a variant upstream of HLA-C. *Nat. Genet.* 41, 1290–1294. doi: 10.1038/ng.486

Torresi, J., Johnson, D., and Wedemeyer, H. (2011). Progress in the development of preventive and therapeutic vaccines for hepatitis C virus. *J. Hepatol.* 54, 1273–1285. doi: 10.1016/j.jhep.2010.09.040

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.

Received: 08 July 2014; accepted: 12 September 2014; published online: 13 October 2014.

*Citation: John M and Gaudieri S (2014) Influence of HIV and HCV on T cell antigen presentation and challenges in the development of vaccines. Front. Microbiol. **5**:514. doi: 10.3389/fmicb.2014.00514*

This article was submitted to Microbial Immunology, a section of the journal Frontiers in Microbiology.

Copyright © 2014 John and Gaudieri. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in
MICROBIOLOGY

PERSPECTIVE ARTICLE
published: 06 October 2014
doi: 10.3389/fmicb.2014.00502

Smarter vaccine design will circumvent regulatory T cell-mediated evasion in chronic HIV and HCV infection

Leonard Moise[1,2], Frances Terry[1], Andres H. Gutierrez[2], Ryan Tassone[2], Phyllis Losikoff[3], Stephen H. Gregory[3], Chris Bailey-Kellogg[4], William D. Martin[1] and Anne S. De Groot[1,2]*

[1] *EpiVax, Inc., Providence, RI, USA*
[2] *Institute for Immunology and Informatics, University of Rhode Island, Providence, RI, USA*
[3] *Department of Medicine, Rhode Island Hospital and the Warren Alpert Medical School at Brown University, Providence, RI, USA*
[4] *Department of Computer Science, Dartmouth College, Hanover, NH, USA*

Edited by:
Shuo Li, Burnet Institute and Monash University, Australia

Reviewed by:
Katie L. Flanagan, Monash University, Australia
Joseph Torresi, University of Melbourne, Australia

****Correspondence:***
Anne S. De Groot, EpiVax, Inc., 146 Clifford Street, Providence, RI 02903, USA
e-mail: dr.annie.degroot@gmail.com

Despite years of research, vaccines against HIV and HCV are not yet available, due largely to effective viral immunoevasive mechanisms. A novel escape mechanism observed in viruses that cause chronic infection is suppression of viral-specific effector $CD4^+$ and $CD8^+$ T cells by stimulating regulatory T cells (Tregs) educated on host sequences during tolerance induction. Viral class II MHC epitopes that share a T cell receptor (TCR)-face with host epitopes may activate Tregs capable of suppressing protective responses. We designed an immunoinformatic algorithm, JanusMatrix, to identify such epitopes and discovered that among human-host viruses, chronic viruses appear more human-like than viruses that cause acute infection. Furthermore, an HCV epitope that activates Tregs in chronically infected patients, but not clearers, shares a TCR-face with numerous human sequences. To boost weak $CD4^+$ T cell responses associated with persistent infection, vaccines for HIV and HCV must circumvent potential Treg activation that can handicap efficacy. Epitope-driven approaches to vaccine design that involve careful consideration of the T cell subsets primed during immunization will advance HIV and HCV vaccine development.

Keywords: HIV, HCV, T cell epitope, immunoinformatics, vaccines, cross-reactivity, regulatory T cells, MHC class II

THE CHALLENGE

Despite modern advances in preventing disease by vaccination, persistent viral infections continue to pose a major challenge to vaccine development. The most prominent examples are HIV-1 and HCV infections, which remain two of the largest global public health challenges today. Highly effective medications are now available, but these are still inaccessible to the majority of at-risk individuals mainly due to their cost, and limitations on access to healthcare in the developing world countries where HIV and HCV are most prevalent. Unfortunately, no vaccine candidate against AIDS or hepatitis C is currently nearing market approval. Only a handful of HIV vaccine efficacy trials have been completed, and none have yet been completed for HCV (Honegger et al., 2014). While a range of strategies to treat infection and prevent transmission have been studied and implemented, it is widely believed that an effective vaccine for these global health threats is essential to stopping new infections worldwide.

Development of effective HIV and HCV vaccines is lagging because traditional strategies for developing vaccines have failed to overcome the ability of the viruses to evade the human immune response. Many obstacles to vaccine development have been uncovered: (i) extensive viral genetic diversity, enabling HIV and HCV to evade humoral as well as cell-mediated immune responses (von Hahn et al., 2007; Haaland et al., 2013), (ii) lack of suitable animal models, (iii) establishment of latent reservoirs following integration into the host genome soon after infection (in the case of HIV; Perreau et al., 2013), and (iv) lack of clear correlates of protective immunity. We recently hypothesized that viruses that tend to cause chronic diseases *mutate their T cell epitopes toward greater homology with the human genome*. The existence of several highly homologous T cell epitopes, some of which induce regulatory T cell (Treg) responses, has been confirmed. We postulate that this is an important means by which HIV and HCV evade effective T cell responses, and that failure to account for such epitopes may have contributed to the failure of certain vaccine approaches undertaken to date. Methods for discovering HIV and HCV Treg-activating epitopes and strategies for improving HIV and HCV vaccines are described briefly here.

VIRUSES FIND NEW MEANS TO EVADE HUMAN IMMUNE RESPONSES

No matter where one stands on the subject of the correlates of immunity to HIV and HCV infection, it is generally acknowledged that strong and broadly reactive HIV- and HCV-specific $CD4^+$ T cell responses are required for control of acute viral infections (Rosenberg et al., 1997; Gerlach et al., 1999). Early collapse of the $CD4^+$ T cell response impairs antibody production and $CD8^+$ T cell responses; thus, an effective vaccine needs to induce long-lived $CD4^+$ T cells capable of sustaining these essential

components of immunity (Lichterfeld et al., 2004; Schulze Zur Wiesch et al., 2012).

$CD4^+$ T cells are activated by virus-specific epitopes presented in the context of class II MHC by antigen presenting cells. Identification of class II MHC epitopes has been an active area of research for characterization of antigen-specific HCV and HIV $CD4^+$ T cell responses in infection and vaccination and for construction of epitope-driven vaccines (De Groot et al., 2004; El-Awady et al., 2013; Karpenko et al., 2014; Mishra et al., 2014; Takei et al., 2014). Long-standing criteria for characterizing class II MHC epitopes include allele coverage in the human population and virus coverage among circulating strains, measured by how well an epitope represents a virus-induced response in the human host. Our group has published and validated methods by which this might be accomplished (De Groot et al., 2004; Koita et al., 2006; Mishra et al., 2014).

A significant epitope property that is beginning to gain wider attention is homology with host sequences. Viral epitopes with substantial homology to self are, at best, inert because of clonal deletion in the development of central tolerance; at worst, they may activate Tregs that suppress protective inflammatory responses and thereby enable viral persistence (Rolland et al., 2007; Frankild et al., 2008; Calis et al., 2012). It is well established that HCV-induced Treg activation is associated with extended chronic infections (Losikoff et al., 2012). Several studies of chronic HCV subjects have shown increased frequencies of natural $CD4^+$ Tregs that express high levels of CD25, produce IL-10 (Cabrera et al., 2004), TGF-β (Bolacchi et al., 2006), and FoxP3 (Li et al., 2007), and suppress IFN-γ production (Sugimoto et al., 2003) and proliferation of HCV-specific $CD8^+$ T cells (Boettler et al., 2005; Rushbrook et al., 2005). In the case of HIV, the role of Tregs in infection is currently being debated and requires further investigation to identify Treg subsets that may be responsible for suppressing non-specific T cell activation (beneficial) and of HIV-specific effector T cell responses (detrimental; Chevalier and Weiss, 2013).

We believe that vaccines for HIV and HCV must account for potential Treg activation that can diminish efficacy, particularly when a strong $CD4^+$ T helper immune response is required. Ideally, vaccines should be carefully designed to reduce or eliminate potential Treg-activating sequences. That is possible today with the availability of immunoinformatic tools to predict class II MHC epitopes that may stimulate Tregs.

For over 20 years and until very recently, T cell epitope-mapping algorithms have focused on the MHC-facing side of epitopes and ignored the T cell receptor (TCR) face; thus, their usefulness was limited to identifying MHC ligands. While MHC binding is necessary to stimulate a T cell response, it is not sufficient. Hence, immunoinformatic-identified MHC ligands have been screened experimentally for T cell activation to validate predictions. Because Tregs are responsive to HIV and HCV epitopes, T cell assays should be performed to ascertain which T cell subsets are activated. Indeed, Treg-activating epitopes have been discovered using overlapping peptide arrays and tetramers in HCV core, NS3, NS4, and NS5 antigens (Li et al., 2007, 2009; Ebinuma et al., 2008; Langhans et al., 2010) and HIV Gag (Angin et al., 2012). These approaches are extremely cumbersome, however, when screens are conducted on a genomic scale with the intent to broadly cover human MHC diversity.

ACCELERATING THE DISCOVERY OF VIRAL CAMOUFLAGE SEQUENCES

An informatic tool that rapidly screens thousands of candidate epitopes could address the problem of viral immune escape, but also needs to consider the vast variability and degeneracy of TCRs, making prediction extremely challenging. Fortunately, the problem can be significantly reduced by searching directly for virus-encoded human homologs that potentially stimulate natural and inducible Tregs, even if some Treg-activating epitopes are not necessarily human homologs. Specifically, pathogen sequences that bind MHC and share the same TCR-face with human MHC ligands may stimulate pre-existing Tregs that emerged from development of central and peripheral tolerance. Shared sequence patterns on the TCR-face are easily searchable.

To better define and rapidly assess viral camouflage epitopes, i.e., those epitopes homologous to human, we developed the JanusMatrix algorithm, which leverages our existing algorithm (EpiMatrix) to define MHC-binding peptide epitopes while searching for cross-conservation at the TCR-face. JanusMatrix can be applied to any viral or bacterial target protein to compare its TCR-faces to others in any genomes of interest. JanusMatrix analyzes the two faces of peptide sequences of pathogen origin for T cell activation potential (Moise et al., 2013). MHC-facing residues are analyzed for MHC binding potential using the EpiMatrix epitope-mapping algorithm (De Groot et al., 1997). We have examined TCR-facing residues for conservation against a variety of sequence databases, including the complete human proteome, the human microbiome, and human pathogens (Moise et al., 2013). For example, we screened a wide range of human-host viruses for TCR-face similarity to self and discovered that chronic viruses generally appear more human-like than viruses that cause acute infection (He et al., 2014), and that H7N9 influenza may evade immune response in a similar way (De Groot et al., 2013, 2014).

Using JanusMatrix, we discovered a promiscuous class II epitope located within non-structural HCV protein p7 that exhibits homology with hundreds of human sequences (**Figure 1**). The epitope induces an increase in $CD4^+CD25^+FoxP3^+$ Treg number and function in peripheral blood leukocyte cultures derived from an HLA-diverse cohort of HCV-infected patients, but not in cultures derived from patients who spontaneously cleared HCV or from non-infected individuals (Losikoff et al., 2014). A human analog of the HCV epitope stimulates Tregs in both HCV-infected and non-infected people, suggesting that tolerance to HCV is promoted by activating Tregs that recognize a common TCR-face. It is well known that HCV and HIV $CD8^+$ and $CD4^+$ T cell epitopes mutate over the course of infection, decreasing MHC binding as a mechanism of viral escape (Harcourt et al., 1998; Norris et al., 2006; Petrovic et al., 2012). The p7 HCV epitope exhibits a novel escape mechanism, evolving a TCR-face similar to that found in autologous T cell epitopes, thus stimulating Treg responses and suppressing immune clearance.

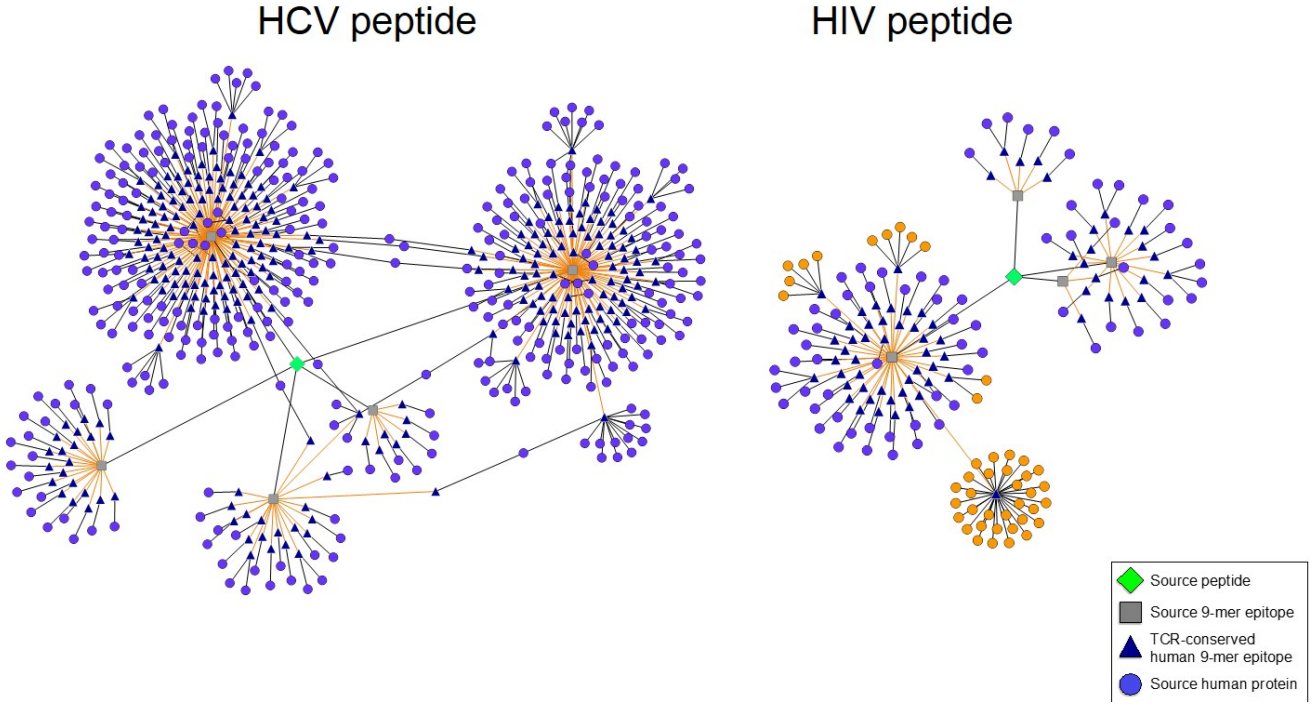

FIGURE 1 | Predicted Treg-activating HIV and HCV sequences possess TCR faces shared by numerous human proteins. Epitope networks are shown, illustrating the abundance of TCR faces one HCV and one HIV peptide share with the human genome as determined by JanusMatrix analysis. The HIV and HCV source peptides are represented by green diamonds, their constituent 9-mer epitopes by gray squares, their cross-conserved partners in the human genome by blue triangles, and the source human proteins by light purple circles. In the HIV peptide (right) a single cross-conserved epitope can be found in 32 different HLA class I alleles; several additional 9-mer epitopes are cross-conserved with 12 other HLA sequences (source protein orange highlight).

At first glimpse, JanusMatrix analysis indicates that HIV also exhibits curious patterns of potential T cell cross-reactivity. While searching HIV envelope sequences for TCR-face conservation in the human genome, we recently uncovered a high frequency of human MHC (HLA) molecule sequences that share a TCR-face with a highly conserved epitope located in the HIV envelope protein (orange circles, **Figure 1**). Because HLA is highly variable in the human population, conservation of this sequence across HLA subtypes is noteworthy. The phenotype of T cells responding to this epitope has not yet been evaluated in our laboratory but an Immune Epitope Database search identified a closely related epitope capable of stimulating $CD4^+$ T cell recognition and proliferation (Atassi and Atassi, 1992). If these $CD4^+$ T cells are T-effector in nature, their activation could restrict HIV expansion (Sanjuán et al., 2013). We believe that it is more likely, however, that T cells that recognize this epitope possess a Treg phenotype, which may promote HIV expansion and/or persistence instead.

These HIV and HCV epitope examples are consistent with our previously published observation that "hit-and-stay" viruses escape protective immune responses by stimulating cross-reactive Tregs (He et al., 2014). This suggests that Treg-activating HCV and HIV sequences may affect HCV and HIV vaccine efficacy. We find similar patterns of cross reactivity in EBV, CMV, and HSV, all viruses that establish chronic infection and for which no vaccine exists.

ADDRESSING VIRAL CAMOUFLAGE IN VACCINE DESIGN

Homology with the human genome represents a novel means by which viruses that seek to establish chronic infections escape human immunity and ensure their survival. Better classification of viral epitopes as either effector- or Treg-activating will improve the design of HIV and HCV vaccines. Knowledge of which epitopes to include or exclude makes it possible to generate virus-specific T cell responses that are essential for protection and that sidestep suppression. One potential solution to the challenge of HCV and HIV vaccine design is to develop epitope-driven subunit vaccines, either as whole antigen protein vaccines, using a structure-based approach, or alternatively, as platform-neutral epitope-based vaccines that do not contain Treg-activating epitopes. Such vaccines would have major advantages over conventional, but as yet unsuccessful approaches because they would simultaneously account for viral and human diversity for the purpose of broad reactivity and promote protective virus-specific T cell responses.

Importantly, the impact of Treg-activating epitopes may have different inhibitory effects depending on the level of viral replication and immune activation in acute or chronic HIV infection. Thus, Treg epitopes to exclude may differ for prophylactic and therapeutic HIV vaccines. With respect to HCV, we believe the maximum effectiveness of an HCV vaccine used either prophylactically or therapeutically would be achieved by always excluding

Treg-activating epitopes because (i) unlike HIV, which infects and replicates in Tregs, HCV is primarily hepatotropic and (ii) a better understanding of Treg function in chronic HCV infection could lead to treatments that are capable of balancing the competing needs for sustained effector T cell-mediated immunity and limited tissue damage moderated by Tregs (Self et al., 2013).

Finally, we are exploring means to fine-tune the epitope content of HIV and HCV vaccines to induce nuanced T cell responses associated with protection. We believe that careful design is needed to improve efficacy. Cross-reactivity at the individual level owing to HLA and HIV or HCV sequence variation may necessitate the development of personalized vaccines that contain T effector, but not Treg epitopes. While personalized vaccines may seem futuristic, tools are available to design such vaccines. As this technology becomes ever more accessible, there will be an even greater incentive to define the means of personalizing vaccines.

REFERENCES

Angin, M., King, M., Altfeld, M., Walker, B. D., Wucherpfennig, K. W., and Addo, M. M. (2012). Identification of HIV-1-specific regulatory T-cells using HLA class II tetramers. *AIDS* 26, 2112–2115. doi: 10.1097/QAD.0b013e328358cc75

Atassi, H., and Atassi, M. Z. (1992). HIV envelope protein is recognized as an alloantigen by human DR-specific alloreactive T cells. *Hum. Immunol.* 34, 31–38. doi: 10.1016/0198-8859(92)90082-X

Boettler, T., Spangenberg, H. C., Neumann-Haefelin, C., Panther, E., Urbani, S., Ferrari, C., et al. (2005). T cells with a $CD4^+CD25^+$ regulatory phenotype suppress in vitro proliferation of virus-specific $CD8^+$ T cells during chronic hepatitis C virus infection. *J. Virol.* 79, 7860–7867. doi: 10.1128/JVI.79.12.7860-7867.2005

Bolacchi, F., Sinistro, A., Ciaprini, C., Demin, F., Capozzi, M., Carducci, F. C., et al. (2006). Increased hepatitis C virus (HCV)-specific $CD4^+CD25^+$ regulatory T lymphocytes and reduced HCV-specific $CD4^+$ T cell response in HCV-infected patients with normal versus abnormal alanine aminotransferase levels. *Clin. Exp. Immunol.* 144, 188–196. doi: 10.1111/j.1365-2249.2006.03048.x

Cabrera, R., Tu, Z., Xu, Y., Firpi, R. J., Rosen, H. R., Liu, C., et al. (2004). An immunomodulatory role for CD4(+)CD25(+) regulatory T lymphocytes in hepatitis C virus infection. *Hepatology* 40, 1062–1071. doi: 10.1002/hep.20454

Calis, J. J., de Boer, R. J., and Keşmir, C. (2012). Degenerate T-cell recognition of peptides on MHC molecules creates large holes in the T-cell repertoire. *PLoS Comput. Biol.* 8:e1002412. doi: 10.1371/journal.pcbi.1002412

Chevalier, M. F., and Weiss, L. (2013). The split personality of regulatory T cells in HIV infection. *Blood* 121, 29–37. doi: 10.1182/blood-2012-07-409755

De Groot, A. S., Ardito, M., Terry, F., Levitz, L., Ross, T., Moise, L., et al. (2013). Low immunogenicity predicted for emerging avian-origin H7N9: implication for influenza vaccine design. *Hum. Vaccin. Immunother.* 9, 950–956. doi: 10.4161/hv.24939

De Groot, A. S., Bishop, E. A., Khan, B., Lally, M., Marcon, L., Franco, J., et al. (2004). Engineering immunogenic consensus T helper epitopes for a cross-clade HIV vaccine. *Methods* 34, 476–487. doi: 10.1016/j.ymeth.2004.06.003

De Groot, A. S., Jesdale, B. M., Szu, E., Schafer, J. R., Chicz, R. M., and Deocampo, G. (1997). An interactive Web site providing major histocompatibility ligand predictions: application to HIV research. *AIDS Res. Hum. Retroviruses* 13, 529–531. doi: 10.1089/aid.1997.13.529

De Groot, A. S., Moise, L., Liu, R., Gutierrez, A. H., Terry, F., Koita, O. A., et al. (2014). Cross-conservation of T-cell epitopes: now even more relevant to (H7N9) influenza vaccine design. *Hum. Vaccin. Immunother.* 10, 256–262. doi: 10.4161/hv.28135

Ebinuma, H., Nakamoto, N., Li, Y., Price, D. A., Gostick, E., Levine, B. L., et al. (2008). Identification and in vitro expansion of functional antigen-specific $CD25^+$ $FoxP3^+$ regulatory T cells in hepatitis C virus infection. *J. Virol.* 82, 5043–5053. doi: 10.1128/JVI.01548-07

El-Awady, M. K., El Gendy, M., Waked, I., Tabll, A. A., El Abd, Y., Bader El Din, N., et al. (2013). Immunogenicity and safety of HCV E1E2 peptide vaccine in chronically HCV-infected patients who did not respond to interferon based therapy. *Vaccine*. doi: 10.1016/j.vaccine.2013.07.074 [Epub ahead of print].

Frankild, S., de Boer, R. J., Lund, O., Nielsen, M., and Kesmir, C. (2008). Amino acid similarity accounts for T cell cross-reactivity and for "holes" in the T cell repertoire. *PLoS ONE* 3:e1831. doi: 10.1371/journal.pone.0001831

Gerlach, J. T., Diepolder, H. M., Jung, M. C., Gruener, N. H., Schraut, W. W., Zachoval, R., et al. (1999). Recurrence of hepatitis C virus after loss of virus-specific CD4(+) T-cell response in acute hepatitis C. *Gastroenterology* 117, 933–941. doi: 10.1016/S0016-5085(99)70353-7

Haaland, R. E., Johnson, J. A., and Tang, J. (2013). Recent advances in research of HIV infection: implications of viral and host genetics on treatment and prevention. *Public Health Genomics* 16, 31–36. doi: 10.1159/000345935

Harcourt, G. C., Garrard, S., Davenport, M. P., Edwards, A., and Phillips, R. E. (1998). HIV-1 variation diminishes CD4 T lymphocyte recognition. *J. Exp. Med.* 188, 1785–1793. doi: 10.1084/jem.188.10.1785

He, L., De Groot, A. S., Gutierrez, A. H., Martin, W. D., Moise, L., and Bailey-Kellogg, C. (2014). Integrated assessment of predicted MHC binding and cross-conservation with self reveals patterns of viral camouflage. *BMC Bioinformatics* 15:S1. doi: 10.1186/1471-2105-15-S4-S1

Honegger, J. R., Zhou, Y., and Walker, C. M. (2014). Will there be a vaccine to prevent HCV infection? *Semin. Liver Dis.* 34, 79–88. doi: 10.1055/s-0034-1371081

Karpenko, L. I., Bazhan, S. I., Antonets, D. V., and Belyakov, I. M. (2014). Novel approaches in polyepitope T-cell vaccine development against HIV-1. *Expert Rev. Vaccines* 13, 155–173. doi: 10.1586/14760584.2014.861748

Koita, O. A., Dabitao, D., Mahamadou, I., Tall, M., Dao, S., Tounkara, A., et al. (2006). Confirmation of immunogenic consensus sequence HIV-1 T-cell epitopes in Bamako, Mali and Providence, Rhode Island. *Hum. Vaccin.* 2, 119–128. doi: 10.4161/hv.2869

Langhans, B., Braunschweiger, I., Arndt, S., Schulte, W., Satoguina, J., Layland, L. E., et al. (2010). Core-specific adaptive regulatory T-cells in different outcomes of hepatitis C. *Clin. Sci. (Lond.)* 119, 97–109. doi: 10.1042/CS20090661

Li, S., Floess, S., Hamann, A., Gaudieri, S., Lucas, A., Hellard, M., et al. (2009). Analysis of $FOXP3^+$ regulatory T cells that display apparent viral antigen specificity during chronic hepatitis C virus infection. *PLoS Pathog.* 5:e1000707. doi: 10.1371/journal.ppat.1000707

Li, S., Jones, K. L., Woollard, D. J., Dromey, J., Paukovics, G., Plebanski, M., et al. (2007). Defining target antigens for $CD25^+$ $FOXP3^+$ IFN-gamma-regulatory T cells in chronic hepatitis C virus infection. *Immunol. Cell Biol.* 85, 197–204. doi: 10.1038/sj.icb.7100020

Lichterfeld, M., Kaufmann, D. E., Yu, X. G., Mui, S. K., Addo, M. M., Johnston, M. N., et al. (2004). Loss of HIV-1-specific $CD8^+$ T cell proliferation after acute HIV-1 infection and restoration by vaccine-induced HIV-1-specific $CD4^+$ T cells. *J. Exp. Med.* 200, 701–712. doi: 10.1084/jem.20041270

Losikoff, P. T., Mishra, S., Terry, F., Gutierrez, A., Ardito, M., et al. (2014). HCV epitope, homologous to multiple human protein sequences, induces a regulatory T cell response in infected patients. *J. Hepatol.* doi: 10.1016/j.jhep.2014.08.026 [Epub ahead of print].

Losikoff, P. T., Self, A. A., and Gregory, S. H. (2012). Dendritic cells, regulatory T cells and the pathogenesis of chronic hepatitis C. *Virulence* 3, 610–620. doi: 10.4161/viru.21823

Mishra, S., Losikoff, P. T., Self, A. A., Terry, F., Ardito, M. T., Tassone, R., et al. (2014). Peptide-pulsed dendritic cells induce the hepatitis C viral epitope-specific responses of naïve human T cells. *Vaccine* 32, 3285–3292. doi: 10.1016/j.vaccine.2014.03.083

Moise, L., Gutierrez, A. H., Bailey-Kellogg, C., Terry, F., Leng, Q., Abdel Hady, K. M., et al. (2013). The two-faced T cell epitope: examining the host–microbe interface with JanusMatrix. *Hum. Vaccin. Immunother.* 9, 1577–1586. doi: 10.4161/hv.24615

Norris, P. J., Stone, J. D., Anikeeva, N., Heitman, J. W., Wilson, I. C., Hirschkorn, D. F., et al. (2006). Antagonism of HIV-specific $CD4^+$ T cells by C-terminal truncation of a minimum epitope. *Mol. Immunol.* 43, 1349–1357. doi: 10.1016/j.molimm.2005.09.004

Perreau, M., Levy, Y., and Pantaleo, G. (2013). Immune response to HIV. *Curr. Opin. HIV AIDS* 8, 333–340. doi: 10.1097/COH.0b013e328361faf4

Petrovic, D., Dempsey, E., Doherty, D. G., Kelleher, D., and Long, A. (2012). Hepatitis C virus—T-cell responses and viral escape mutations. *Eur. J. Immunol.* 42, 17–26. doi: 10.1002/eji.201141593

Rolland, M., Nickle, D. C., Deng, W., Frahm, N., Brander, C., Learn, G. H., et al. (2007). Recognition of HIV-1 peptides by host CTL is related to HIV-1 similarity to human proteins. *PLoS ONE* 2:e823. doi: 10.1371/journal.pone.0000823

Rosenberg, E. S., Billingsley, J. M., Caliendo, A. M., Boswell, S. L., Sax, P. E., Kalams, S. A., et al. (1997). Vigorous HIV-1-specific $CD4^+$ T cell responses associated with control of viremia. *Science* 278, 1447–1450. doi: 10.1126/science.278.5342.1447

Rushbrook, S. M., Ward, S. M., Unitt, E., Vowler, S. L., Lucas, M., Klenerman, P., et al. (2005). Regulatory T cells suppress in vitro proliferation of virus-specific $CD8^+$ T cells during persistent hepatitis C virus infection. *J. Virol.* 79, 7852–7859. doi: 10.1128/JVI.79.12.7852-7859.2005

Sanjuán, R., Nebot, M. R., Peris, J. B., and Alcamí, J. (2013). Immune activation promotes evolutionary conservation of T-cell epitopes in HIV-1. *PLoS Biol.* 11:e1001523. doi: 10.1371/journal.pbio.1001523

Schulze Zur Wiesch, J., Ciuffreda, D., Lewis-Ximenez, L., Kasprowicz, V., Nolan, B. E., Streeck, H., et al. (2012). Broadly directed virus-specific $CD4^+$ T cell responses are primed during acute hepatitis C infection, but rapidly disappear from human blood with viral persistence. *J. Exp. Med.* 209, 61–75. doi: 10.1084/jem.20100388

Self, A. A., Losikoff, P. T., and Gregory, S. H. (2013). Divergent contributions of regulatory T cells to the pathogenesis of chronic hepatitis C. *Hum. Vaccin. Immunother.* 9, 1569–1576. doi: 10.4161/hv.24726

Sugimoto, K., Ikeda, F., Stadanlick, J., Nunes, F. A., Alter, H. J., and Chang, K. M. (2003). Suppression of HCV-specific T cells without differential hierarchy demonstrated ex vivo in persistent HCV infection. *Hepatology* 38, 1437–1448. doi: 10.1016/j.hep.2003.09.026

Takei, S., Omoto, C., Kitagawa, K., Morishita, N., Katayama, T., Shigemura, K., et al. (2014). Oral administration of genetically modified *Bifidobacterium* displaying HCV-NS3 multi-epitope fusion protein could induce an HCV-NS3-specific systemic immune response in mice. *Vaccine* 32, 3066–3074. doi: 10.1016/j.vaccine.2014.03.022

von Hahn, T., Yoon, J. C., Alter, H., Rice, C. M., Rehermann, B., Balfe, P., et al. (2007). Hepatitis C virus continuously escapes from neutralizing antibody and T-cell responses during chronic infection in vivo. *Gastroenterology* 132, 667–678. doi: 10.1053/j.gastro.2006.12.008

Conflict of Interest Statement: Several of the coauthors on this manuscript are employees of EpiVax (Anne S. De Groot, Frances Terry, Leonard Moise, William D. Martin). Anne S. De Groot and William D. Martin are majority stockholders and Leonard Moise holds stock options. These authors recognize the presence of a potential conflict of interest and affirm that the information represented in this paper is original and unbiased observations.

Received: 02 July 2014; accepted: 08 September 2014; published online: 06 October 2014.

Citation: Moise L, Terry F, Gutierrez AH, Tassone R, Losikoff P, Gregory SH, Bailey-Kellogg C, Martin WD and De Groot AS (2014) Smarter vaccine design will circumvent regulatory T cell-mediated evasion in chronic HIV and HCV infection. Front. Microbiol. 5:502. doi: 10.3389/fmicb.2014.00502

This article was submitted to Microbial Immunology, a section of the journal Frontiers in Microbiology.

Copyright © 2014 Moise, Terry, Gutierrez, Tassone, Losikoff, Gregory, Bailey-Kellogg, Martin and De Groot. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 30 June 2014
doi: 10.3389/fmicb.2014.00314

Association of antibodies to *Plasmodium falciparum* reticulocyte binding protein homolog 5 with protection from clinical malaria

Chris Y. H. Chiu[1,2], Julie Healer[1], Jennifer K. Thompson[1], Lin Chen[1], Aiki Kaul[3], Laxman Savergave[3], Arjun Raghuwanshi[3], Connie S. N. Li Wai Suen[1], Peter M. Siba[4], Louis Schofield[1,5], Ivo Mueller[1,6], Alan F. Cowman[1,2] and Diana S. Hansen[1,2]*

[1] The Walter and Eliza Hall Institute of Medical Research, Parkville, VIC, Australia
[2] Department of Medical Biology, The University of Melbourne, Parkville, VIC, Australia
[3] Gennova Biopharmaceuticals, Pune, India
[4] Vector Borne Disease Unit, Papua New Guinea Institute of Medical Research, Goroka, New Guinea
[5] Australian Institute of Tropical Health and Medicine, James Cook University, Douglas, QLD, Australia
[6] Barcelona Center for International Health, University of Barcelona, Barcelona, Spain

Edited by:
Magdalena Plebanski, Monash University, Australia

Reviewed by:
Hajime Hisaeda, Gunma University, Japan
Kendall A. Smith, Weill Medical College of Cornell University, USA

***Correspondence:**
Diana S. Hansen, The Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, VIC 3052, Australia
e-mail: hansen@wehi.edu.au

Emerging evidence suggests that antibodies against merozoite proteins involved in *Plasmodium falciparum* invasion into the red blood cell (RBC) play an important role in clinical immunity to malaria. The protein family of parasite antigens known as *P. falciparum* reticulocyte binding protein-like homolog (PfRh) is required for RBC invasion. PfRh5 is the only member within the PfRh family that cannot be genetically deleted, suggesting it plays an essential role in parasite survival. This antigen forms a complex with the cysteine-rich *P. falciparum* Rh5 interacting protein (PfRipr), on the merozoite surface during RBC invasion. The PfRh5 ectodomain sequence and a C-terminal fragment of PfRipr were cloned and expressed in *Escherichia coli* and baculovirus-infected cells, respectively. Immunization of rabbits with these recombinant proteins induced antibodies able to inhibit growth of various *P. falciparum* strains. Antibody responses to these proteins were investigated in a treatment–re-infection study conducted in an endemic area of Papua New Guinea (PNG) to determine their contribution to naturally acquired immunity. Antibody titers to PfRh5 but not PfRipr showed strong association with protection against *P. falciparum* clinical episodes. When associations with time-to-first infection were analyzed, high antibody levels against PfRh5 were also found to be associated with protection from high-density infections but not from re-infection. Together these results indicate that PfRh5 is an important target of protective immunity and constitutes a promising vaccine candidate.

Keywords: malaria, *Plasmodium falciparum*, reticulocyte binding protein homolog 5, antibodies, immunity

INTRODUCTION

Malaria is one of the most serious infectious diseases of humans causing 500 million clinical cases annually, with nearly 25% of the global burden occurring in the Asia-Pacific. The blood stage of the *Plasmodium* parasite is entirely responsible for malaria-associated pathology (Miller et al., 2002). Fatalities are associated with a spectrum of disease syndromes including acute respiratory distress, hypoglycemia, renal failure, pulmonary oedema and cerebral involvement. The most susceptible population to severe malaria are children under the age of 5, who have experienced few parasitic infections. After years of repeated exposure, individuals living in endemic areas develop clinical immunity. This form of protection does not result in sterilizing immunity but prevents clinical episodes by significantly reducing parasite burden. Naturally acquired immunity predominantly targets blood-stage parasites and appears to require antibody responses since passive transfer of sera from clinically immune individuals protects non-immune recipients from high parasitemia and disease symptoms (Cohen et al., 1961).

During blood-stage replication, *Plasmodium falciparum* merozoites invade erythrocytes through a complex multistep process that requires initial contact of the parasite with the red blood cell (RBC) surface followed by apical reorientation of the merozoite, tight junction formation and final entry into the erythrocyte (reviewed in Cowman and Crabb, 2006). These invasion steps depend on interactions between specific parasite proteins and their receptors on the erythrocyte surface. Two families of invasion ligands have been identified in *P. falciparum*: the erythrocyte-binding antigens (EBAs) and the *P. falciparurm* reticulocyte binding protein-like homologs (PfRhs; reviewed in Cowman and Crabb, 2006). EBAs are orthologs of the Duffy-binding protein of *P. vivax* and include EBA-140, EBA-175, and EBA-181. They consist of an N-terminal cysteine-rich domain, a highly conserved domain, a C-terminal cysteine-rich domain and a transmembrane and cytoplasmic domain (reviewed in Cowman and Crabb, 2006). EBAs are located in the micronemes and are secreted onto the parasite surface just before invasion. Whereas EBA-175 has been shown to interact with glycophorin A on the surface of the erythrocyte

(ref), EBA-140 binds to glycophorin C (Maier et al., 2009). The receptor for EBA-181 has not been identified. The PfRhs family consists of five proteins located in the parasite's rhoptries. Members of this family include: PfRh1, PfRh2a, PfRh2b, PfRh4, and PfRh5. So far only the host receptor for PfRh4 (Tham et al., 2010; complement receptor 1) and PfRh5 (Crosnier et al., 2011; basigin) have been identified. Except for PfRh5 (Baum et al., 2009), all the other members of this family are large type-1 transmembrane proteins.

PfRh5 is considerably smaller than the other PfRh proteins and lacks a transmembrane domain (Baum et al., 2009). After its release from the rhoptries, PfRh5 forms a complex with a cysteine-rich antigen named *P. falciparum* Rh5 interacting protein (PfRipr), which facilitates its expression on the merozoite's surface for erythrocyte invasion (Chen et al., 2011). The genes encoding both PfRh5 and PfRipr are refractory to gene targeted deletion, suggesting essential roles for these antigens in parasite invasion (Baum et al., 2009; Chen et al., 2011).

P. falciparum invasion ligands are targets of inhibitory antibodies that prevent parasite invasion and subsequent replication in the erythrocyte (reviewed in Cowman and Crabb, 2006). Thus these molecules have been proposed as vaccine candidates. With this in view, PfRh5 has recently received considerable attention, since unlike many other merozoite antigens, it has limited genetic diversity among *P. falciparum* isolates (Bustamante et al., 2013). Moreover, rabbit antisera raised against PfRh5 have been shown to inhibit parasite replication *in vitro* (Baum et al., 2009; Douglas et al., 2011). Parasite growth inhibition was observed across a wide range of laboratory-adapted parasite lines, suggesting that PfRh5 could be an effective vaccine target (Bustamante et al., 2013; Douglas et al., 2014; Reddy et al., 2014). Similarly, antibodies to PfRipr have been shown to inhibit parasite growth *in vitro* (Chen et al., 2011).

To date, it is unclear whether PfRipr is recognized by naturally acquired antibodies from individuals residing in malaria-endemic areas. Moreover, despite promising results in experimental animals and *in vitro* assays on the potential for PfRh5 as a leading vaccine candidate, there are conflicting reports on whether this antigen is the target of naturally acquired immunity, with studies suggesting suggesting that PfRh5 has poor natural immunogenicity (Douglas et al., 2011) or that anti-PfRh5 responses predict protection from clinical malaria (Tran et al., 2014). To further address this question, we have expressed stable, soluble forms of PfRh5 and PfRipr. The association between antibody responses to the PfRh5 and PfRipr recombinant proteins with reduced risk to re-infection and symptomatic disease was investigated in a treatment–reinfection study in a malaria-endemic area. Our main results indicate that in a population of children who are actively acquiring immunity to malaria, anti-PfRh5 antibody responses are associated with protection against *P. falciparum* clinical episodes and high-density infections.

MATERIALS AND METHODS

STUDY POPULATION AND ETHICS STATEMENT

Plasma samples were obtained from a prospective treatment–reinfection study of 206 children aged from 5 to 14 years conducted in Madang province of Papua New Guinea (PNG). Full details of this cohort have been previously described (Michon et al., 2007). Briefly, venous blood was collected at study enrolment into heparinized tubes and plasma was stored at −80°C. All participants received a 7-day treatment of oral artesunate to clear existing infections. During a 6-month follow up period, participants were monitored every two weeks for symptomatic illness and/or parasitemia (active surveillance) and when a child presented with symptoms at the local Mugil Health Centre (passive surveillance). Re-infection was detected by post-PCR ligase detection reaction–fluorescent microsphere assay (LDR-FMA) and light microscopy (LM) on Giemsa stained blood smears. Re-infections were categorized to (1) PCR-detectable, (2) LM-detectable, (3) LM-detectable re-infection with >500 parasites per μl, and (4) LM-detectable re-infection with >5000 parasites per μl. Clinical episode was defined as the presence of fever with ≥37.5°C and >5000 parasites/μl. The study was approved by the Medical Research Advisory Committee (MRAC), PNG Ministry of Health, The Walter and Eliza Hall Institute Human Research Ethics Committee and the institutional review board of the Veteran's Affairs Medical Center (Cleveland, OH, USA). Written consent was obtained from parents/guardians of all participants.

EXPRESSION OF RECOMBINANT PROTEINS AND ANTIBODY GENERATION

The PfRh5 ectodomain sequence was codon-optimized for *Escherichia coli* expression and cloned into the pET-303 vector (Invitrogen, MA, USA). The C-terminally HIS tagged expression product was refolded from the insoluble pellet material under standard conditions. PfRipr (AA604-1086) was cloned into pTri-Ex 2 vector for expression in baculovirus-infected Hi-5 cells (Life Technologies, MA, USA). HIS-FLAG tagged PfRiPr protein was purified from the cell culture supernatant by incubation with M2-FLAG beads (Sigma, MO, USA), followed by elution with FLAG peptide in NaCl-Tris buffer pH 8.0. The peptide was removed by dialysis prior to immunization. Purity and integrity of recombinant proteins were assessed in SDS–polyacrylamide gels (Invitrogen, MA, USA). Rabbits were immunized twice with 100 μg PfRh5 in GLA-SE adjuvant (a kind gift from Darrick Carter, IDRI), or three times with 200 μg PfRipr in Freund's adjuvant. Total IgG was purified from immune sera as described (Healer et al., 2013). All experiments were performed in compliance with the Walter and Eliza Hall Institute Animal Ethics Committee requirements.

GROWTH INHIBITION ASSAYS

Growth inhibition assays (GIA) using IgG isolated from immunized rabbits were performed as described (Healer et al., 2013). Briefly, serial dilutions of purified IgG starting at 2 mg/ml were added to *P. falciparum*-infected RBC (3D7, W2mef, and FCR3) at a parasitemia of 0.5%. Parasitemia was counted after 48 h and specific growth inhibition calculated relative to parasites grown in non-immune IgG. Specific growth inhibition was calculated relative to parasites grown in non-immune IgG.

ELISA

Microtiter plates were coated with recombinant PfRh5 (2 μg/ml) and PfRipr (0.5 μg/ml) in carbonate buffer pH 9.6 by overnight incubation at 4°C. Empty sites were blocked with 5% skim milk for 1 h at 37°C. After washing with 0.05% Tween-PBS, 100 μl of 1:2 serially diluted plasma samples were added to plates and incubated at 37°C for 1 h. The plates were washed three times and incubated with a peroxidase-conjugated mouse anti-human antibody (Southern Biotech, AL, USA). Bound complexes were detected by reaction with tetramethy-benzidine (KBL, MD, USA) and H_2O_2. Absorbance was read at 450 nm. Plasma samples from malaria naïve anonymous Australian blood donors were included as negative controls. Antibody titers were calculated as the plasma dilution with an optical density (OD) value higher than that observed for negative controls at a 1/100 dilution.

STATISTICAL ANALYSES

Statistical analysis was performed using STATA 9.2 (STATA-Corp., College Station, TX, USA). For analysis of associations with clinical outcomes, participants with no detectable parasitemia by PCR or LM during follow up were regarded as non-exposed and excluded from the analysis. Demographic and clinical variables were assessed as potential confounders. Kaplan–Meier method and log-rank test were used to explore associations between antibody titers and time to *P. falciparum* re-infection or clinical episodes. Cox proportional hazards modeling was used to calculate hazard ratios (HR) for time-to-first *P. falciparum* infection by PCR, LM, and time-to-first infection of >500 and >5000 parasites/μL. Poisson model was used to obtain incidence rate ratio (IRR) for the incidence of clinical malaria episodes throughout the study period. HR and IRR were adjusted for identified confounders. Differences in antibody titers between categorical variables were assessed using Wilcoxon rank sum test or Kruskal–Wallis tests.

RESULTS

RECOMBINANT PfRh5 AND PfRipr ARE IMMUNOGENIC AND INDUCE PARASITE GROWTH INHIBITORY ANTIBODIES

The PfRh5 ecotodomain sequence and the C-terminal PfRipr fragment from 3D7 were cloned and expressed in *E. coli* and baculovirus-infected insect cells, respectively. HIS-FLAG tagged proteins were purified with M2-FLAG beads. SDS-PAGE analysis of the purified recombinant proteins revealed highly pure protein preparations with predominant bands at 63 kDa corresponding to PfRh5 and 65 kDa corresponding to PfRipr (**Figure 1A**).

To determine if the recombinant proteins were immunogenic, rabbits were immunized twice with 100 μg PfRh5 in GLA-SE adjuvant or three times with 200 μg PfRiPr in Freund's adjuvant. Seroconversion of immunized rabbits was successfully confirmed by ELISA using PfRh5 and PfRipr recombinant proteins (data not shown). Total IgG was purified from immune sera 2 weeks after the last immunization and added to *P. falciparum* trophozoite cultures to determine their capacity to inhibit parasite growth. **Figures 1B,C** shows that both anti-PfRh5 and anti-PfRipr antibodies inhibit growth of *P. falciparum* 3D7, W2Mef, and FCR3 in a dose dependent manner. Incubation with 2 mg/ml of both antibodies resulted in ∼40% of growth inhibition of all parasite strains tested. Thus immunization with these novel recombinant proteins induces antibodies that prevent growth of multiple *P. falciparum* clones.

ANTI-PfRh5 ANTIBODIES ARE ASSOCIATED WITH PROTECTION FROM HIGH PARASITEMIA AND REDUCE INCIDENCE OF MALARIA CLINICAL EPISODES

A preliminary screen conducted in the Madang Province of PNG suggested that antibodies to PfRh5 and to the EGF-like domain of PfRipr show an intermediate association with protection from clinical malaria (Richards et al., 2013). To further explore this hypothesis, antibody titers to the recombinant PfRh5 and PfRipr proteins described above, were measured by ELISA in a longitudinal cohort of PNG children. Similar to other endpoints previously examined in this cohort (Michon et al., 2007; Richards et al., 2010; Hill et al., 2013), antibody responses to PfRh5 were heterogeneous and thus divided into terciles for analysis. The relationship between antibody levels amongst low (L), medium (M), and high (H) responders and time-to-reinfection of different parasite densities was investigated in Kaplan–Meier survival curves and a log-rank test was used to determine the difference between groups. **Figure 2** shows that anti-PfRh5 responses protected from high-density (≥5000 parasites/μL) parasitemia (H vs L: $p < 0.0001$, M vs L: $p = 0.019$) but not against re-infection *per se* (assessed by PCR or LM). The association between antibody titers and the risk of acquiring new *P. falciparum* infections was also analyzed by Cox regression model. In agreement with previous studies with this cohort (Michon et al., 2007; Richards et al., 2010; Hill et al., 2013), age (<9 years and ≥9 years) and location of residence were identified as confounders. Thus HRs were adjusted for those variables. Anti-PfRh5 antibody responses did not reduce the risk of *P. falciparum* re-infection by PCR, LM or moderate density infection (>500 parasites/μL). However, a reduced risk of developing a high-density infection (>5000 parasites/μL) was observed for high anti-PfRh5 titers, even after adjustment for age and location of participants (**Table 1**).

The association of anti-PfRh5 antibody responses with time-to-first clinical episode was also determined. Anti-PfRh5 antibody levels were found to be significantly associated with a protection from clinical malaria, as medium and high antibody responders showed 61.7 and 82.2% reduced risk of experiencing a clinical episode than low responders after the adjustment for confounders (M vs L: adjusted HR = 0.383, 95% CI = 0.216–0.678, $p = 0.001$; H vs L: adjusted HR = 0.178, 95% CI = 0.082–0.389, $p < 0.001$; **Table 2**). A Poisson regression was used to test for associations between antibody levels and overall incidence of clinical disease. Increased anti-PfRh5 antibody responses were found to be strongly associated with reduced incidence of malaria clinical episode during the 6 month follow-up period (M vs L: adjusted IRR = 0.539, 95% CI = 0.344–0.842, $p = 0.007$; H vs L: adjusted IRR = 0.257, 95% CI = 0.134–0.492, $p < 0.001$; **Table 3**). Moreover, anti-PfRh5 antibody titers were found to be significantly higher in individuals that did not experience clinical episodes compared to those that experienced 1, 2, or 3 malaria clinical episodes over the follow up period (**Figure 3**). Thus together these results indicate that naturally acquired anit-PfRh5 antibodies protect from parasitemia of high density and clinical illness.

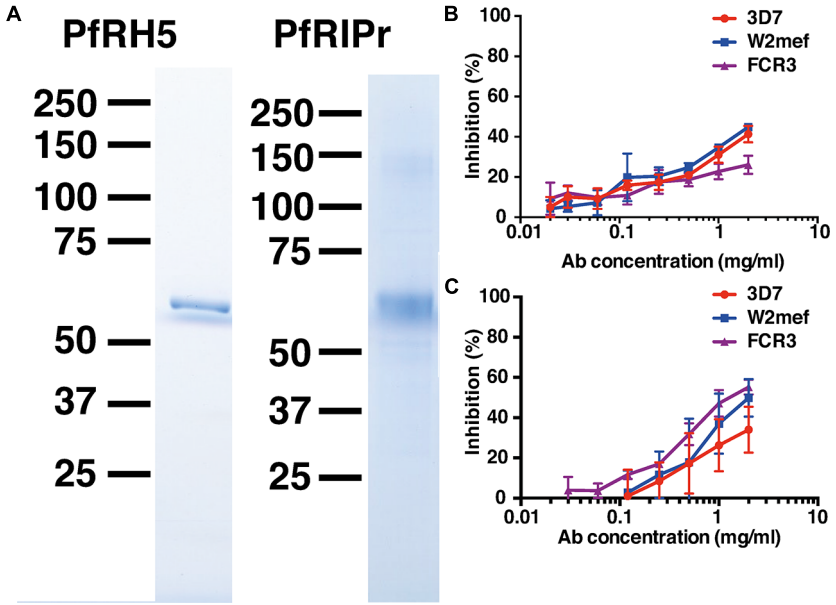

FIGURE 1 | Immunization of rabbits with recombinant PfRh5 and PfRip5 induces strain-transcending parasite growth inhibitory antibodies. The PfRh5 ectodomain sequence and the C-terminal PfRipr fragment were cloned and expressed in *E. coli* and baculovirus-infected Hi-5 cells, respectively. SDS-PAGE analysis **(A)** of the purified recombinant proteins. Total IgG purified from sera of PfRh5 **(B)** and PfRipr **(C)** immunized rabbits was added to *P. falciparum* 3D7, W2Mef, and FCR3 cultures to determine their capacity to inhibit parasite growth.

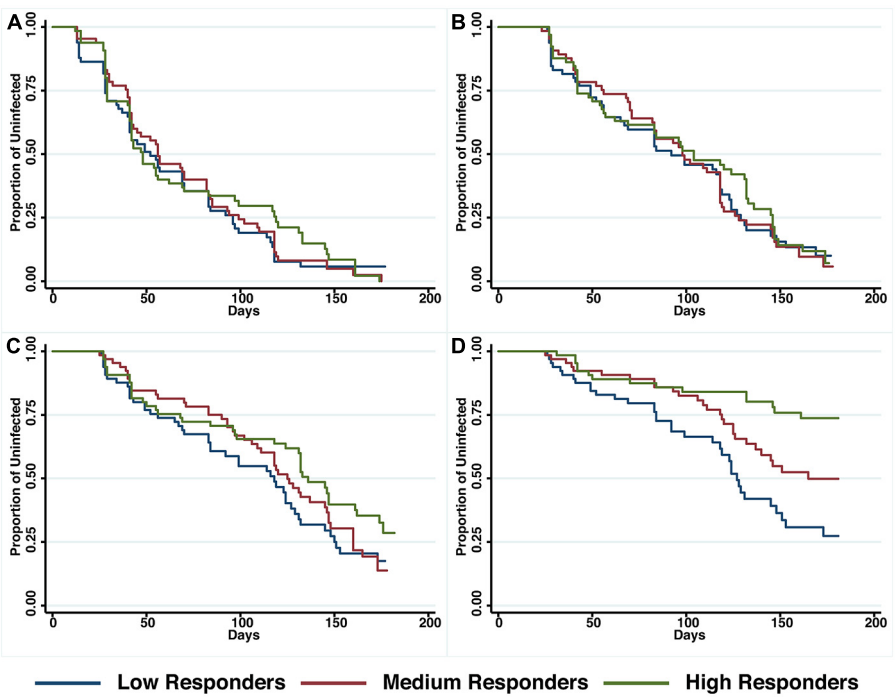

FIGURE 2 | Probability of remaining free of malaria re-infection amongst individuals with different anti-PfRh5 antibody levels. Time-to-first PCR-positive *P. falciparum* blood-stage infection **(A)**, LM-positive **(B)**, LM-positive with ≥500 parasites/μL **(C)** and LM-positive with ≥5000 parasites/μl **(D)** in individuals with low, medium or high anti-PfRH5-specific antibody titers at study enrolment, as determined by Kaplan–Meier survival analysis. Log-rank was used to determine differences among responder groups, $p < 0.0001$ between high and low responders and $p = 0.019$ between medium and low responders with LM ≥ 5000 parasites/μl.

Table 1 | Associations between anti-PfRh5 antibody titers and risk of acquiring *P. falciparum* infections of varying density.

		Anti-PfRh5 responses					
		Medium vs low			High vs low		
		HR	95% CI	P	HR	95% CI	P
PCR	Unadjusted	0.95	(0.66–1.35)	0.76	0.88	(0.61–1.27)	0.51
PCR	Adjusted	0.98	(0.68–1.42)	0.93	0.87	(0.57–1.33)	0.53
LM	Unadjusted	0.99	(0.68–1.45)	0.96	0.89	(0.61–1.31)	0.56
LM	Adjusted	1.10	(0.74–1.64)	0.64	1.05	(0.67–1.65)	0.83
LM500	Unadjusted	0.87	(0.58–1.30)	0.49	**0.63**	**(0.41–0.97)**	**0.03**
LM500	Adjusted	1.00	(0.64–1.54)	0.99	0.85	(0.51–1.42)	0.55
LM5000	Unadjusted	**0.56**	**(0.34–0.93)**	**0.03**	**0.27**	**(0.15–0.50)**	**<0.001**
LM5000	Adjusted	0.61	(0.36–1.05)	0.07	**0.37**	**(0.18–0.72)**	**<0.001**

Hazard ratio (HRs) were determined to assess associations with anti-PhRh5 antibody responses and time-to-first PCR detectable, LM detectable, ≥500 parasites/μl, and ≥5000 parasites/μl P. falciparum infections. HRs were adjusted for age and location of participants. Significant associations are shown in bold.

Table 2 | Association between IgG responses to PfRh5 and PfRipr and time-to-first clinical episode.

	PfRh5						PfRipr					
	M vs L			H vs L			M vs L			H vs L		
	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P
Unadjusted	**0.45**	**(0.26–0.77)**	**<0.01**	**0.17**	**(0.08–0.35)**	**<0.01**	0.90	(0.51–1.60)	0.73	0.55	(0.30–1.00)	0.053
Adjusted	**0.38**	**(0.22–0.68)**	**<0.01**	**0.18**	**(0.08–0.39)**	**<0.01**	0.90	(0.50–1.61)	0.72	0.64	(0.35–1.17)	0.150

Hazard ratios (HRs) were determined to assess associations with anti-PhRh5 and anti-PfRipr antibody responses and time to P. falciparum clinical episodes with ≥5000 parasites/μl. Values represent unadjusted and adjusted ratios (age and location) ±95% confidence intervals (CI). Significant associations are shown in bold.

Table 3 | Association between IgG responses to PfRh5 and PfRipr and incidence of malaria clinical episodes.

	PfRh5						PfRipr					
	M vs L			H vs L			M vs L			H vs L		
	IRR	95% CI	P	IRR	95% CI	P	IRR	95% CI	P	IRR	95% CI	P
Unadjusted	**0.55**	**(0.35–0.85)**	**<0.01**	**0.21**	**(0.12–0.39)**	**<0.01**	1.03	(0.64–1.65)	0.90	0.81	(0.49–1.33)	0.41
Adjusted	**0.54**	**(0.34–0.84)**	**<0.01**	**0.26**	**(0.13–0.49)**	**<0.01**	1.05	(0.65–1.69)	0.83	0.87	(0.53–1.44)	0.58

Incidence rate ratios (IRR) were determined to assess associations between antibody responses and overall incidence of clinical episodes for all individuals. Values represent unadjusted and adjusted ratios (age and location) ± 95% confidence intervals (CI). Significant associations are shown in bold.

ANTI-PfRipr ANTIBODIES ARE NOT ASSOCIATED WITH REDUCED RISK OF *P. falciparum* RE-INFECTION AND SYMPTOMATIC MALARIA

The relationship between anti-PfRipr antibody titers and time to reinfection of different parasite densities was investigated in Kaplan–Meier survival curves and a log-rank test was used to determine the difference between high, medium and low responders. **Figure 4** shows that only high responders (H vs L: $p = 0.01$) appeared to be protected from parasitemia of high density (>5000 parasites/μl) but not from moderate-density parasitemia (>500 parasites/μl) or re-infection. Before adjustment for confounders, high responders showed a reduced risk to develop a high-density infection (H vs L, HR: 0.49, 95% CI 0.28–0.86, $p = 0.02$). However, this association did not reach statistical significance after adjustment for age and location of the study participants (H vs L, HR: 0.38, 95% CI 0.33–1.05, $p = 0.07$).

The association of anti-PfRipr antibody levels with risk of experiencing a malaria clinical episode was also determined. The risk of developing clinical episodes (**Table 2**) as well as the overall incidence of malaria episodes throughout the 6 month follow-up

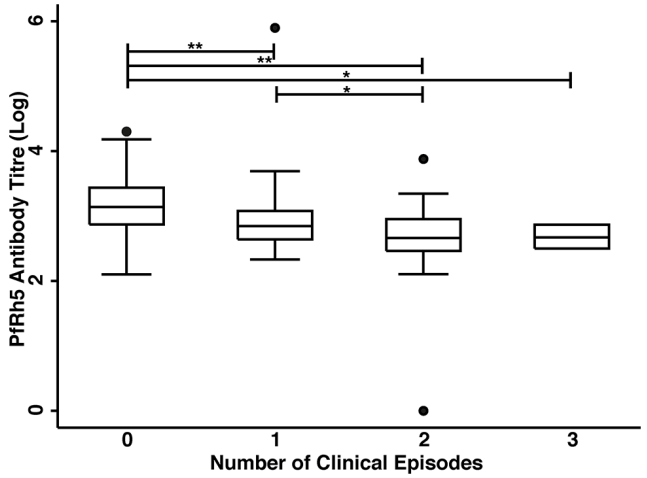

FIGURE 3 | High anti-PfRh5 antibody levels in individuals protected from clinical malaria. Anti-PfRh5 antibody titers were stratified on the basis of the number of clinical episodes during the 6 month follow-up period. Statistical significance was determined by Kruskal–Wallis test and by Wilcoxon rank sum test (*$p < 0.01$, **$p < 0.001$).

period (**Table 3**) were similar amongst all responders, suggesting anti-PfRipr antibodies do not play an important role in clinical outcome.

DISCUSSION

Antibodies that prevent *P. falciparum* invasion into erythrocytes are an important effector mechanism mediating immunity against blood-stage malaria parasites. Over the past 10 years, a large body of data has been generated towards understanding the molecular basis of merozoite invasion into the erythrocyte and many parasite antigens have been characterized. From those antigens, PfRh5 has been identified as an attractive vaccine candidate since it appears to be essential for parasite survival (Baum et al., 2009), has limited sequence polymorphisms and upon animal immunization induces broadly growth inhibitory antibodies (Douglas et al., 2011; Bustamante et al., 2013; Reddy et al., 2014). Consistent with those findings, immunization of rabbits with the PfRh5 recombinant protein described here induced strong antibody responses able to inhibit parasite growth across three different *P. falciparum* lines.

Previous studies suggested that PfRh5 is not highly immunogenic in humans exposed to malaria (Douglas et al., 2011) and that antibody responses to this antigen are rapidly lost at the end of high transmission season. However, a recent study conducted in Mali reported that naturally acquired anti-PfRh5 antibody responses predict protection from clinical malaria (Tran et al., 2014). Consistent with those findings, our study revealed strong associations between anti-PfRh5 antibody titers and protection from symptomatic malaria and high-density parasitemia but not re-infection. Moreover, anti-PfRh5 antibodies at study baseline were found to

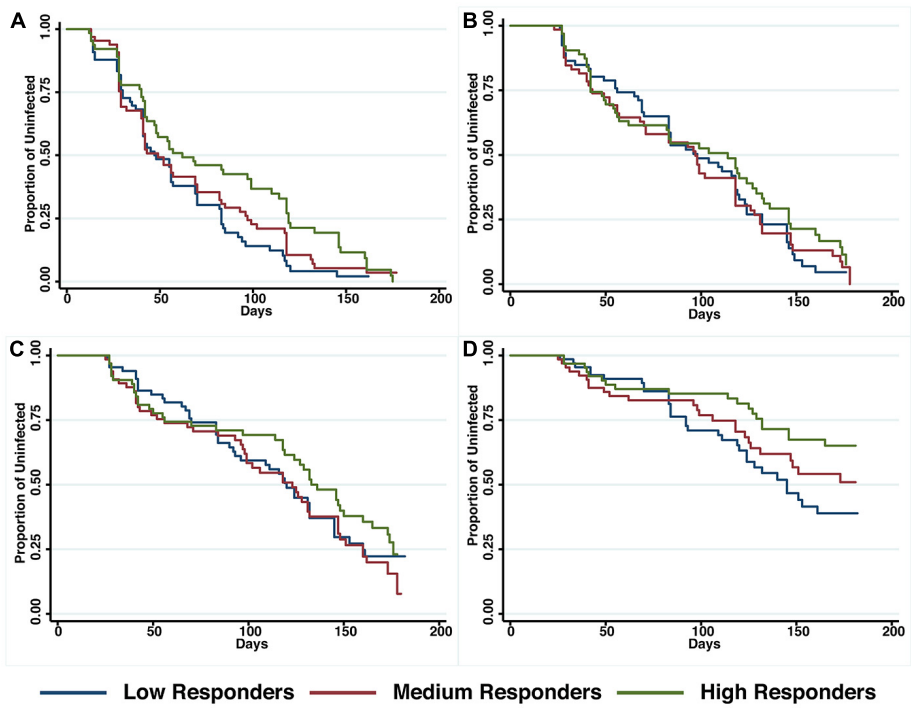

FIGURE 4 | Probability of remaining free of malaria re-infection amongst individuals with different anti-PfRipr antibody levels. Time-to-first PCR-positive *P. falciparum* blood-stage infection **(A)**, LM-positive **(B)**, LM-positive with ≥500 parasites/μL **(C)** and LM-positive with ≥5000 parasites/μl **(D)** in individuals with low, medium, or high anti-PfRipr-specific antibody titers at study enrolment, as determined by Kaplan–Meier survival analysis. Log-rank was used to determine differences among responder groups, $p = 0.01$ between high and low responders with ≥5000 parasites/μl.

reduce the overall incidence of clinical episodes throughout the 6-month follow-up period.

PfRh5 exists in a membrane-associated complex with the cysteine-rich protein PfRipr. We have previously found antibodies to *E. coli* expressed C-terminal and N-terminal fragments of PfRipr inhibit merozoite attachment to the erythrocytes as well as *in vitro* parasite growth (Chen et al., 2011). Here immunization of rabbits with a baculovirus-expressed C-terminal fragment of PfRipr induced strong antibody responses, able to inhibit the *in vitro* growth of several *P. falciparum* lines to a similar level than that observed for anti-PfRh5 rabbit antibodies. Recombinant PfRipr was readily recognized by plasma from PNG participants in our cohort study. However, unlike anti-PfRh5 responses, antibodies against PfRipr showed only a modest association with protection from high-density parasitemia, which did not reach statistical significance after adjustment for confounder variables. Interestingly, despite the close association of PfRipr and PfRh5 on the surface of the merozoite, antibody responses against the two antigens appeared to be only weakly correlated (data not shown). To date, antibody responses to PfRipr have not been extensively investigated. Further work in other settings is required to determine whether this *Plasmodium* antigen is an important target of naturally acquired immunity to malaria.

There is an urgent need for a blood stage component in an anti-malarial vaccine to reduce parasite burdens responsible for morbidity and mortality in susceptible individuals. Development of a blood stage vaccine against malaria has been largely delayed mainly due to the high level of polymorphism in the few antigenic targets that were tested in clinical trials (Lyon et al., 2008; Ogutu et al., 2009). Thus it has been suggested that an effective blood stage vaccine should include more than one (preferably conserved) antigenic components to circumvent parasite phenotypic diversity. This report and others (Douglas et al., 2011; Bustamante et al., 2013; Reddy et al., 2014) demonstrated that immunization with PfRh5 results in the induction of strain-transcending parasite growth inhibitory antibodies. Similarly, we have recently found that immunization of rabbits with a highly conserved region of EBA-175 (region III–V) also induces potent, parasite strain-transcending growth inhibitory antibodies, which appear to be active at concentrations even lower than those described for anti-PfRh5 antibodies (Healer et al., 2013). Further studies are now required to investigate whether co-immunization with PfRh5 and the III–V EBA-175 region results in synergistic interactions and induces robust antibody responses required for the development of an effective malaria vaccine.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: Chris Y. H. Chiu, Jennifer K. Thompson, Julie Healer, Alan F. Cowman, and Diana Silvia Hansen. Performed the experiments and analyzed the data: Chris Y. H. Chiu, Jennifer K. Thompson, Julie Healer, and Diana Silvia Hansen. Contributed essential reagents/materials/analysis tools: Lin Chen, Connie S. N. Li Wai Suen, Peter M. Siba, Lin Chen, Ivo Mueller, and Alan F. Cowman. Interpreted data and wrote the paper: Chris Y. H. Chiu, and Diana Silvia Hansen.

ACKNOWLEDGMENTS

Supported by the Australian Government National Health and Medical Research Council IRIISS and Project Grants 1031212, 1058665. The funders had no role in study design, data collection, and analysis, decision to publish, or preparation of the manuscript.

REFERENCES

Baum, J., Chen, L., Healer, J., Lopaticki, S., Boyle, M., Triglia, T., et al. (2009). Reticulocyte-binding protein homologue 5–an essential adhesin involved in invasion of human erythrocytes by *Plasmodium falciparum*. *Int. J. Parasitol.* 39, 371–380. doi: 10.1016/j.ijpara.2008.10.006

Bustamante, L. Y., Bartholdson, S. J., Crosnier, C., Campos, M. G., Wanaguru, M., Nguon, C., et al. (2013). A full-length recombinant *Plasmodium falciparum* PfRH5 protein induces inhibitory antibodies that are effective across common PfRH5 genetic variants. *Vaccine* 31, 373–379. doi: 10.1016/j.vaccine.2012.10.106

Chen, L., Lopaticki, S., Riglar, D. T., Dekiwadia, C., Uboldi, A. D., Tham, W.-H., et al. (2011). An EGF-like protein forms a complex with PfRh5 and is required for invasion of human erythrocytes by Plasmodium falciparum. *PLoS Pathog.* 7:e1002199. doi: 10.1371/journal.ppat.1002199

Cohen, S., Mcgregor, I. A., and Carrington, S. (1961). Gamma-globulin and acquired immunity to human malaria. *Nature* 192, 733–737. doi: 10.1038/192733a0

Cowman, A. F., and Crabb, B. S. (2006). Invasion of red blood cells by malaria parasites. *Cell* 124, 755–766. doi: 10.1016/j.cell.2006.02.006

Crosnier, C., Bustamante, L. Y., Bartholdson, S. J., Bei, A. K., Theron, M., Uchikawa, M., et al. (2011). Basigin is a receptor essential for erythrocyte invasion by Plasmodium falciparum. *Nature* 480, 534–537. doi: 10.1038/nature10606

Douglas, A. D., Williams, A. R., Illingworth, J. J., Kamuyu, G., Biswas, S., Goodman, A. L., et al. (2011). The blood-stage malaria antigen PfRH5 is susceptible to vaccine-inducible cross-strain neutralizing antibody. *Nat. Commun.* 2, 601. doi: 10.1038/ncomms1615

Douglas, A. D., Williams, A. R., Knuepfer, E., Illingworth, J. J., Furze, J. M., Crosnier, C., et al. (2014). Neutralization of *Plasmodium falciparum* merozoites by antibodies against PfRH5. *J. Immunol.* 192, 245–258. doi: 10.4049/jimmunol.1302045

Healer, J., Thompson, J. K., Riglar, D. T., Wilson, D. W., Chiu, Y.-H. C., Miura, K., et al. (2013). Vaccination with conserved regions of erythrocyte-binding antigens induces neutralizing antibodies against multiple strains of *Plasmodium falciparum*. *PLoS ONE* 8:e72504. doi: 10.1371/journal.pone.0072504

Hill, D. L., Eriksson, E. M., Li Wai Suen, C. S. N., Chiu, C. Y., Ryg-Cornejo, V., Robinson, L. J., et al. (2013). Opsonising antibodies to *P. falciparum* merozoites associated with immunity to clinical malaria. *PLoS ONE* 8:e74627. doi: 10.1371/journal.pone.0074627

Lyon, J. A., Angov, E., Fay, M. P., Sullivan, J. S., Girourd, A. S., Robinson, S. J., et al. (2008). Protection induced by *Plasmodium falciparum* MSP1(42) is strain-specific, antigen and adjuvant dependent, and correlates with antibody responses. *PLoS ONE* 3:e2830. doi: 10.1371/journal.pone.0002830

Maier, A. G., Baum, J., Smith, B., Conway, D. J., and Cowman, A. F. (2009). Polymorphisms in erythrocyte binding antigens 140 and 181 affect function and binding but not receptor specificity in *Plasmodium falciparum*. *Infect. Immun.* 77, 1689–1699. doi: 10.1128/IAI.01331-08

Michon, P., Cole-Tobian, J. L., Dabod, E., Schoepflin, S., Igu, J., Susapu, M., et al. (2007). The risk of malarial infections and disease in Papua New Guinean children. *Am. J. Trop. Med. Hyg.* 76, 997–1008.

Miller, L. H., Baruch, D. I., Marsh, K., and Doumbo, O. K. (2002). The pathogenic basis of malaria. *Nature* 415, 673–679. doi: 10.1038/415673a

Ogutu, B. R., Apollo, O. J., McKinney, D., Okoth, W., Siangla, J., Dubovsky, F., et al. (2009). Blood stage malaria vaccine eliciting high antigen-specific antibody concentrations confers no protection to young children in Western Kenya. *PLoS ONE* 4:e4708. doi: 10.1371/journal.pone.0004708

Reddy, K. S., Pandey, A. K., Singh, H., Sahar, T., Emmanuel, A., Chitnis, C. E., et al. (2014). Bacterially expressed full-length recombinant *Plasmodium falciparum* RH5 protein binds erythrocytes and elicits potent strain-transcending parasite-neutralizing antibodies. *Infect. Immun.* 82, 152–164. doi: 10.1128/IAI.00970-13

Richards, J. S., Arumugam, T. U., Reiling, L., Healer, J., Hodder, A. N., Fowkes, F. J. I., et al. (2013). Identification and prioritization of merozoite antigens as targets of protective human immunity to *Plasmodium falciparum* malaria for vaccine and biomarker development. *J. Immunol.* 191, 795–809. doi: 10.4049/jimmunol.1300778

Richards, J. S., Stanisic, D. I., Fowkes, F. J. I., Tavul, L., Dabod, E., Thompson, J. K., et al. (2010). Association between naturally acquired antibodies to erythrocyte-binding antigens of *Plasmodium falciparum* and protection from malaria and high-density parasitemia. *Clin. Infect. Dis.* 51, e50–e60. doi: 10.1086/656413

Tham, W.H., Wilson, D. W., Lopaticki, S., Schmidt, C. Q., Tetteh-Quarcoo, P. B., Barlow, P. N., et al. (2010). Complement receptor 1 is the host erythrocyte receptor for *Plasmodium falciparum* PfRh4 invasion ligand. *Proc. Natl. Acad. Sci. U.S.A.* 107, 17327–17332. doi: 10.1073/pnas.1008151107

Tran, T. M., Ongoiba, A., Coursen, J., Crosnier, C., Diouf, A., Huang, C.-Y., et al. (2014). Naturally acquired antibodies specific for *Plasmodium falciparum* reticulocyte-binding protein homologue 5 inhibit parasite growth and predict protection from malaria. *J. Infect. Dis.* 209, 789–798. doi: 10.1093/infdis/jit553

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.

Received: 07 May 2014; accepted: 09 June 2014; published online: 30 June 2014.
*Citation: Chiu CYH, Healer J, Thompson JK, Chen L, Kaul A, Savergave L, Raghuvanshi A, Li Wai Suen CSN, Siba PM, Schofield L, Mueller I, Cowman AF and Hansen DS (2014) Association of antibodies to Plasmodium falciparum reticulocyte binding protein homolog 5 with protection from clinical malaria. Front. Microbiol. **5**:314. doi: 10.3389/fmicb.2014.00314*
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Microbiology.
Copyright © 2014 Chiu, Healer, Thompson, Chen, Kaul, Savergave, Raghuvanshi, Li Wai Suen, Siba, Schofield, Mueller, Cowman and Hansen. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in
MICROBIOLOGY

MINI REVIEW ARTICLE
published: 27 May 2014
doi: 10.3389/fmicb.2014.00249

Malaria drives T cells to exhaustion

Michelle N. Wykes[1]*, Joshua M. Horne-Debets[1,2], Chiuan-Yee Leow[1] and Deshapriya S. Karunarathne[1]

[1] Molecular Immunology Laboratory, QIMR Berghofer Medical Research Institute, Brisbane, QLD, Australia
[2] The School of Medicine, University of Queensland, Brisbane, QLD, Australia

Edited by:
Magdalena Plebanski, Monash University, Australia

Reviewed by:
Hajime Hisaeda, Gunma University, Japan
Mutsa Tatenda Madondo, Monash University, Australia
Ian Andrew Cockburn, Australian National University, Australia

***Correspondence:**
Michelle N. Wykes, Molecular Immunology Laboratory, QIMR Berghofer Medical Research Institute, 300 Herston Road, Brisbane, QLD 4006, Australia
e-mail: michelle.wykes@qimrberghofer.edu.au

Malaria is a significant global burden but after >30 years of effort there is no vaccine on the market. While the complex life cycle of the parasite presents several challenges, many years of research have also identified several mechanisms of immune evasion by *Plasmodium* spp. Recent research on malaria, has investigated the programmed cell death-1 (PD-1) pathway which mediates exhaustion of T cells, characterized by poor effector functions and recall responses and in some cases loss of the cells by apoptosis. Such studies have shown exhaustion of $CD4^+$ T cells and an unappreciated role for $CD8^+$ T cells in promoting sterile immunity against blood stage malaria. This is because PD-1 mediates up to a 95% reduction in numbers and functional capacity of parasite-specific $CD8^+$ T cells, thus masking their role in protection. The role of T cell exhaustion during malaria provides an explanation for the absence of sterile immunity following the clearance of acute disease which will be relevant to future malaria-vaccine design and suggests the need for novel therapeutic solutions. This review will thus examine the role of PD-1-mediated T cell exhaustion in preventing lasting immunity against malaria.

Keywords: $CD8^+$ T cell, malaria, exhaustion, PD-1, chronic disease, $CD4^+$ T cells, B cells, PD-L1

INTRODUCTION

Malaria is a mosquito-borne infectious disease of humans caused by parasitic protozoans of the genus *Plasmodium*. There are five species that infect humans and annual deaths from the malaria parasite *Plasmodium falciparum* (*Pf*) alone are estimated at 780,000, with approximately 225 million clinical infections worldwide (Schwartz et al., 2012). The *Anopheles* mosquito, serves as a transmission vector for the parasite. An infected mosquito introduces sporozoites into the blood or lymph of bitten individuals when taking a blood meal. These sporozoites then migrate to the host's liver, where they begin to invade and multiply. During the first week of infection, sporozoites develop into merozoites and then exit the hepatocytes to invade red blood cells progressively, resulting in massive destruction of red blood cells. This eventually leads to anemia in infected subjects due to the loss of erythrocyte and severe symptoms associated with malaria.

Over the last 10 years, more than 40 vaccines were developed to control the morbidity of malaria which then reached the clinical trial stage (Schwartz et al., 2012). Most of these vaccines were specifically designed to target liver or blood stage parasites, the majority through protection by antibodies and/or $CD4^+$ T cells. Although some of them showed potent protection at pre-clinical trials (Schwartz et al., 2012), none to date conferred complete protection against both clinical and severe malaria. The only vaccine to reach stage 3b, RTS,S/AS01E, showed approximately 50% efficacy for 18 months (Alonso et al., 2005) but immunity was significantly diminished within 4 years (Olotu et al., 2013). Notably, protection declined over time and with increasing malaria exposure.

To explain why these vaccines have been unsuccessful, it is important to understand natural immunity against malaria. Perhaps most informative has been an intensive longitudinal study of immunity in Malian children and adults (Tran et al., 2013). In this study, 251 healthy children and adults who were free of blood-stage *Plasmodium* infections, by polymerase chain reaction (PCR), were enrolled just prior to an intense 6-month malaria season. Subsequent clinical malaria episodes were detected by weekly active surveillance or self-referral. Asymptomatic infections were detected by blood-smear microscopy and PCR analysis of blood collected every 2 weeks for 7 months. These studies found that while the risk of symptomatic malaria decreased with increasing age, the risk *P. falciparum* infections did not change. This means that despite years of exposure to intense *Pf* transmission, there was no evidence of acquired, sterile immunity to the parasite in this population, even as clinical immunity to blood-stage malaria was clearly acquired (Tran et al., 2013). Significantly, this indicates that while anti-malarial immune responses to protect against symptoms were effectively induced, sterile immunity against the parasite was not acquired in the same time frame.

NATURALLY ACQUIRED IMMUNITY

The factors that regulate anti-parasite immunity (i.e., those processes that kill parasites and reduce parasite biomass in the body) are not completely understood. For decades it has been known that antibodies play an important role in protection against malaria, and it has been assumed that antibody responses alone can provide sufficient protection to control this disease. This was predominantly based on studies where the transfer of IgG from malaria-immune individuals to non-immune individuals markedly reduced parasite burden (Cohen et al., 1961). Studies indicating that antibodies protect against malaria have led to a large-scale effort to develop a B cell targeted vaccine against malaria, with limited success to date. In endemic settings, memory antibody responses against *Plasmodium* parasites

are short-lived and require constant parasite exposure (Crompton et al., 2010). Furthermore, a year-long study in malaria-endemic Mali, found that both *Pf*-specific memory B cells (MBC) and antibody titers increased after acute malaria and then, after 6 months of decreased *Pf* exposure, contracted to a point slightly higher than pre-infection levels (Weiss et al., 2010). Further, the 19-kDa carboxyl-terminal fragment of merozoite surface protein 1 ($MSP1_{19}$) was a leading malaria vaccine candidate, tested in Kenya which has holoendemic transmission of *Pf*. In these trials, vaccination with $MSP1_{19}$ generated very high titers of antibodies, but did not protect against infection (Ogutu et al., 2009). An evaluation of this vaccine using an experimental mouse model found that vaccination with $MSP1_{19}$ generated $MSP1_{19}$-specific MBC capable of secreting antibodies in response to the vaccine ($MSP1_{19}$), but not infection (Wykes et al., 2005). In fact, infection caused apoptosis of MBC. Subsequent studies showed malarial infections result in a decrease in the proportion of dendritic cells (DCs) that expressed the B-cell survival factor, BAFF, resulting in a decreased ability of these DCs to support MBC survival (Liu et al., 2012). A review proposes a model of how the parasite may mediate these effects by direct interaction with B cells and modulation of the host's BAFF-immune pathway (Scholzen and Sauerwein, 2013).

$CD4^+$ T cells consist of several helper-subtypes which shape immune responses against particular pathogens. During malaria, $CD4^+$ T cell subsets have multiple roles in protection, pathogenesis and also escape from immune responses. $CD4^+$ T cells have been demonstrated to be the major source of both interferon-γ (IFN-γ) and tumor necrosis factor alpha (TNF-α) during experimental malaria in mice (Muxel et al., 2011) which are implicated in both protection and pathology of this disease. Studies in mice infected with *Plasmodium chabaudi* malaria have shown that IFN-γ and TNF-α cooperatively induce nitric oxide synthase expression in the spleen to control peak parasite burden (Jacobs et al., 1996). Similarly, in humans, early IFN-γ responses to *Pf* correlate with better anti-parasite immunity (McCall et al., 2010). IFN-γ contributes to a vast network of protective responses against malaria, summarized in McCall and Sauerwein (2010). Of particular note is a study which investigated the effects of chronic malaria on MSP1-specific transgenic $CD4^+$ T cells (Stephens and Langhorne, 2010). These parasite-specific T cells were seeded into Thy1.1 congenic mice which were then infected with 10^5 *P. chabaudi* infected red cells. One half of the mice were treated with Chloroquine on days 30–34 to clear chronic malaria. After 60 days, flow cytometric analysis of transgenic T cells found that approximately 25% of memory $CD44^+IL\text{-}7R^+$ $CD4^+$ T cells were lost in untreated mice compared to drug-treated mice which had cleared the infection (Stephens and Langhorne, 2010). This study highlights that ongoing infections cause a loss of some parasite-specific memory T cells capable of protection from re-infection.

The loss of $CD4^+$ T cells during malaria was perhaps first suggested when up to 99% of parasite-specific T cells labeled with the fluorescent dye 5-(and -6)-carboxy-fluorescein succinimidyl ester (CSFE), were found to be deleted only following infection of mice (Hirunpetcharat and Good, 1998). Subsequent studies also found deletion of T cells specific for the malaria vaccine, $MSP1_{19}$ during malaria infections (Wipasa et al., 2001). Further studies excluded a role for TNF or Fas pathways but implicated IFN-γ in loss of these cells (Xu et al., 2002). However, FOXP3-expressing $CD4^+CD25^+$ regulatory T cells were also shown to correlate with more rapid parasite growth in human malaria infections (Walther et al., 2005) which may explain why protective cells subside. Mouse models also showed IL-10 produced by these cells was responsible for poor immunity (Couper et al., 2008). Finally, other studies identified elevated numbers of a highly suppressive subset of regulatory T cells in patients with severe malaria (Minigo et al., 2009).

The role of $CD8^+$ T cell-mediated immunity against blood-stage malaria has been largely overlooked, with research being limited to protection against liver-stage infection, the pathogenesis of cerebral malaria (Hafalla et al., 2006) and damage to splenic architecture (Beattie et al., 2006). This view was adopted because parasites and infected erythrocytes have no known antigen presentation machinery and therefore T cells supposedly cannot interact directly with these potential targets (Langhorne et al., 2008). However, early studies in experimental animal models found that depletion of $CD8^+$ T lymphocytes during blood stage *P. chabaudi* infections, significantly delayed clearance of the infection (Podoba and Stevenson, 1991). Other groups showed that naive mice, transfused with $CD8^+$ T cells derived from mice that survived two successive bouts of lethal *P. yoelii* infections with drug cure, subsequently survived lethal blood stage malaria challenge (Imai et al., 2010). More recent studies in experimental mouse models have shown that malaria parasites can parasitize erythroblasts, which have the capacity to activate $CD8^+$ T cells (Imai et al., 2013). What has remained unclear is that while these studies showed $CD8^+$ T cells had potential to protect against blood stage malaria, direct studies did not find a clear role for these cells in protection against blood stage disease.

PROGRAMMED CELL DEATH 1

Programmed cell death-1 (PD-1) is a member of the extended family of molecules that are known to down-regulate T cell function. PD-1 has two known ligands, PD-L1 (B7-H1; Dong et al., 1999; Freeman et al., 2000) and PD-L2 (B7-DC; Latchman et al., 2001; Tseng et al., 2001), which both belong to the B7 co-signaling molecule family. Expression of PD-1 can be observed on T cells, B cells, natural killer T cells, DCs, and activated monocytes (Keir et al., 2008). PD-1 is not expressed on resting T cells but is inducible upon activation (Agata et al., 1996). Functional effects of PD-1 ligation can be observed within a few hours after T cell activation but PD-1 cell surface protein up-regulation requires 24 h (Chemnitz et al., 2004). When PD-1 is engaged simultaneously with T cell receptor signals, it can trigger an inhibitory signal, although no signal transduction occurs when PD-1 is cross-linked alone (Sharpe et al., 2007). In general, interactions between PD-1 on T cells and its ligand, PD-L1, control the induction and maintenance of peripheral T-cell tolerance and negatively regulate proliferation and cytokine production by T cells during immune responses to pathogens or cancer (Sharpe et al., 2007).

A main feature of potent immunity against intracellular pathogens is the development of an optimal T cell response which shows rapid proliferative potential, low apoptosis and polyfunctionality (Lukens et al., 2008). During acute infections, optimal

functioning T cells clear the pathogen, eventually leading to development of robust memory T cells. Those T cells have the ability to mount rapid recall response and re-establish polyfunctional effector mechanisms upon antigen re-exposure (Wherry, 2011). T cell exhaustion is defined by inferior effector function, sustained expression of inhibitory receptors (such as PD-1), poor recall responses and a transcriptional state distinct from that of functional effector or memory T cells (Wherry, 2011). In many chronic infectious diseases, antigen specific T cells become functionally impaired or exhausted. For example, viruses, such as human immunodeficiency virus (HIV) and hepatitis C virus (HCV) have been shown to induce T cell exhaustion mediated by PD-1 (Day et al., 2006; Urbani et al., 2006). Significantly, blockade of the PD-1 pathway improved immunity (Freeman et al., 2006; Urbani et al., 2008). PD-1-related exhaustion has also been implicated in other chronic protozoan infections, such as Leishmaniasis (Liang et al., 2006; Joshi et al., 2009). Further, the apicomplexan parasite, *Toxoplasma gondii* has been shown to induce PD-1 expression on $CD8^+$ T cells, but function was restored by experimental blockade of the PD-1 pathway during chronic murine infections (Bhadra et al., 2011). Together these data have established T cell exhaustion by PD-1 as a key mechanism in chronicity of infectious diseases.

EXHAUSTION OF $CD4^+$ T CELLS DURING MALARIA

PD-1 has been implicated in the pathogenesis of malaria. One of the first studies to examine PD-1 expression during malaria used a mouse model to show PD-1 expression on IL-$7R^{lo}$-expressing $CD4^+$ and $CD8^+$ T cells (Chandele et al., 2011). These PD-1-expressing cells (especially $CD8^+$ T cells) were almost completely lost within 30 days of infection (Chandele et al., 2011). The study did not however measure functional responses to identify T cell exhaustion. Similarly, subsequent studies showed that PD-1 was also expressed on $CD4^+$ (Butler et al., 2012; Illingworth et al., 2013) and $CD8^+$ T cells (Illingworth et al., 2013) in blood of *Pf*-infected individuals in Mali and Kenya, but no functional evidence of exhaustion was provided.

To validate these observations, a murine model of blood stage malaria was adopted to explore the effects of increased expression of PD-1 and LAG-3 on $CD4^+$ T cells (Butler et al., 2012). The combined blockade of PD-L1 and LAG-3 inhibitory molecules with antibodies, during *P. yoelii* and *P. chabaudi* malaria in mice accelerated clearance of parasitemia (Butler et al., 2012). This dual blockade of PD-L1 and Lag-3 improved $CD4^+$ follicular T helper cell (T_{FH}) numbers which correlated with enhanced antibody-mediated immunity (Butler et al., 2012). Moreover, infected mice treated with the anti-malarial drug chloroquine at day 8 and 9 post-infection, showed a lower level of $CD4^+$ T cell dysfunction (Butler et al., 2012). These studies showed that lymphocyte exhaustion modulated immunity against malaria.

Subsequent studies used mice with a deletion of PD-1 (PD-1KO) to conclusively determine if PD-1 had a role in modulating immunity, given that PD-L1 can interact specifically with both B7-1 (Butte et al., 2007) and PD-1 (Iwai et al., 2003) to inhibit T cell activation. *P. chabaudi* malaria was investigated as this infection develops into chronic infections. It was shown that PD-1 mediated a reduction in the capacity of parasite-specific $CD4^+$ T cells to proliferate and secrete IFN-γ and TNF-α during the chronic phase of malaria (day 35) indicating exhaustion of these cells (Horne-Debets et al., 2013). However, in contrast to the combined PD-L1/Lag-3 blockade study, no changes to T_{FH} numbers were observed. One likely explanation for this apparent contradiction is that PD-1 KO mice compared with wild type (WT) mice had a significantly higher proportion of regulatory T follicular cells (T_{FR} cells; Horne-Debets et al., 2013). T_{FR} cells are known to be suppressive *in vitro* and to limit the numbers of T_{FH} cells and germinal centers (GC) B cells *in vivo* (Linterman et al., 2011). Alternatively, since PD-L1 can also interact specifically with B7-1 to inhibit T cell activation (Butte et al., 2007), this pathway may control T_{FH} numbers in PD-1 KO mice.

B CELLS AND EXHAUSTION

Antibodies are known to have a key role in controlling blood-stage infections (Cohen et al., 1961). Investigations into the mechanism of protection, found mice deficient in mature B cells developed a chronic relapsing parasitemia, confirming the need for antibodies to control chronic malaria (von der Weid et al., 1996). For antibodies to be protective, they have to undergo processes of class switching, somatic mutation, and affinity selection within GC where antibodies of the highest affinity are generated and selected. The formation of GC requires T_{FH} which also contribute to B cell differentiation into plasma and memory cells (Crotty, 2011).

The combined blockade of PD-L1 and LAG-3 increased numbers of $CD4^+$ T_{FH} and GC B cells along with higher antibody titers which contributed to better control of blood stage of malaria (Butler et al., 2012). In contrast, PD-1 KO mice showed no significant improvement in numbers of germinal center B cells, plasma cells or antibody titers (Horne-Debets et al., 2013). The absence of any improvement in B cell function in PD-1KO mice could be explained by an absence of improvement in T_{FH} numbers or inhibitory signals to LAG-3 expressed on B cells (Kisielow et al., 2005).

EXHAUSTION OF $CD8^+$ T CELLS DURING MALARIA

PD-1-mediated cellular exhaustion has been best associated with exhaustion of $CD8^+$ T cells. However, as described earlier, a role for $CD8^+$ T cells in the clearance of blood-stage malaria is not widely acknowledged although their role in pathogenesis of cerebral malaria and damage to splenic architecture (Beattie et al., 2006) are known. Critically, PD-1 was recently shown to mediate a 95% loss in the numbers and functional capacity of parasite-specific $CD8^+$ T cells during the acute phase of malaria, which exacerbated the infection leading to chronic malaria (Horne-Debets et al., 2013). This study examined the progression of chronic malaria in PD-1 KO mice compared to WT where 100% of mice develop chronic infections. Interestingly, <30% of the PD-1 KO mice developed chronic infections, and parasitemia levels in these mice were >100-fold lower than those in the WT mice. However, depletion of $CD8^+$ T cells in PD-1 KO mice, increased peak parasitemia by 2-fold and 100% of the PD-1 KO mice developed chronic malaria (Horne-Debets et al., 2013). Overall, PD-1-mediated 80% reduction in numbers of tetramer$^+$$CD8^+$$CD62L^-$ T cells and 95% reduction

in capacity of $CD8^+$ cells to proliferate in response to parasites, during the chronic phase of malaria (Horne-Debets et al., 2013). Of particular note is that even though PD-1 KO mice had more functional $CD4^+$ T cells than WT mice and similar titers of parasite-specific antibodies, they still developed chronic malaria if $CD8^+$ T cells were depleted. Finally, PD-1 KO mice had more granzyme B-expressing $CD8^+$ T cells than WT mice suggesting that cytotoxic-killing of infected cells was involved. These observations highlight the crucial role of $CD8^+$ T cells in protection against chronic malaria. In contrast, a previous study had found blockade of PD-L1 augmented experimental cerebral malaria which is mediated by pathogenic $CD8^+$ T cells (Hafalla et al., 2012), indicating the pathway protects against cerebral malaria. The clinical significance of these findings are highlighted by studies in Kenya which found human $CD8^+$ T cells from individuals infected with malaria, express PD-1 (Illingworth et al., 2013). Thus the role of $CD8^+$ T cells requires particular consideration as it may explain why despite years of exposure to intense *Pf* transmission there was no evidence of acquired, sterile immunity (Tran et al., 2013). It may be that antibodies and $CD4^+$ T cells provide protection against symptomatic malaria but $CD8^+$ T cells are required for sterile immunity. Thus with PD-1 mediated exhaustion of $CD8^+$ T cells, sterile immunity is never acquired as recently reported (Tran et al., 2013).

CONCLUSION

Antibodies and $CD4^+$ T cells are known to protect against blood-stage *Plasmodium* spp. infections. However, there is a growing body of evidence that show that $CD8^+$ T cells have a role in protection. Furthermore, there is also increasing evidence that lymphocyte exhaustion mediates loss of protection by $CD4^+$ and $CD8^+$ T cells. Finally, studies have shown MBC numbers (Weiss et al., 2010) and antibody (Crompton et al., 2010) titers specific for the parasite decline following malarial infections. Accordingly, the likelihood of making a malaria vaccine is declining. The new frontier may be the development of therapeutics to block *Plasmodium*-mediated pathogenesis.

ACKNOWLEDGMENTS

The authors acknowledge the support of The NH&MRC (Australia) and Queensland Tropical Health Alliance. Michelle N. Wykes was supported by an ARC Future Fellowship. Joshua M. Horne-Debets is supported by an Australian Post-Graduate Award through the University of Queensland, School of Medicine.

REFERENCES

Agata, Y., Kawasaki, A., Nishimura, H., Ishida, Y., Tsubata, T., Yagita, H., et al. (1996). Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. *Int. Immunol.* 8, 765–772. doi: 10.1093/intimm/8.5.765

Alonso, P. L., Sacarlal, J., Aponte, J. J., Leach, A., Macete, E., Aide, P., et al. (2005). Duration of protection with RTS,S/AS02A malaria vaccine in prevention of *Plasmodium falciparum* disease in Mozambican children: single-blind extended follow-up of a randomised controlled trial. *Lancet* 366, 2012–2018. doi: 10.1016/S0140-6736(05)67669-6

Beattie, L., Engwerda, C. R., Wykes, M., and Good, M. F. (2006). CD8+ T lymphocyte-mediated loss of marginal metallophilic macrophages following infection with *Plasmodium chabaudi chabaudi* AS. *J. Immunol.* 177, 2518–2526. doi: 10.4049/jimmunol.177.4.2518

Bhadra, R., Gigley, J. P., Weiss, L. M., and Khan, I. A. (2011). Control of *Toxoplasma* reactivation by rescue of dysfunctional CD8+ T-cell response via PD-1-PDL-1 blockade. *Proc. Natl. Acad. Sci. U. S. A.* 108, 9196–9201. doi: 10.1073/pnas.1015298108

Butler, N. S., Moebius, J., Pewe, L. L., Traore, B., Doumbo, O. K., Tygrett, L. T., et al. (2012). Therapeutic blockade of PD-L1 and LAG-3 rapidly clears established blood-stage *Plasmodium* infection. *Nat. Immunol.* 13, 188–195. doi: 10.1038/ni.2180

Butte, M. J., Keir, M. E., Phamduy, T. B., Sharpe, A. H., and Freeman, G. J. (2007). Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses. *Immunity* 27, 111–122. doi: 10.1016/j.immuni.2007.05.016

Chandele, A., Mukerjee, P., Das, G., Ahmed, R., and Chauhan, V. S. (2011). Phenotypic and functional profiling of malaria-induced CD8 and CD4 T cells during blood-stage infection with *Plasmodium yoelii*. *Immunology* 132, 273–286. doi: 10.1111/j.1365-2567.2010.03363.x

Chemnitz, J. M., Parry, R. V., Nichols, K. E., June, C. H., and Riley, J. L. (2004). SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation. *J. Immunol.* 173, 945–954. doi: 10.4049/jimmunol.173.2.945

Cohen, S., McGregor, I., and Carrington, S. (1961). Gamma-globulin and acquired immunity to human malaria. *Nature* 192, 733–737. doi: 10.1038/192733a0

Couper, K. N., Blount, D. G., Wilson, M. S., Hafalla, J. C., Belkaid, Y., Kamanaka, M., et al. (2008). IL-10 from CD4CD25Foxp3CD127 adaptive regulatory T cells modulates parasite clearance and pathology during malaria infection. *PLoS Pathog.* 4:e1000004. doi: 10.1371/journal.ppat.1000004

Crompton, P. D., Kayala, M. A., Traore, B., Kayentao, K., Ongoiba, A., Weiss, G. E., et al. (2010). A prospective analysis of the Ab response to *Plasmodium falciparum* before and after a malaria season by protein microarray. *Proc. Natl. Acad. Sci. U.S.A.* 107, 6958–6963. doi: 10.1073/pnas.1001323107

Crotty, S. (2011). Follicular helper CD4 T cells (TFH). *Annu. Rev. Immunol.* 29, 621–663. doi: 10.1146/annurev-immunol-031210-101400

Day, C. L., Kaufmann, D. E., Kiepiela, P., Brown, J. A., Moodley, E. S., Reddy, S., et al. (2006). PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* 443, 350–354. doi: 10.1038/nature05115

Dong, H., Zhu, G., Tamada, K., and Chen, L. (1999). B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. *Nat. Med.* 5, 1365–1369. doi: 10.1038/70932

Freeman, G. J., Long, A. J., Iwai, Y., Bourque, K., Chernova, T., Nishimura, H., et al. (2000). Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J. Exp. Med.* 192, 1027–1034. doi: 10.1084/jem.192.7.1027

Freeman, G. J., Wherry, E. J., Ahmed, R., and Sharpe, A. H. (2006). Reinvigorating exhausted HIV-specific T cells via PD-1-PD-1 ligand blockade. *J. Exp. Med.* 203, 2223–2227. doi: 10.1084/jem.20061800

Hafalla, J. C., Claser, C., Couper, K. N., Grau, G. E., Renia, L., de Souza, J. B., et al. (2012). The CTLA-4 and PD-1/PD-L1 inhibitory pathways independently regulate host resistance to *Plasmodium*-induced acute immune pathology. *PLoS Pathog.* 8:e1002504. doi: 10.1371/journal.ppat.1002504

Hafalla, J. C., Cockburn, I. A., and Zavala, F. (2006). Protective and pathogenic roles of CD8+ T cells during malaria infection. *Parasite Immunol.* 28, 15–24. doi: 10.1111/j.1365-3024.2006.00777.x

Hirunpetcharat, C., and Good, M. F. (1998). Deletion of *Plasmodium berghei*-specific CD4+ T cells adoptively transferred into recipient mice after challenge with homologous parasite. *Proc. Natl. Acad. Sci. U.S.A.* 95, 1715–1720. doi: 10.1073/pnas.95.4.1715

Horne-Debets, J. M., Faleiro, R., Karunarathne, D. S., Liu, X. Q., Lineburg, K. E., Poh, C. M., et al. (2013). PD-1 dependent exhaustion of CD8(+) T cells drives chronic malaria. *Cell Rep.* 5, 1204–1213. doi: 10.1016/j.celrep.2013.11.002

Illingworth, J., Butler, N. S., Roetynck, S., Mwacharo, J., Pierce, S. K., Bejon, P., et al. (2013). Chronic exposure to *Plasmodium falciparum* is associated with phenotypic evidence of B and T cell exhaustion. *J. Immunol.* 190, 1038–1047. doi: 10.4049/jimmunol.1202438

Imai, T., Ishida, H., Suzue, K., Hirai, M., Taniguchi, T., Okada, H., et al. (2013). CD8(+) T cell activation by murine erythroblasts infected with malaria parasites. *Sci. Rep.* 3:1572. doi: 10.1038/srep01572

Imai, T., Shen, J., Chou, B., Duan, X., Tu, L., Tetsutani, K., et al. (2010). Involvement of CD8+ T cells in protective immunity against murine blood-stage infection with *Plasmodium yoelii* 17XL strain. *Eur. J. Immunol.* 40, 1053–1061. doi: 10.1002/eji.200939525

Iwai, Y., Terawaki, S., Ikegawa, M., Okazaki, T., and Honjo T. (2003). PD-1 inhibits antiviral immunity at the effector phase in the liver. *J. Exp. Med.* 198, 39–50. doi: 10.1084/jem.20022235

Jacobs, P., Radzioch, D., and Stevenson, M. M. (1996). A Th1-associated increase in tumor necrosis factor alpha expression in the spleen correlates with resistance to blood-stage malaria in mice. *Infect. Immun.* 64, 535–541.

Joshi, T., Rodriguez, S., Perovic, V., Cockburn, I. A., and Stager S. (2009). B7-H1 blockade increases survival of dysfunctional CD8(+) T cells and confers protection against *Leishmania donovani* infections. *PLoS Pathog.* 5:e1000431. doi: 10.1371/journal.ppat.1000431

Keir, M. E., Butte, M. J., Freeman, G. J., and Sharpe, A. H. (2008). PD-1 and its ligands in tolerance and immunity. *Annu. Rev. Immunol.* 26, 677–704. doi: 10.1146/annurev.immunol.26.021607.090331

Kisielow, M., Kisielow, J., Capoferri-Sollami, G., and Karjalainen, K. (2005). Expression of lymphocyte activation gene 3 (LAG-3) on B cells is induced by T cells. *Eur. J. Immunol.* 35, 2081–2088. doi: 10.1002/eji.200526090

Langhorne, J., Ndungu, F. M., Sponaas, A. M., and Marsh, K. (2008). Immunity to malaria: more questions than answers. *Nat. Immunol.* 9, 725–732. doi: 10.1038/ni.f.205

Latchman, Y., Wood, C. R., Chernova, T., Chaudhary, D., Borde, M., Chernova, I., et al. (2001). PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat. Immunol.* 2, 261–268. doi: 10.1038/85330

Liang, S. C., Greenwald, R. J., Latchman, Y. E., Rosas, L., Satoskar, A., Freeman, G. J., et al. (2006). PD-L1 and PD-L2 have distinct roles in regulating host immunity to cutaneous *leishmaniasis*. *Eur. J. Immunol.* 36, 58–64. doi: 10.1002/eji.200535458

Linterman, M. A., Pierson, W., Lee, S. K., Kallies, A., Kawamoto, S., Rayner, T. F., et al. (2011). Foxp3+ follicular regulatory T cells control the germinal center response. *Nat. Med.* 17, 975–982. doi: 10.1038/nm.2425

Liu, X. Q., Stacey, K. J., Horne-Debets, J. M., Cridland, J. A., Fischer, K., Narum D., et al. (2012). Malaria infection alters the expression of B-cell activating factor resulting in diminished memory antibody responses and survival. *Eur. J. Immunol.* 42, 3291–3301. doi: 10.1002/eji.201242689

Lukens, J. R., Cruise, M. W., Lassen, M. G., and Hahn Y. S. (2008). Blockade of PD-1/B7-H1 interaction restores effector CD8+ T cell responses in a hepatitis C virus core murine model. *J. Immunol.* 180, 4875–4884. doi: 10.4049/jimmunol.180.7.4875

McCall, M. B., Hopman, J., Daou, M., Maiga, B., Dara, V., Ploemen, I., et al. (2010). Early interferon-gamma response against *Plasmodium falciparum* correlates with interethnic differences in susceptibility to parasitemia between sympatric Fulani and Dogon in Mali. *J. Infect. Dis.* 201, 142–152. doi: 10.1086/648596

McCall, M. B., and Sauerwein, R. W. (2010). Interferon-gamma–central mediator of protective immune responses against the pre-erythrocytic and blood stage of malaria. *J. Leukoc. Biol.* 88, 1131–1143. doi: 10.1189/jlb.0310137

Minigo, G., Woodberry, T., Piera, K. A., Salwati, E., Tjitra, E., Kenangalem, E., et al. (2009). Parasite-dependent expansion of TNF receptor II-positive regulatory T cells with enhanced suppressive activity in adults with severe malaria. *PLoS Pathog.* 5:e1000402. doi: 10.1371/journal.ppat.1000402

Muxel, S. M., Freitas do Rosario, A. P., Zago, C. A., Castillo-Mendez, S. I., Sardinha, L. R., Rodriguez-Malaga, S. M., et al. (2011). The spleen CD4+ T cell response to blood-stage *Plasmodium chabaudi* malaria develops in two phases characterized by different properties. *PLoS ONE* 6:e22434. doi: 10.1371/journal.pone.0022434

Ogutu, B. R., Apollo, O. J., McKinney, D., Okoth, W., Siangla, J., Dubovsky, F., et al. (2009). Blood stage malaria vaccine eliciting high antigen-specific antibody concentrations confers no protection to young children in Western Kenya. *PLoS ONE* 4:e4708. doi: 10.1371/journal.pone.0004708

Olotu, A., Fegan, G., Wambua, J., Nyangweso, G., Awuondo, K. O., Leach, A., et al. (2013). Four-year efficacy of RTS,S/AS01E and its interaction with malaria exposure. *N. Engl. J. Med.* 368, 1111–1120. doi: 10.1056/NEJMoa1207564

Podoba, J. E., and Stevenson, M. M. (1991). CD4+ and CD8+ T lymphocytes both contribute to acquired immunity to blood-stage *Plasmodium chabaudi* AS. *Infect. Immun.* 59, 51–58.

Scholzen, A., and Sauerwein, R. W. (2013). How malaria modulates memory: activation and dysregulation of B cells in *Plasmodium* infection. *Trends Parasitol.* 29, 252–262. doi: 10.1016/j.pt.2013.03.002

Schwartz, L., Brown, G. V., Genton, B., and Moorthy, V. S. (2012) A review of malaria vaccine clinical projects based on the WHO rainbow table. *Malar. J.* 11, 11. doi: 10.1186/1475-2875-11-11

Sharpe, A. H., Wherry, E. J., Ahmed, R., and Freeman, G. J. (2007). The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. *Nat. Immunol.* 8, 239–245. doi: 10.1038/ni1443

Stephens, R., and Langhorne, J. (2010). Effector memory Th1 CD4 T cells are maintained in a mouse model of chronic malaria. *PLoS Pathog.* 6:e1001208. doi: 10.1371/journal.ppat.1001208

Tran, T. M., Li, S., Doumbo, S., Doumtabe, D., Huang, C. Y., Dia, S., et al. (2013). An intensive longitudinal cohort study of Malian children and adults reveals no evidence of acquired immunity to *Plasmodium falciparum* infection. *Clin. Infect. Dis.* 57, 40–47. doi: 10.1093/cid/cit174

Tseng, S. Y., Otsuji, M., Gorski, K., Huang, X., Slansky, J. E., Pai, S. I., et al. (2001). B7-DC, a new dendritic cell molecule with potent costimulatory properties for T cells. *J. Exp. Med.* 193, 839–846. doi: 10.1084/jem.193.7.839

Urbani, S., Amadei, B., Tola, D., Massari, M., Schivazappa, S., Missale, G., et al. (2006). PD-1 expression in acute hepatitis C virus (HCV) infection is associated with HCV-specific CD8 exhaustion. *J. Virol.* 80, 11398–11403. doi: 10.1128/JVI.01177-06

Urbani, S., Amadei, B., Tola, D., Pedrazzi, G., Sacchelli, L., Cavallo, M. C., et al. (2008). Restoration of HCV-specific T cell functions by PD-1/PD-L1 blockade in HCV infection: effect of viremia levels and antiviral treatment. *J. Hepatol.* 48, 548–558. doi: 10.1016/j.jhep.2007.12.014

von der Weid, T., Honarvar, N., and Langhorne, J. (1996). Gene-targeted mice lacking B cells are unable to eliminate a blood stage malaria infection. *J. Immunol.* 156, 2510–2516.

Walther, M., Tongren, J. E., Andrews, L., Korbel, D., King, E., Fletcher H., et al. (2005). Upregulation of TGF-beta, FOXP3, and CD4+CD25+ regulatory T cells correlates with more rapid parasite growth in human malaria infection. *Immunity* 23, 287–296. doi: 10.1016/j.immuni.2005.08.006

Weiss, G. E., Traore, B., Kayentao, K., Ongoiba, A., Doumbo, S., Doumtabe, D., et al. (2010). The *Plasmodium falciparum*-specific human memory B cell compartment expands gradually with repeated malaria infections. *PLoS Pathog.* 6:e1000912. doi: 10.1371/journal.ppat.1000912

Wherry, E. J. (2011). T cell exhaustion. *Nat. Immunol.* 12, 492–499. doi: 10.1038/ni.2035

Wipasa, J., Xu, H., Stowers, A., and Good, M. F. (2001). Apoptotic deletion of Th cells specific for the 19-kDa carboxyl-terminal fragment of merozoite surface protein 1 during malaria infection. *J. Immunol.* 167, 3903–3909. doi: 10.4049/jimmunol.167.7.3903

Wykes, M. N., Zhou, Y. H., Liu, X. Q., and Good, M. F. (2005). *Plasmodium yoelii* can ablate vaccine-induced long-term protection in mice. *J. Immunol.* 175, 2510–2516. doi: 10.4049/jimmunol.175.4.2510

Xu, H., Wipasa, J., Yan, H., Zeng, M., Makobongo, M. O., Finkelman, F. D., et al. (2002). The mechanism and significance of deletion of parasite-specific CD4(+) T cells in malaria infection. *J. Exp. Med.* 195, 881–892. doi: 10.1084/jem.20011174

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.

Received: 28 March 2014; accepted: 07 May 2014; published online: 27 May 2014.
Citation: Wykes MN, Horne-Debets JM, Leow C-Y and Karunarathne DS (2014) Malaria drives T cells to exhaustion. Front. Microbiol. ***5****:249. doi: 10.3389/fmicb.2014.00249*
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Microbiology.
Copyright © 2014 Wykes, Horne-Debets, Leow and Karunarathne. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 16 July 2014
doi: 10.3389/fmicb.2014.00365

The dichotomy of pathogens and allergens in vaccination approaches

***Fiona J. Baird*[1,2] ** and *Andreas L. Lopata*[1,2]**

[1] *Centre for Biodiscovery & Molecular Development of Therapeutics, Centre for Biosecurity in Tropical Infectious Diseases, Australian Institute of Tropical Health & Medicine, James Cook University, Townsville, QLD, Australia*
[2] *Molecular Immunology Group, School of Pharmacy and Molecular Biology, James Cook University, Townsville, QLD, Australia*

Edited by:
Shuo Li, Burnet Institute, Australia

Reviewed by:
Koubun Yasuda, Hyogo College of Medicine, Japan
Hari Mohan Saxena, Guru Angad Dev Veterinary and Animal Sciences University, India

****Correspondence:***
Fiona J. Baird, Molecular Immunology Group, School of Pharmacy and Molecular Biology, James Cook University, 1 James Cook Drive, Townsville, QLD 4811, Australia
e-mail: fiona.baird@jcu.edu.au

Traditional prophylactic vaccination to prevent illness is the primary objective of many research activities worldwide. The golden age of vaccination began with an approach called variolation in ancient China and the evolution of vaccines still continues today with modern developments such as the production of Gardasil™ against HPV and cervical cancer. The historical aspect of how different forms of vaccination have changed the face of medicine and communities is important as it dictates our future approaches on both a local and global scale. From the eradication of smallpox to the use of an experimental vaccine to save a species, this review will explore these successes in infectious disease vaccination and also discuss a few significant failures which have hampered our efforts to eradicate certain diseases. The second part of the review will explore designing a prophylactic vaccine for the growing global health concern that is allergy. Allergies are an emerging global health burden. Of particular concern is the rise of food allergies in developed countries where 1 in 10 children is currently affected. The formation of an allergic response results from the recognition of a foreign component by our immune system that is usually encountered on a regular basis. This may be a dust-mite or a prawn but this inappropriate immune response can result in a life-time of food avoidance and lifestyle restrictions. These foreign components are very similar to antigens derived from infectious pathogens. The question arises: should the allergy community be focussing on protective measures rather than ongoing therapeutic interventions to deal with these chronic inflammatory conditions? We will explore the difficulties and benefits of prophylactic vaccination against various allergens by means of genetic technology that will dictate how vaccination against allergens could be utilized in the near future.

Keywords: vaccine, vaccine history, allergy, food allergy, allergen, prophylactic vaccination, vaccine challenges

INTRODUCTION

Globally, the burden of disease and infection is diverse and inescapable. It is a shared affliction for humanity and one that is constantly moderated by better hygiene, enhanced education, and improved vaccines and therapeutic interventions. In terms of healthcare, it is always more beneficial to prevent a disease or infection from occurring than to treat and cure it. The development of vaccines is dependent on the knowledge of: what pathogen causes the disease; how it establishes itself in the host; how the host's innate and cell-mediated immunity responds to pathogens; and how it maintains ongoing protection after the disease using antibodies. Whilst there are many successful vaccines currently available, there are still no registered vaccines for some globally prevalent infectious diseases such as malaria and human immunodeficiency virus (HIV). Although we have made enormous progress in medicine over the last 300 years since the practice of vaccination first began, there are still diseases that are killing millions of people globally which desperately require a vaccine. Furthermore, there is a multitude of autoimmune conditions such as food allergies which may benefit from a traditional prophylactic vaccination approach. This review will explore the progression of traditional vaccines from empirical vaccines to the more recent novel vaccines and how recent advancements could change the field of allergy research.

A BRIEF HISTORY OF VACCINATION

The first crude attempt at disease control was the procedure of variolation where the inoculated person stood a good chance at surviving both the procedure and later exposure to the pathogen. Variolation consisted of directly transferring the infection from a sick person to a healthy person, through direct contact or by infectious matter such as pus, saliva or blood (Dinc and Ulman, 2007). This form of vaccination is believed to have begun in either ancient China or India, but was only brought to the UK by the wife of a British diplomat, Lady Wortley-Montagu in 1721 (Dinc and Ulman, 2007).

Lady Wortley-Montagu had observed that harem girls in Constantinople had pox-free faces which were attributed to them being variolated; hence she had her son variolated in Istanbul in 1718 to save him from experiencing what she had as a young adult – smallpox. Later she also variolated her daughter in London; however, this was only after she had confirmed that it did not

result in death or disease in eleven orphans and six convicted murderers from Newgate Prison (Dinc and Ulman, 2007). Lady Wortley-Montagu was so impressed that she implored her surgeon, Dr. Charles Maitland, to learn the technique and demonstrate it to the Royal British Court (Stewart and Devlin, 2006). After this demonstration, 200 upper-class members of British society, including members of the royal family, underwent the procedure, and in 1729 a further 897 more inoculations were performed with only 17 deaths post-procedure which is infinitely fewer than smallpox mortality at the time (Dinc and Ulman, 2007).

Even though Lady Wortley-Montagu was severely criticized for bringing the procedure to Britain, it was slowly implemented throughout the UK over the following years, and in 1757 a young boy named Edward Jenner would be variolated against smallpox (Dinc and Ulman, 2007). This ultimately saved countless lives from smallpox; the most devastating disease of the time. However, there were two issues with variolation: (1) it could impair the patient or even kill them if the dosage was incorrect or if they were not physically fit enough to withstand the infection, and (2) whilst the patient would be protected from further infections, they would become contagious during the active infection (Bazin, 2003).

Although variolation was popular in the cities, in the English countryside there were many rumors that if you contracted cowpox you were protected from the deadly smallpox. Subsequently, a farmer named Benjamin Jesty in Yetminster, England, inoculated his wife and two sons with cowpox in the hope of surviving a smallpox epidemic (Pead, 2003, 2006). Even though his wife became very ill, she and the whole family survived and went on to survive many smallpox epidemics in the area. This transpired a full 20 years before scientist Jenner began his experiment with a boy called James Phipps (Pead, 2003); however, Jesty was recognized for his contribution in 1805 by a published statement and a portrait commissioned by the Original Vaccine Pock Institution, London (Pead, 2006). It is believed that Jenner was also aware of the rumors of cowpox protecting against smallpox, and that this was the inspiration for his experiment, resulting in him being the first to document that a person infected with cowpox would survive subsequent exposure to smallpox (Stewart and Devlin, 2006). This technique evolved into using cow inoculums as the vaccine, which did provide immunity to smallpox although not to the same degree as natural disease or variolation. This discovery was heralded as the new age of vaccines and instigated new research into other common diseases.

A couple of centuries later, medicine would again make another considerable leap forward with the separate works of scientists Louis Pasteur and Robert Koch, and the publication of germ theory. The most famous of these works would be Pasteur's and his attenuation of the bacteria *Pasteurella multocida,* which causes fowl cholera, by exposing the cultures to air and room temperature for extended periods of time (Bordenave, 2003). He demonstrated that whilst the bacteria were avirulent, they provided full protection from the virulent strain of the bacteria, which was a revolutionary idea at the time. Pasteur also went on to attenuate the rabies virus by passage through rabbits (Bazin, 2003). Koch, on the other hand, would discover the bacterial agents of anthrax, tuberculosis and cholera whilst also compiling postulates with fellow scientist Jacob Henle that would transform the world of microbiology (Kaufmann and Schaible, 2005). All of these discoveries led to the development of immunology and non-empirical vaccines.

The first whole cell vaccine was produced by Salmon and Smith in 1886 and was based on a *Salmonella* strain that was killed by heat and injected into pigeons to provide immunity (Bazin, 2003). Around the same time, others were investigating bacterial components and methods to purify them. This was the beginning of traditional vaccine methodology. During this era there were many great innovations in the field of immunology and vaccinology, such as the discovery of toxins and the consequent inactivation of toxins by heat and formalin, killed vaccines, adjuvants, sub-unit or acellular vaccines, tissue culture and live attenuated vaccines. With the establishment of molecular biology and genetic engineering in the late 1950s, a new era began where vaccine development no longer needed to be empirical and bacterial components could be produced artificially or even *in vivo* by unrelated vectors.

VACCINES IN THE MODERN ERA

What makes a good vaccine?

The traditional definition of a vaccine is one that protects against a particular (or group of) infectious agent(s); however, these days there are many vaccines that could be designated as therapeutic agents against diseases such as cancer (Bergman et al., 2006), although the goal is still to prevent illness. In this review we will focus on prophylactic vaccines. The global market for vaccines is estimated to be around US$8 billion per year whilst the cost to develop each vaccine from concept to commercialism is around US$300–800 million (Plotkin, 2005). The reason for the high expenditure is that each vaccine has to be rigorously tested before commercial release and the average time it takes to fully develop a vaccine is between 15 and 20 years (Arntzen et al., 2005). A successful vaccine is measured by its effectiveness, its spectrum of protection, the duration of immunity and the strength of immunological memory that it induces. Secondary considerations of a good vaccine are its stability, ease of administration and storage, achievable mass production and its toxicity.

Biotechnology is a rapidly developing area which allows continued improvement into the exploration of antigens suitability as vaccine candidates. Choosing the right antigen is a core decision in the development of a vaccine candidate as some antigens that are immunogenic *in vivo* may not elicit long term protection. The same antigen may also vary in structure and sequence between strains, limiting its usefulness. Some antigens are also hard to express and purify on a large scale which is required for mass production (Mora et al., 2003). This is where novel vaccine methodology hopes to improve how vaccines are made and administered; this will be examined subsequently.

Routes of administration

The oldest technique for vaccination is that of subcutaneous delivery via scarification and one of the newest techniques is

intramuscular injection (Bazin, 2003). Whilst the intramuscular route of vaccination is quite standard today in developed countries, it is an inconvenient method of application as it requires sterile needles and syringes, and usually a medical physician to administer it. This is the major drawback of vaccines that rely on intramuscular injections to be effective. In one study, a viral vectored vaccine was found to elicit stronger systemic and detectable mucosal responses via a single intramuscular injection than if it was applied via the oral route. The oral route proved to stimulate suboptimal T-cell responses and did induce a higher level of mucosal antibody than the intramuscular route (Lin et al., 2007).

Nasal and oral administration routes of a vaccine are more desirable than intramuscular as they are non-invasive, painless, not required to be sterile and do not require a physician for administration. This final point is most important as it is one of the reasons that third world countries have the lowest level of immunizations in the world (Costantino et al., 2007). Nasal immunization would place the vaccine in contact with the large surface of the nasal mucosa which consists of the nasal-associated lymphoid tissue (NALT), which can lead to both humoral and cellular immune responses (Zuercher et al., 2002; Costantino et al., 2007). The most well-known nasal vaccine is FluMist®; a live cold-adapted influenza virus. It can be given as one or two doses from a syringe sprayer, is licensed for use in the USA for persons aged 5–50 and has shown high efficacy from its inception (Plotkin, 2005; Costantino et al., 2007). However, one of the detriments of a nasal vaccine is that an unpleasant taste and nasal discomfort can occur often discouraging repeated use (Atmar et al., 2007).

Oral administration is a practical method of application if it can be achieved without diminishing the effectiveness of the vaccine, and immunity can be achieved with a single dose. The objective of oral vaccines is to mimic a natural infection and provide mucosal immunity. Orally delivered vaccines can induce suboptimal T-cell responses with high levels of mucosal antibody than the intramuscular route; however, the vaccine must be very stable as it will have to survive the acidic environment of the stomach before it reaches the M cells of the intestinal wall where it can be processed by antigen-presenting cells (APCs; Lin et al., 2007).

Adjuvants

Adjuvants are defined as compounds that influence the immune system into mounting a Th1 or Th2 response and whilst doing so, greatly enhances the magnitude of immune response against the antigen (Marciani, 2003). They are an important aspect of vaccines due to their tendency to make an ineffective antigen become effective. It is vital that adjuvants have the following properties: a non-toxic nature or have minimal toxicity at the dosage to elicit effective adjuvanticity; able to stimulate a strong humoral and/or T-cell immune response; provide good immunological memory or long-term protection; not induce autoimmunity; are non-mutagenic, non-carcinogenic, non-teratogenic, and non-pyrogenic; and be stable under broad ranges of storage time, temperature and pH levels (Marciani, 2003). The most popular adjuvants are aluminum-based and were first described and published in 1926 (Glenny et al., 1926). There are three adjuvants that are currently licensed for human use: aluminum hydroxide, also known as alum (Davies and Flower, 2007); monophosphoryl lipid A (MPL) and AS03 consisting of D,L-alpha-tocopherol (vitamin E), squalene and polysorbate 80 (U.S. Food and Drug Administration, 2014). It is well established that aluminum adjuvants stimulate the production of IgE and a Th2 immune profile, yet for some diseases this would not be adequate protection against pathogens as a Th1 response would be required (Lindblad, 2004). MPL and AS03 have demonstrated clinical efficacy when used in a HPV and influenza vaccine, respectively (Mohan et al., 2013).

Adjuvants are used to lengthen the dissemination time of the antigen from the site of injection which allows the antigen to be released over a prolonged period improving the effectiveness of the vaccine. This feature is called the depot effect and is traditionally associated with aluminum-based adjuvants (Lindblad, 2004; Mohan et al., 2013); however, recently the depot effect has been questioned as reducing the dissemination time of the antigen does not alter the magnitude of the immune response (Hutchison et al., 2012) along with other evidence suggesting other modes of action (reviewed in De De Gregorio et al., 2013). Other methods in which adjuvants improve the immune response are to form complexes with the antigen and to target the vaccine towards specific receptors. For example, the use of mannose in the adjuvant is recognized by pattern recognition receptors (PRRs) that initiate endocytosis and antigen processing (Stahl and Ezekowitz, 1998). The use of pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) and CpG-DNA, and of synthetic low-MW imidazoquinolines in adjuvants, all trigger innate immune responses that lead to a Th1 or Th2 response in the vaccinated person (Marciani, 2003). Other adjuvants consist of cytokines (Cheng et al., 2007) and glycolipids (Singh et al., 1999; Ko et al., 2005) and other immunomodulators (Morrow et al., 2004) that bind to highly specific receptors on T cells which activate them.

TRADITIONAL VACCINE METHODOLOGY

The early development of vaccines focused on using killed organisms, inactivated toxins or modified organisms, but currently there are many different approaches to vaccine development, which will be examined subsequently. As these approaches were empirical in design, these types of vaccines, whilst being successful, are now viewed as being traditional vaccines. These can be divided into three different types: (a) killed vaccines; (b) attenuated vaccines; and (c) sub-unit vaccines.

Killed vaccines

A killed or "inactivated" vaccine is developed by the pathogen being grown and then being made inactive by means of heat, chemical or radiation treatment and was the basis of most vaccines until the 1980s. This results in the pathogen being unable to cause disease whilst providing the immune system with stimulation via its normal antigenic epitopes on its cell surface. One major disadvantage of this approach is that, whilst these vaccines are immunogenic, they do not replicate *in vivo* infectivity limiting the spectrum of the immunity acquired as the agent is incapable of

going through its normal antigenic variation over the course of the infection. This results in decreased immunity and a requirement of booster shots to maintain immunity (Moylett and Hanson, 2003). Another disadvantage of this vaccine type is that during the inactivation process the antigenic epitopes can be modified resulting in a less efficacious vaccine (Tano et al., 2007). Despite these limitations, killed vaccines are commonly used today with the typhoid, Salk poliomyelitis, and seasonal influenza vaccines still being administered (Bazin, 2003; Palese, 2006).

Attenuated vaccines

Among the more efficacious of the traditional vaccines are the attenuated ones. In this case, a pathogen is subjected to altered growth conditions, is passage through a host or is genetically modified to eliminate its virulence, yet retaining its ability to replicate albeit at a greatly reduced rate. These vaccines are more successful at eliciting a robust lifelong immunity than other traditional vaccines. This can be attributed to their ability to cause an asymptomatic infection which stimulates both the humoral and cellular branches of the immune system.

However, this ability to replicate carries the greatest risk as the vaccine can persist in immune-compromised persons or the elderly due to limited immune responses. A benefit to these vaccines is they express their own immunogenic antigens which stimulate the immune system strongly thereby negating the need for an adjuvant to be used (Loessner et al., 2008). The most commonly used attenuated vaccine is the MMR vaccine which protects children worldwide against measles, mumps and rubella and with subsequent boosters provides lifelong immunity (Vandermeulen et al., 2007). Attenuated vaccines have further progressed into carrier vaccines where they can deliver heterologous antigens (Bachtiar et al., 2003; Lotter et al., 2008; Schoen et al., 2008). For live carrier vaccines that deliver multiple heterologous antigens, there is a risk that the host immune system will dampen the immune response to the heterologous antigens by misdirecting the immune response against the carrier (Berzofsky et al., 2004). However, if the immunity induced is cell-mediated the response can be enhanced by pre-existing immunity to the carrier strain (Saxena et al., 2013).

Subunit vaccines

Traditionally, it was thought that the only way to protect against a disease was to use the whole organism to vaccinate the host. However, it was elucidated that specific parts of the organisms, when purified or isolated, demonstrated immunogenic properties. These components could be the capsule, the flagella or even an outer membrane protein of the cell wall. These types of vaccines are known as subunit vaccines or acellular vaccines. These vaccines are not able to cause the disease and in comparison to whole cell killed vaccines they are not as efficacious. This is both an advantage as they are safe for immune-compromised patients and a disadvantage as they do not elicit long-term immunity and will often require multiple vaccinations to maintain immunity (Schmitt et al., 2008). An advantage of this type of vaccine is that it can be engineered to protect against various strains of the organism. An example of a successful subunit vaccine is the *Haemophilus influenzae* type b (HiB) conjugate vaccine which consists of a polysaccharide-protein conjugate. This vaccine has eliminated or significantly reduced this disease in children in regions of South America (Ribeiro et al., 2007; Franco-Paredes et al., 2008) and Africa (Adegbola et al., 2005; Muganga et al., 2007) where it was once endemic. In the UK, the success of this vaccination program was compromised by a highly publicized paper (which has now been retracted) that linked autism to early childhood vaccination which lead to a rise in HiB infections as parents chose not to vaccinate; however, subsequent booster campaigns by the NHS has seen a reduction in infection rates again (Ladhani et al., 2008). A recent meta-analysis covering studies involving over 1.2 million children has discredited any link between vaccinations or vaccine components thimerosal or mercury to the development of autism or autism spectrum disorders (Taylor et al., 2014).

DNA VACCINES – A NEXT GENERATION EXAMPLE

There are multiple novel types of vaccines that are currently under development, such as bacterial ghosts (Szostak et al., 1996; Jawale and Lee, 2014) and nanovaccines (Cho et al., 2014). However, one that holds great promise and has had documented successes is DNA vaccines. DNA vaccines differ from traditional vaccines as they do not consist of a protein or a cell component but only the DNA that encodes an immunogenic antigen within a plasmid vector. The plasmid can be administered by injection, gene gun, electroporation, or aerosol delivery, upon which the host's immune cells, usually dendritic cells, will sample the plasmid and express the encoded antigens. These antigens are then degraded by the cell into peptides and presented via MHC class I and class II molecules depending on the mode of administration and the cell type. From this, both antibody and cellular responses can be induced (Forde, 2005).

The first reported use of a plasmid DNA vaccine outside of trial or experimental conditions was in 2003 and was a desperate attempt to save an endangered species from extinction (Bouchie, 2003). The vaccine was for the highly endangered California condors against the lethal West Nile virus. West Nile virus had emerged in New York in 1999 and spread to 41 out of the 50 US states killing birds from 138 species in a matter of years. It was believed that if the virus spread to California, the remaining 200 or so condors would face extinction. The US Centers for Disease Control and Prevention (CDC) expedited the manufacture of an experimental vaccine and permitted the condors to be vaccinated with it (Bouchie, 2003). The DNA vaccine expressed West Nile virus pre-membrane/membrane and envelope proteins. The vaccinated condors were monitored and it was observed that the DNA vaccination stimulated protective immunity in adults, nestlings and newly hatched chicks. Following two intramuscular vaccinations, the condors demonstrated excellent neutralizing antibodies 60 days post-vaccination with a continued increase until approximately 1 year post-vaccination. It was also noted that the birds did not show any unusual behaviors, health changes or side effects post-vaccination (Chang et al., 2007). This vaccine has also demonstrated efficacy in other bird species such as the American robins (*Turdus migratorius*) (Kilpatrick et al., 2010) and the fish crows (*Corvus ossifragus*; Turell et al., 2003). The first two DNA vaccines for veterinary use were granted US approval in

2005 for West Nile virus vaccine for horses and haematopoietic necrosis vaccine for farm-reared Atlantic salmon (Chalmers, 2006). Even though there are no currently approved DNA vaccines for human use, as of May, 2014 there are 128 open trials listed on Clinicaltrials.Gov (2014) that involve DNA-based vaccines and therapies

GLOBAL VACCINE SUCCESS

The global eradication of smallpox is, to date; the most successful vaccination campaign in history. Smallpox has existed for many thousands of years and spread through the world following the migration of humans to new settlements (Barquet and Domingo, 1997). As mentioned previously, Edward Jenner is famously credited with developing a smallpox vaccination using the cow-pox virus (vaccinia virus) and published many observations on both the successful and adverse events (Jenner, 1809) associated with his vaccination protocol. Small pox was an indiscriminate disease that is caused by two virus variants Variola major and Variola minor and was responsible for 300–500 million deaths before its eradication (Theves et al., 2014). The smallpox vaccine that was developed by Jenner produces both neutralizing antibodies and cell mediated responses that are protective against other members of the Orthopoxvirus genus (Barquet and Domingo, 1997). After years of vaccination success but with deaths from smallpox still common, the World Health Assembly, the executive body of the [World Health Organization (WHO), 2013] set a target to eradicate smallpox. This was only achievable as humans are the only reservoir for the virus and the vaccine had demonstrated high efficacy (Fenner et al., 1988). In the late 1960s, the efforts of the WHO were strengthened with more funding and new surveillance protocols.

The last natural occurrence of smallpox occurred in Somalia, where cook Ali Maow Maalin developed the rash on October 26th 1977, but tragically it was not the last global smallpox death [World Health Organization (WHO), 1980]. Medical photographer Janet Parker became the last person to die of smallpox in the world when she was accidently exposed to it in her workplace at the University of Birmingham and unfortunately a lapse in obtaining her booster vaccination led to her being susceptible at the time of exposure (Barquet and Domingo, 1997). Eradication of smallpox was declared on May 8, 1980 by the WHO when the Final Report of Global Commission for Certification of Smallpox Eradication was published [World Health Organization (WHO), 1980]. As of 2014, two depositories of smallpox still exist at the CDC in the USA and the State Research Center of Virology and Biotechnology VECTOR in Koltsovo, Russia. The destruction of these viral stocks has been delayed and debated since the declaration of eradication occurred in, 1980. Discovery of smallpox victims during building excavations often fuels these debates although no viable virus has been recovered from these corpses, so the risk of a modern smallpox outbreak is improbable (Reardon, 2014; Theves et al., 2014). The WHO is again debating the existence of these stocks in May, 2014 (Reardon, 2014).

Another successful vaccine that has been implemented globally is those against poliomyelitis – the Salk, and Sabin vaccines. There are three different poliovirus serotypes and all of them can lead to serious disability in children, even death by acute flaccid paralysis [World Health Organization (WHO), 2014a]. Due to its moderate mortality rates, its long-term severe disability consequences and like smallpox, humans being the only natural reservoir for the virus, the World Health Assembly set a target of eradication by the year 2000. This project is known as the Global Polio Eradication Initiative. Poliovirus Type 2 infection has not been observed since 1999 in India and Type 3 since 2012. However, in 2014, poliovirus Type 1 is still endemic in regions of Nigeria, Pakistan, and Afghanistan [World Health Organization (WHO), 2014a]. The reasons behind these persisting endemics will be discussed later.

There are two vaccines, an oral live attenuated vaccine known as the Sabin vaccine and the inactivated poliovirus vaccine also known as the Salk vaccine [World Health Organization (WHO), 2014a]. The Sabin vaccine was derived from passages of the poliovirus strains through rats and mice and then through cell cultures more than 50 times resulting in an attenuated forms of the virus types that all induced good antibody levels (Sabin, 1957; Baicus, 2012). In 1972, Sabin donated his vaccine strains to the WHO which increased the number of vaccine recipients from 5 to 80% [Baicus, 2012; World Health Organization (WHO), 2014a]. The Sabin vaccine is no longer in use in the USA or UK as the only poliomyelitis cases reported in the populations were vaccine-associated paralytic poliomyelitis where the vaccine strain has caused an outbreak but it is still used in some developing countries due to its ease of administration and cost (US$0.14 a dose vs US$2–3 a dose for Salk vaccine; Willyard, 2014). There are now plans to eliminate the Sabin vaccine entirely in the 124 countries that still use it by 2015 (Willyard, 2014).

The Salk vaccine is grown in monkey kidney cells and inactivated with formalin (Salk et al., 1954) and was introduced in the USA in 1955 and by 1961, the incidence of poliomyelitis had decreased from 13.9 cases per 100,000 in 1954 to 0.8 cases per 100,000 in 1961 [Baicus, 2012; World Health Organization (WHO), 2014a]. Besides preventing deaths, the main benefit to come from polio vaccination is the cost savings to the healthcare system which is estimated at US$40–50 billion for the period between 1988 and 2035 in the USA alone [World Health Organization (WHO), 2014a]. Most countries that have been certified polio-free still have rare isolated cases which have come from travelers importing the virus from endemic areas, for example in Australia had one such case in 2007 (Paterson and Durrheim, 2013). However, the Global Polio Eradication Initiative has a new timeline for eradication and with a new strategy of phasing out the Sabin vaccines, hopefully the world will be certified polio-free in 2018 (Willyard, 2014).

A more recent vaccine accomplishment is the pneumococcal conjugate vaccine (PCV) against *Streptococcus pneumoniae* (pneumococcus) infections which include acute otitis media, sinusitis, pneumonia and invasive pneumococcal diseases such as meningitis and sepsis. The first conjugate vaccine was a heptavalent vaccine which protects against seven different serotypes of pneumococcus and it was licensed in the USA in 2000 (Black et al., 2000; Lee et al., 2014). Since that time, 10- (Domingues et al., 2014), 13- (Spijkerman et al., 2013), and 23-valent (Grabenstein and Manoff, 2012) vaccines have been licensed with all producing strong immunity against a broad

spectrum of strains. In the USA, all age groups from children under 5 years to adults over 65 years had dramatic reductions in incidence of pneumococcal infections over a seven year period after the PCV was available (Pilishvili et al., 2010).

It is predicted that if the heptavalent PCV was implemented in China it would prevent 4222 cases of invasive pneumococcal disease, 4,061,524 cases of otitis media and 472,527 cases of pneumonia, as well as preventing an additional 2682 deaths from pneumococcal disease; however, the implementation cost would be estimated at US$6.44 billion (Che et al., 2014). The current overall cost of pneumococcal disease in the unvaccinated population in China is estimated to be US$3.5 billion (Che et al., 2014). Following the introduction of PCV in the USA, an estimated 211,000 serious pneumococcal infections and 13,000 deaths were prevented in the period of 2000–2008 (Pilishvili et al., 2010). The influence of this vaccine on public health is in its early stages and has already had impacts on child mortality in over 88 countries that have included various PCV on their recommended immunization schedule (Whitney et al., 2014).

There are other vaccines that have been successfully implemented in the past decade. The most recent and highly publicized vaccine is the quadrivalent human papillomavirus vaccine against cervical cancer, marketed as Gardasil®, which prevents the premalignant disease that leads to cervical cancers and fulfills all the above criteria of being a successful vaccine (Zhou et al., 1991; Govan, 2008). Initially the cost of Gardasil® was extremely prohibitive at US$120 per dose with three doses required; however, in collaboration with GAVI Alliance, the cost from the supplier has dropped to US4.50 per dose which increases its affordability and likelihood of being implemented in developing countries (Anon, 2013). As the cost of the vaccine decreases and more people are immunized this vaccine which has been included in over 30 countries immunization schedules, in conjunction with regular Pap screening, may lead to a long-term reduction in cervical cancer incidence (Harper et al., 2010; Ribeiro-Muller and Muller, 2014).

VACCINE FAILURES AND CHALLENGES

Historically there have been more vaccine failures than successes and unfortunately those failures can be publicized and instill fear in the general public long after the event. One such failure is one that occurred early in the rollout of the Salk polio vaccine is known as the Cutter incident. In April, 1955 a few weeks after Salk's polio vaccine had been declared safe and efficacious, there were reports from California that five children had become paralyzed after receiving the vaccine (Offit, 2005). These vaccines were traced to Cutter which was one of the five pharmaceutical companies that were granted a license to produce the vaccine in the USA (Nathanson and Langmuir, 1995). It was found that two production batches failed the deactivation steps; so live virulent poliovirus was found in 120,000 doses of the vaccine. Of the children vaccinated from this pool, 40,000 developed abortive polio, 51 suffered from permanent paralysis and five died (Nathanson and Langmuir, 1995). Unfortunately this was not the end of the tragedy, a polio outbreak followed where a further 113 people in close contact with the vaccinated children were infected and subsequently paralyzed, and a further five deaths (Nathanson and Langmuir, 1995; Offit, 2005). This incident halted the implementation of the polio vaccine program and severely affected public confidence in the vaccination, not only in the USA but as far reaching as New Zealand (Day, 2009), Germany, the UK and Sweden (Axelsson, 2012) and in the end, it caused the USA to recommend Sabin's vaccine in the long term which, barring manufacturing failures, proved to be the more risky of the two vaccines as it could revert to full virulence and cause outbreaks of vaccine-associated paralytic poliomyelitis (Offit, 2005; Fitzpatrick, 2006).

Following its emergence in 1981, HIV infections and its subsequent disease acquired immunodeficiency syndrome (AIDS) has become a global pandemic with millions of deaths and over 34 million people living with HIV (De Cock et al., 2012). According to the [World Health Organization (WHO, 2014c)] and the Joint United Nations Programme on Hiv/Aids (2013) the pandemic appears to have peaked as AIDS-related deaths have decreased by 25% in the past decade as well as new infections decreasing by 20% since 2006. This is the combined effect of the development of anti-retroviral drugs, and better education about the transmission of this disease. However, a vaccine is desperately needed to prevent new infections and to stop this pandemic from affecting future generations.

Multiple HIV vaccines have been tested in clinical trials with limited success (Johnson et al., 2013). In the last decade, the most prominent vaccine trial failures was that of the Merck STEP phase II test of concept and efficacy trial for an Adenovirus5 (Ad5) vaccine. It showed that the MRKAd5 HIV-1 gag/pol/nef vaccine was highly immunogenic and elicited a higher magnitude of HIV-specific CD8+ T cells than any of the other HIV candidate vaccines over the past 15 years but it did not prevent HIV infection or reduce viral loads in infected patients (Buchbinder et al., 2008). In fact, more disturbingly, there was an increase in the number of HIV-1 infections in male recipients of the vaccine compared to the controls (McElrath et al., 2008). This trial was immediately ceased when the independent data and safety monitoring board determined that the study could not demonstrate efficacy (Buchbinder et al., 2008).

One of the reasons behind the failure of the Merck STEP clinical trial was the pre-existing neutralizing antibodies against Ad5. A recent study confirmed that the international epidemiology of pre-existing immunity to different adenovirus types can severely compromise its efficacy as only 14.8% of the 1904 participants were seronegative for neutralizing antibodies against Ad5 (Mast et al., 2010). This indicates that naturally acquired infections from virulent forms of the vaccine vectors can limit their usefulness in the same species. However, choosing a virus from a different species for which no prior exposure is possible but may sound too risky to be accepted by the general population. It was also found that whilst the group of men that became more susceptible to HIV infection post-vaccination were seropositive against the Ad5 vector, they were also uncircumcised and had sexual relations with the same sex implying that pre-existing immunity may not be the sole factor that caused this vaccine failure (Gray et al., 2010; Duerr et al., 2012). Whilst this phase II trial failure was a major setback for the HIV research community, it raised fundamental

questions about the pathogenesis of HIV and also gave insight into immunological mechanisms that were previously unexplored (Johnson et al., 2013; Fauci et al., 2014). The search for a HIV vaccine is ongoing and as of May 2014, there are 92 open HIV vaccine trials according to Clinicaltrials.Gov (2014).

Another infectious disease that is under surveillance by health departments worldwide is a double-stranded RNA virus called rotavirus. Rotavirus causes acute enteritis resulting in severe, dehydrating diarrhea in infants and young children and is very transmissible through close contact [Bishop et al., 1976; World Health Organization (WHO), 2013]. In the pre-vaccination era, rotavirus caused 111 million cases of illness with 25 million medical visits, 2 million hospitalizations and between 352,000 and 592,000 rotavirus gastroenteritis-associated deaths worldwide annually with most of these occurring in low income countries (Parashar et al., 2003). The first rotavirus vaccine was RotaShield which was developed by Wyeth-Lederle Vaccines and Pediatrics, Philadelphia, as an oral vaccine and showed high efficacy at 80% protection from severe illness; hence it was recommended for all infants in the USA once it was approved by the Food and Drug Administration (FDA) on August 31, 1998 (American Academy of Pediatrics, 1998). Over the eleven month period after the vaccine was approved until July 7, 1999, 15 cases of intussusception, a type of intestinal blockage requiring surgical intervention, were reported and linked to the vaccination. In consultation with the FDA, Wyeth-Lederle Vaccines withdrew Rotashield from the market on October 15, 1999. Before this withdrawal, the cases of confirmed intussusception had risen to 101 (Delage, 2000) and fortuitously, because there were no deaths caused by this vaccine, physician trust in vaccine safety measures were not compromised by this withdrawal (McPhillips et al., 2001).

In 2006, two new oral rotavirus vaccines were released onto the market: Rotarix® – a live monovalent attenuated human strain by GlaxoSmithKline Biologicals (Vesikari et al., 2004; Keating, 2006b) and RotaTeq® – a live pentavalent human-bovine reassortant vaccine by Merck & Co. Inc. (Clark et al., 2004; Keating, 2006a). After 6 years of use, a Cochrane Review found that both of these vaccines are efficacious with no increased risk of adverse side effects such as intussusception (Soares-Weiser et al., 2012). However, in 2013, a small increase in risk was confirmed when the data was analyzed comparing the risk of intussusception in the post-vaccine period with other periods (Haber et al., 2013; Quinn et al., 2014).

A year later, the vaccines are still on the market albeit with an intussusception warning even though there is an estimated up to sixfold increase with the use of these two rotavirus vaccines. So far the Vaccine Safety Datalink has reported that Rotarix® has had 66 intussusception cases in 200,000 doses, whilst RotaTeq® had eight cases for 1.3 million doses administered with most occurring within 7 days after the first dose [World Health Organization (WHO), 2014b]. Currently the risk of intussusception is estimated to be 1–2 per 100,000 infants vaccinated [World Health Organization (WHO), 2013]. However, the general view is that there are great benefits to vaccination against rotavirus as the infant mortality rates in countries that have added this to their vaccination schedule have significantly decreased (Buttery et al., 2014) and this is reflected in the WHO's Global Advisory Committee on Vaccine Safety in their weekly epidemiological record [World Health Organization (WHO), 2014b] stated this in regards to the new intussusception risk: "the findings remain reassuring that the risk of intussusception following current rotavirus vaccines remains small compared to the benefits of preventing the impact of severe diarrhea." Surveillance of such adverse effects requires long-term study in order to make sound decisions about the appropriateness of the vaccine. There may come a time where the relative risk is too high and the vaccine is withdrawn like Rotashield which had a rate of intussusception of 1 in 10,000 infant doses [World Health Organization (WHO), 2013], even though it provided strong immunological protection. This is one of the hardest aspects in vaccine development to plan for and may lead to public distrust in future vaccines, if it is not done expediently when those risks increases.

POLITICAL AND GLOBAL ASPECTS OF VACCINE USAGE

When a vaccine is designed, it is assumed that if it proves effective it will be used in various countries around the world to vaccinate the population; however, this is not always the case. Within each country there are government agencies, industry and community health advocates, and outside agencies such as the WHO that will make recommendations for vaccination strategies. Often this process will result in a successful vaccination strategy such as the global eradication of smallpox (Stewart and Devlin, 2006), but it can also lead to confusion and scepticism in the chosen strategy. One such example was the choice of pertussis vaccine for a national vaccination campaign in the Netherlands.

Originally, the Dutch government chose to use a whole cell vaccine based on the *Bordetella pertussis* bacterium; however, after speculation that the vaccine could cause brain damage, alternative vaccines were sought. At this time, acellular vaccines comprising three to five bacterial components were being used by many countries in Europe as they were comparable in protection to the whole cell vaccines and demonstrated minimal side effects (Blume and Zanders, 2006). Over the course of 7 years, the debate over the new vaccine became very convoluted as many government agencies, drug companies, and consumer groups presented opposing studies and evidence. There was also external pressure from neighboring countries and global non-profit groups including the WHO and United Nations Children's Fund (UNICEF) for the Dutch government to make a decision. Concurrently, many parents had lost faith in the old vaccine strategy; hence an epidemic of pertussis ensued. To combat the growing epidemic the Dutch government chose an acellular vaccine which was used in primary vaccinations in 2005; however, the Health Minister advised that this decision was not based on recommendations and evidence provided by the Dutch Health Council, but on the need to appease parents and re-establish their confidence in the vaccine strategy (Blume and Zanders, 2006). By contrast, in areas where any disease is endemic and the health system is overwhelmed, often the vaccination strategy proposed by governing bodies will be accepted by the population and acquiesced as mandatory (Chalmers, 2006).

Unfortunately this has not worked in areas such as Pakistan, Nigeria, and Afghanistan where the eradication of polio has failed

due in part to misinformation, violence, politics, and mistrust about vaccination. There is a distinct divide in these populations between vaccine acceptors and non-acceptors which is based in the abundance of misinformation about the vaccine, religious beliefs and the emotional fear about the agenda; however, if there is an outbreak many non-acceptors will accept the vaccination as the fear of disease outweighs the perceived risks (Murele et al., 2014). Socio-cultural, educational and perceptual factors are particularly strong in these regions and in some cases targeting male authority figures could improve vaccination uptake (Murele et al., 2014); however, in other regions maternal education and empowerment has been suggested as a strong motivator in vaccine acceptance (Larson et al., 2014).

Violence is another contributing factor to this program's failure particularly when there are fatal attacks on vaccination workers in Pakistan and Nigeria (Abimbola et al., 2013). In Afghanistan, both the Taliban regime and the militant Islamist terrorist group Al Qaeda support the Global Polio Eradication Initiative; however, factions within these groups can disrupt it as they view it as a Westernization issue, rather than a health one (Abimbola et al., 2013). In Nigeria and Pakistan, militants can gain international media attention by attacking polio health workers (Riaz and Rehman, 2013) and spreading propaganda that immunization programs are actually covert sterilization campaigns to reduce the Muslim population, which puts more fear into the local communities than the disease itself (Abimbola et al., 2013; Willyard, 2014).

All of the aforementioned issues affect the successful eradication of infectious diseases with well documented epidemiology and pathology. However, there exist conditions and disorders where the mechanisms of development and ongoing chronic pathology are yet to be fully ascertained. One such condition causing concern among health professionals globally is allergy.

ALLERGY AND VACCINE POTENTIAL

Allergy is a hypersensitivity disease characterized by the production of IgE antibodies against antigenic components (i.e., allergens) that can enter the body via the respiratory and gastrointestinal tract, the skin, an insect sting or injection of a drug (Sicherer and Sampson, 2014). The clinical reactions experienced by sensitized patients vary in different target organs and include rhinitis, urticaria, and allergic asthma to life-threatening anaphylactic shock (Sampson, 2003, 2004). The acute symptoms of allergy are usually due to the release of inflammatory mediators by tissue-bound mast cells and circulation basophils. These inflammatory mediators include histamine, platelet-activating factor, leukotrienes, mast cell proteases, and a range of cytokines. Mediators are released when allergen binds to IgE antibody attached to FεRI receptors on the cell surface, causing degranulation. Studies show a skewing towards a Th2 response, with elevated levels of IL-4, IL-5, and IL-13, while tolerant individuals usually have higher levels of the Th1 cytokines IFN-gamma and TNF-alpha, and the regulatory cytokine IL-10 (Andre et al., 1996; Noma et al., 1996; Schade et al., 2003; Turcanu et al., 2003; Tiemessen et al., 2004). The class switch to produce IgE antibody occurs during primary sensitization in allergic patients and seems to be driven by IL-4, which is a direct product of Th2 cells and other effector cells of the allergic immune response. The activation of allergen-specific T cells is achieved by the presentation of allergens via APCs, including dendritic cells (Grainger et al., 2014; Nagai et al., 2014).

As the prevalence and potential fatality of this disease has increased, so have the efforts to find effective therapies and prophylaxis also intensified (Valenta et al., 2010). Specific immunotherapy (SIT) is effective for desensitization against inhalant allergens; however, it is not advised as a therapy against food allergy because of the high risk of adverse side-effects (Sabato et al., 2014). Oral administration of antigens usually leads to tolerance, and has been effective in decreasing allergic sensitization to antibiotics and other medications (Stevenson, 2000, 2003). Obviously native food allergens cannot be administered in this way, but it may be possible for hypoallergenic or CpG-conjugated derivatives. Microencapsulation provides a promising way of delivering allergens without degradation in the stomach (Litwin et al., 1996), thereby inducing oral tolerance, and has already been applied in clinical trials (TePas et al., 2004). Conjugation or co-administration of recombinant allergens with Th1-inducing heat-killed bacteria has yielded good protective results in mice (Li et al., 2003a,b) and allergic dogs (Frick et al., 2005). Various approaches have been attempted to develop safe and effective DNA vaccines and are discussed in the following section.

DNA VACCINES AND ALLERGY

DNA vaccines, as demonstrated in the California condors, can induce protective immune responses against infectious diseases. Plasmid DNA injected intramuscularly, intraperitoneally or with a gene gun results in transcription and translation of encoded genes and elicits an antibody response in the host (Tang et al., 1992; Ulmer et al., 1993; Hsu et al., 1996b). This method of immunization preferentially induces a Th1 immune response and suppression of IgE (Raz et al., 1996; Yoshida et al., 2000). These effects appear to be mediated by both CD8+ and CD4+ cells (Hsu et al., 1996a; Lee et al., 1997; Peng et al., 2002), and plasmid DNA requires immunostimulatory sequences such as CpG for optimal immunogenicity (Sato et al., 1996; Adel-Patient et al., 2001; Jilek et al., 2001; Hartl et al., 2004). Unmethylated CpG motifs either in bacterial DNA or as synthetic oligodeoxynucleotides (CpG-ODN) are recognized by the mammalian immune system via toll-like receptor 9 (and possibly other PRRs) and trigger a Th1 response (Hartmann and Krieg, 1999; Stacey et al., 2000; Bauer et al., 2001). Experiments in murine models of allergic asthma, rhino sinusitis, and conjunctivitis show that administration of CpG-ODN alone prevents symptoms and reduces already established disease by reducing Th2 immune responses and IgE (Kline et al., 1998, 1999; Magone et al., 2000; Serebrisky et al., 2000). Allergen/CpG-ODN conjugates have been shown to be less allergenic and more immunogenic than native allergen (Tighe et al., 2000; Horner et al., 2002). The major allergen from ragweed, Amb a 1, linked to an immunostimulatory DNA sequence promoted Th1 cytokine expression and down regulated Th2 expression *in vitro* (Simons et al., 2004), reversed established airway hyperreactivity in a murine model of asthma (Marshall et al., 2001; Santeliz et al., 2002)

and yielded promising results in Phase II clinical trials (Tulic et al., 2004).

Genetic immunization to specific allergens using plasmid DNA offers a powerful solution to the major problems associated with protein immunization, such as cross-linking of IgE antibody on effector cells or even *de novo* synthesis of IgE antibodies to the immunized protein itself. However, genetic vaccination may lead to an uncontrolled synthesis of allergens in the vaccinated host (Slater et al., 1998) and has been a major hurdle for application in allergic patients. Three approaches are currently used to prevent this from occurring: (a) cutting the allergen-coding gene into fragments, lacking the antigenic determinant but containing the original T cell epitope repertoire, (b) the use of hypoallergenic protein derivatives, or (c) fusing allergen with proteins that promote immune responses.

Several allergens have been tested in DNA vaccination approaches using murine models, including Ara h 2 (peanut), bovine beta-lactoglobulin (cow's milk), Cry j 1 (Japanese cedar), phospholipase A2 (bee venom), Der f 11 and Der p 1 (dust-mite), and Bet v 1 and Phl p 2 (grass; Roy et al., 1999; Toda et al., 2000; Adel-Patient et al., 2001; Jilek et al., 2001; Kwon et al., 2001; Peng et al., 2002; Hochreiter et al., 2003; Ludwig-Portugall et al., 2004). Most studies observed elicitation of a Th1 response and increased IL-10 production. Mice vaccinated against phospholipase A2 were protected against fatal anaphylaxis following allergen challenge (Jilek et al., 2001), while mice receiving an oral DNA vaccine containing the peanut allergen Ara h 2 (Roy et al., 1999) experienced significantly less severe and delayed allergic reactions upon subsequent sensitization and challenge. However, prophylactic effects, while promising, are not sufficient to aid patients who have existing food allergy. In mice pre-sensitized to phospholipase A (bee venom), therapeutic gene vaccination prevented only 30% of mice from anaphylaxis (Jilek et al., 2001).

In addition to direct DNA vaccination, these approaches provide the option of co-delivering genes or adjuvant molecules with immunomodulatory properties together with the antigen sequence (Hartl et al., 2004; Mutschlechner et al., 2009). Allergen–allergen hybrid molecules may combine different allergens from one complex allergen source or use allergens from different sources as demonstrated for grass pollen (Linhart et al., 2005; Wallner et al., 2009). Furthermore, hybrid molecules using only T cell epitopes have been successfully used (Linhart et al., 2008). Vaccination of mice with a plasmid containing the cDNA for OVA fused to the cDNA of IL-18 (Allergen–cytokine fusion protein), a potent Th1 inducer, reversed established airway hyperreactivity, while a plasmid containing OVA alone had only a prophylactic effect (Maecker et al., 2001).

Ubiquitination of allergens represents another routine approach for destroying IgE-binding epitopes on proteins to produce hypoallergenic DNA vaccines. This approach has been applied for the production of a DNA-based vaccine encoding an ubiquitinated version of *Linhart* v 1, the major allergen from birch pollen (Bauer et al., 2006). It was demonstrated in a murine study that this vaccine did not produce any detectable antibody response, but T cell reactivity was preserved as well as allergic reactions prevented.

In summary, several novel therapeutic and prophylactic therapies against allergy are currently under investigation (Nieuwenhuizen and Lopata, 2005; Flicker et al., 2013; Weiss et al., 2013). Genetic immunization has proven a powerful method to induce anti-allergic immune responses. The underlying functional principle described seems to be based on the recruitment of allergen-specific Th1 cells, CD8+ cells and the establishment of a Th1 cytokine milieu. This response can be protective by preventing the development of a Th2-biased response towards allergens, as well as balance an ongoing Th2-type response in a more therapeutic application. More studies are needed to increase our understanding of the pathophysiology and immunological mechanisms of allergy, and to characterize the molecular structure and epitopes of allergens, to develop safer and more effective ways of combating this debilitating and potentially life-threatening disease.

CONCLUSION

The advent of vaccination changed global society and our everyday lives dramatically, especially in conjunction with improved healthcare, infrastructure and technology. Over the last century with increasing knowledge of the immune system and infectious diseases, infant mortality associated with infectious diseases dropped, in developed countries debilitating illnesses like polio disappeared from public view, and the youth of today did not experience the threat or fear of death via infectious diseases. However, some diseases such as HIV and malaria are yet to have efficacious vaccines developed and successfully complete Phase III clinical trials. So the fight continues against these known enemies and with each failure, we learn more. The list of global health threats consists of many incurable infectious diseases; immunological disorders such as allergy should be added to that list. Currently, therapeutic interventions are adequate, but with population and allergy prevalence increasing there is a strong need for a prophylactic vaccine. Although the establishment of allergy is not fully elucidated, researchers should be mining the already long history of infectious disease vaccines to create new avenues of allergen vaccine development.

ACKNOWLEDGMENTS

Financial support from the Australian Research Council (ARC) to Andreas L. Lopata is acknowledged. Andreas L. Lopata is a holder of an ARC Future Fellowship. Financial support from a JCU Centre of Biodiscovery & Molecular Development of Therapeutics Seed Grant and JCU Faculty of Medicine, Health and Molecular Sciences Research Grant to Fiona J. Baird and the ABRS to Andreas L. Lopata is acknowledged.

REFERENCES

Abimbola, S., Malik, A. U., and Mansoor, G. F. (2013). The final push for polio eradication: addressing the challenge of violence in Afghanistan, Pakistan, and Nigeria. *PLoS Med.* 10:e1001529. doi: 10.1371/journal.pmed.1001529

Adegbola, R. A., Secka, O., Lahai, G., Lloyd-Evans, N., Njie, A., Usen, S., et al. (2005). Elimination of *Haemophilus influenzae* type b (Hib) disease from The Gambia after the introduction of routine immunisation with a Hib conjugate vaccine: a prospective study. *Lancet* 366, 144–150. doi: 10.1016/S0140-6736(05)66788-8

Adel-Patient, K., Creminon, C., Boquet, D., Wal, J. M., and Chatel, J. M. (2001). Genetic immunisation with bovine beta-lactoglobulin cDNA induces a preventive

and persistent inhibition of specific anti-BLG IgE response in mice. *Int. Arch. Allergy Immunol.* 126, 59–67. doi: 10.1159/000049495

American Academy of Pediatrics. (1998). Prevention of rotavirus disease: guidelines for use of rotavirus vaccine. American Academy of Pediatrics. *Pediatrics* 102, 1483–1491. doi: 10.1542/peds.102.6.1483

Andre, F., Pene, J., and Andre, C. (1996). Interleukin-4 and interferon-gamma production by peripheral blood mononuclear cells from food-allergic patients. *Allergy* 51, 350–355.

Anon. (2013). GAVI injects new life into HPV vaccine rollout. *Lancet* 381, 1688. doi: 10.1016/S0140-6736(13)61058-2

Arntzen, C., Plotkin, S., and Dodet, B. (2005). Plant-derived vaccines and antibodies: potential and limitations. *Vaccine* 23, 1753–1756. doi: 10.1016/j.vaccine.2005.01.090

Atmar, R. L., Keitel, W. A., Cate, T. R., Munoz, F. M., Ruben, F., and Couch, R. B. (2007). A dose–response evaluation of inactivated influenza vaccine given intranasally and intramuscularly to healthy young adults. *Vaccine* 25, 5367–5373. doi: 10.1016/j.vaccine.2007.05.002

Axelsson, P. (2012). The Cutter incident and the development of a Swedish polio vaccine, 1952–1957. *Dynamis* 32, 311–328. doi: 10.4321/S0211-95362012000200003

Bachtiar, E. W., Sheng, K.-C., Fifis, T., Gamvrellis, A., Plebanski, M., Coloe, P. J., et al. (2003). Delivery of a heterologous antigen by a registered Salmonella vaccine (STM1). *FEMS Microbiol. Lett.* 227, 211–217. doi: 10.1016/S0378-1097(03)00683-9

Baicus, A. (2012). History of polio vaccination. *World J. Virol.* 1, 108–114. doi: 10.5501/wjv.v1.i4.108

Barquet, N., and Domingo, P. (1997). Smallpox: the triumph over the most terrible of the ministers of death. *Ann. Intern. Med.* 127, 635–642. doi: 10.7326/0003-4819-127-8_Part_1-199710150-00010

Bauer, M., Redecke, V., Ellwart, J. W., Scherer, B., Kremer, J. P., Wagner, H., et al. (2001). Bacterial CpG-DNA triggers activation and maturation of human $CD11c^-$, $CD123^+$ dendritic cells. *J. Immunol.* 166, 5000–5007. doi: 10.4049/jimmunol.166.8.5000

Bauer, R., Scheiblhofer, S., Kern, K., Gruber, C., Stepanoska, T., Thalhamer, T., et al. (2006). Generation of hypoallergenic DNA vaccines by forced ubiquitination: preventive and therapeutic effects in a mouse model of allergy. *J. Allergy Clin. Immunol.* 118, 269–276. doi: 10.1016/j.jaci.2006.03.033

Bazin, H. (2003). A brief history of the prevention of infectious diseases by immunisations. *Comp. Immunol. Microbiol. Infect. Dis.* 26, 293–308. doi: 10.1016/S0147-9571(03)00016-X

Bergman, P. J., Camps-Palau, M. A., Mcknight, J. A., Leibman, N. F., Craft, D. M., Leung, C., et al. (2006). Development of a xenogeneic DNA vaccine program for canine malignant melanoma at the Animal Medical Center. *Vaccine* 24, 4582–4585. doi: 10.1016/j.vaccine.2005.08.027

Berzofsky, J. A., Terabe, M., Oh, S., Belyakov, I. M., Ahlers, J. D., Janik, J. E., et al. (2004). Progress on new vaccine strategies for the immunotherapy and prevention of cancer. *J. Clin. Invest.* 113, 1515–1525. doi: 10.1172/JCI21926

Bishop, R. F., Hewstone, A. S., Davidson, G. P., Townley, R. R., Holmes, I. H., and Ruck, B. J. (1976). An epidemic of diarrhoea in human neonates involving a reovirus-like agent and 'enteropathogenic' serotypes of *Escherichia coli*. *J. Clin. Pathol.* 29, 46–49. doi: 10.1136/jcp.29.1.46

Black, S., Shinefield, H., Fireman, B., Lewis, E., Ray, P., Hansen, J. R., et al. (2000). Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Northern California Kaiser Permanente Vaccine Study Center Group. *Pediatr. Infect. Dis. J.* 19, 187–195. doi: 10.1097/00006454-200003000-00003

Blume, S., and Zanders, M. (2006). Vaccine independence, local competences and globalisation: lessons from the history of pertussis vaccines. *Soc. Sci. Med.* 63, 1825–1835. doi: 10.1016/j.socscimed.2006.04.014

Bordenave, G. (2003). Louis Pasteur (1822–1895). *Microbes Infect.* 5, 553–560. doi: 10.1016/S1286-4579(03)00075-3

Bouchie, A. (2003). DNA vaccine deployed for endangered condors. *Nat. Biotechnol.* 21, 11.

Buchbinder, S. P., Mehrotra, D. V., Duerr, A., Fitzgerald, D. W., Mogg, R., Li, D., et al. (2008). Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. *Lancet* 372, 1881–1893. doi: 10.1016/S0140-6736(08)61591-3

Buttery, J. P., Standish, J., and Bines, J. E. (2014). Intussusception and rotavirus vaccines: consensus on benefits outweighing recognised risk. *Pediatr. Infect. Dis. J.* 33, 772–773. doi: 10.1097/inf.0000000000000359

Chalmers, W. S. K. (2006). Overview of new vaccines and technologies. *Vet. Microbiol.* 117, 25–31. doi: 10.1016/j.vetmic.2006.04.006

Chang, G.-J. J., Davis, B. S., Stringfield, C., and Lutz, C. (2007). Prospective immunization of the endangered *California condors* (*Gymnogyps californianus*) protects this species from lethal West Nile virus infection. *Vaccine* 25, 2325–2330. doi: 10.1016/j.vaccine.2006.11.056

Che, D., Zhou, H., He, J., and Wu, B. (2014). Modeling the impact of the 7-valent pneumococcal conjugate vaccine in Chinese infants: an economic analysis of a compulsory vaccination. *BMC Health Serv. Res.* 14:56. doi: 10.1186/1472-6963-14-56

Cheng, G., Zhao, X., Yan, W., Wang, W., Zuo, X., Huang, K., et al. (2007). Alpha interferon is a powerful adjuvant for a recombinant protein vaccine against foot-and-mouth disease virus in swine, and an effective stimulus of in vivo immune response. *Vaccine* 25, 5199–5208. doi: 10.1016/j.vaccine.2007.04.089

Cho, H., Lee, H. J., Heo, Y. K., Cho, Y., Gwon, Y. D., Kim, M. G., et al. (2014). Immunogenicity of a trivalent human papillomavirus L1 DNA-encapsidated, non-replicable baculovirus nanovaccine. *PLoS ONE* 9:e95961. doi: 10.1371/journal.pone.0095961

Clark, H. F., Lawley, D., Shrager, D., Jean-Guillaume, D., Offit, P. A., Whang, S. Y., et al. (2004). Infant immune response to human rotavirus serotype G1 vaccine candidate reassortant WI79-9: different dose response patterns to virus surface proteins VP7 and VP4. *Pediatr. Infect. Dis. J.* 23, 206–211. doi: 10.1097/01.inf.0000115503.55212.bf

Clinicaltrials.Gov (2014). www.clinicaltrials.gov [Online]. Bethesda, MD: National Library of Medicine (US). Available at: https://clinicaltrials.gov/ct2/home [accessed May 7, 2014].

Costantino, H. R., Illum, L., Brandt, G., Johnson, P. H., and Quay, S. C. (2007). Intranasal delivery: physicochemical and therapeutic aspects. *Int. J. Pharm.* 337, 1–24. doi: 10.1016/j.ijpharm.2007.03.025

Davies, M. N., and Flower, D. R. (2007). Harnessing bioinformatics to discover new vaccines. *Drug Discov. Today* 12, 389–395. doi: 10.1016/j.drudis.2007.03.010

Day, A. (2009). 'An American tragedy'. the Cutter incident and its implications for the Salk polio vaccine in New Zealand 1955–1960. *Health History* 11, 42–61.

De Cock, K. M., Jaffe, H. W., and Curran, J. W. (2012). The evolving epidemiology of HIV/AIDS. *AIDS* 26, 1205–1213. doi: 10.1097/QAD.0b013e328354622a

De Gregorio, E., Caproni, E., and Ulmer, J. B. (2013). Vaccine adjuvants: mode of action. *Front. Immunol.* 4:214. doi: 10.3389/fimmu.2013.00214

Delage, G. (2000). Rotavirus vaccine withdrawal in the United states: the role of postmarketing surveillance. *Can. J. Infect. Dis.* 11, 10–12.

Dinc, G., and Ulman, Y. I. (2007). The introduction of variolation 'A La Turca' to the West by Lady Mary Montagu and Turkey's contribution to this. *Vaccine* 25, 4261–4265. doi: 10.1016/j.vaccine.2007.02.076

Domingues, C. M., Verani, J. R., Montenegro Renoiner, E. I., De Cunto Brandileone, M. C., Flannery, B., De Oliveira, L. H., et al. (2014). Effectiveness of ten-valent pneumococcal conjugate vaccine against invasive pneumococcal disease in Brazil: a matched case–control study. *Lancet Respir. Med.* 2, 464–471. doi: 10.1016/s2213-2600(14)70060-8

Duerr, A., Huang, Y., Buchbinder, S., Coombs, R. W., Sanchez, J., Del Rio, C., et al. (2012). Extended follow-up confirms early vaccine-enhanced risk of HIV acquisition and demonstrates waning effect over time among participants in a randomized trial of recombinant adenovirus HIV vaccine (step study). *J. Infect. Dis.* 206, 258–266. doi: 10.1093/infdis/jis342

Fauci, A. S., Marovich, M. A., Dieffenbach, C. W., Hunter, E., and Buchbinder, S. P. (2014). Immune activation with HIV vaccines. *Science* 344, 49–51. doi: 10.1126/science.1250672

Fenner, F., Henderson, D. A., Arita, I., Jezek, Z., and Id, L. (1988). *Smallpox and Its Eradication*. Geneva: World Health Organization.

Fitzpatrick, M. (2006). The Cutter incident: how America's first polio vaccine led to a growing vaccine crisis. *J. R. Soc. Med.* 99, 156. doi: 10.1258/jrsm.99.3.156

Flicker, S., Linhart, B., Wild, C., Wiedermann, U., and Valenta, R. (2013). Passive immunization with allergen-specific IgG antibodies for treatment and prevention of allergy. *Immunobiology* 218, 884–891. doi: 10.1016/j.imbio.2012.10.008

Forde, G. M. (2005). Rapid-response vaccines – does DNA offer a solution? *Nat. Biotechnol.* 23, 1059–1062. doi: 10.1038/nbt0905-1059

Franco-Paredes, C., Lammoglia, L., Hernandez, I., and Santos-Preciado, J. I. (2008). Epidemiology and outcomes of bacterial meningitis in Mexican children: 10-year experience (1993–2003). *Int. J. Infect. Dis.* 12, 380–386. doi: 10.1016/j.ijid.2007.09.012

Frick, O. L., Teuber, S. S., Buchanan, B. B., Morigasaki, S., and Umetsu, D. T. (2005). Allergen immunotherapy with heat-killed *Listeria monocytogenes* alleviates peanut and food-induced anaphylaxis in dogs. *Allergy* 60, 243–250. doi: 10.1111/j.1398-9995.2004.00711.x

Glenny, A. T., Pope, C. G., Waddington, H., and Wallace, U. (1926). Immunological notes XVII to XXIV. *J. Pathol.* 29, 31–40. doi: 10.1002/path.1700290106

Govan, V. A. (2008). A novel vaccine for cervical cancer: quadrivalent human papillomavirus (types 6, 11, 16 and 18) recombinant vaccine (Gardasil). *Ther. Clin. Risk Manag.* 4, 65–70.

Grabenstein, J. D., and Manoff, S. B. (2012). Pneumococcal polysaccharide 23-valent vaccine: long-term persistence of circulating antibody and immunogenicity and safety after revaccination in adults. *Vaccine* 30, 4435–4444. doi: 10.1016/j.vaccine.2012.04.052

Grainger, J. R., Askenase, M. H., Guimont-Desrochers, F., Da Fonseca, D. M., and Belkaid, Y. (2014). Contextual functions of antigen-presenting cells in the gastrointestinal tract. *Immunol. Rev.* 259, 75–87. doi: 10.1111/imr.12167

Gray, G., Buchbinder, S., and Duerr, A. (2010). Overview of STEP and Phambili trial results: two phase IIb test-of-concept studies investigating the efficacy of MRK adenovirus type 5 gag/pol/nef subtype B HIV vaccine. *Curr. Opin. HIV AIDS* 5, 357–361. doi: 10.1097/COH.0b013e32833d2d2b

Haber, P., Patel, M., Pan, Y., Baggs, J., Haber, M., Museru, O., et al. (2013). Intussusception after rotavirus vaccines reported to US VAERS, 2006–2012. *Pediatrics* 131, 1042–1049. doi: 10.1542/peds.2012-2554

Harper, D. M., Vierthaler, S. L., and Santee, J. A. (2010). Review of Gardasil. *J. Vaccines Vaccin.* 1, 1000107. doi: 10.4172/2157-7560.1000107

Hartl, A., Weiss, R., Hochreiter, R., Scheiblhofer, S., and Thalhamer, J. (2004). DNA vaccines for allergy treatment. *Methods* 32, 328–339. doi: 10.1016/j.ymeth.2003.08.014

Hartmann, G., and Krieg, A. M. (1999). CpG DNA and LPS induce distinct patterns of activation in human monocytes. *Gene Ther.* 6, 893–903. doi: 10.1038/sj.gt.3300880

Hochreiter, R., Ferreira, F., Thalhamer, J., and Hammerl, P. (2003). TH1-promoting DNA immunization against allergens modulates the ratio of IgG1/IgG2a but does not affect the anaphylactic potential of IgG1 antibodies: no evidence for the synthesis of nonanaphylactic IgG1. *J. Allergy Clin. Immunol.* 112, 579–584. doi: 10.1016/S0091-6749(03)01623-3

Horner, A. A., Takabaysahi, K., Zubeldia, J. M., and Raz, E. (2002). Immunostimulatory DNA-based therapeutics for experimental and clinical allergy. *Allergy* 57(Suppl. 72), 24–29. doi: 10.1034/j.1398-9995.57.s72.12.x

Hsu, C. H., Chua, K. Y., Tao, M. H., Huang, S. K., and Hsieh, K. H. (1996a). Inhibition of specific IgE response in vivo by allergen-gene transfer. *Int. Immunol.* 8, 1405–1411. doi: 10.1093/intimm/8.9.1405

Hsu, C. H., Chua, K. Y., Tao, M. H., Lai, Y. L., Wu, H. D., Huang, S. K., et al. (1996b). Immunoprophylaxis of allergen-induced immunoglobulin E synthesis and airway hyperresponsiveness in vivo by genetic immunization. *Nat. Med.* 2, 540–544. doi: 10.1038/nm0596-540

Hutchison, S., Benson, R. A., Gibson, V. B., Pollock, A. H., Garside, P., and Brewer, J. M. (2012). Antigen depot is not required for alum adjuvanticity. *FASEB J.* 26, 1272–1279. doi: 10.1096/fj.11–184556

Jawale, C. V., and Lee, J. H. (2014). *Salmonella enterica* Serovar enteritidis ghosts carrying the *Escherichia coli* heat-labile enterotoxin B subunit are capable of inducing enhanced protective immune responses. *Clin. Vaccine Immunol.* 21, 799–807. doi: 10.1128/cvi.00016-14

Jenner, E. (1809). Two cases of small-pox infection communicated to the foetus in utero under peculiar circumstances, with additional remarks. *Med. Chir. Trans.* 1, 271–277.

Jilek, S., Barbey, C., Spertini, F., and Corthesy, B. (2001). Antigen-independent suppression of the allergic immune response to bee venom phospholipase A(2) by DNA vaccination in CBA/J mice. *J. Immunol.* 166, 3612–3621. doi: 10.4049/jimmunol.166.5.3612

Johnson, J. A., Barouch, D. H., and Baden, L. R. (2013). Nonreplicating vectors in HIV vaccines. *Curr. Opin. HIV AIDS* 8, 412–420. doi: 10.1097/COH.0b013e328363d3b7

Joint United Nations Programme on Hiv/Aids. (2013). *Global Report: UNAIDS Report on the Global AIDS Epidemic 2013.* Geneva: Joint United Nations Programme on HIV/AIDS.

Kaufmann, S. H. E., and Schaible, U. E. (2005). 100th anniversary of Robert Koch's Nobel Prize for the discovery of the tubercle bacillus. *Trends Microbiol.* 13, 469–475. doi: 10.1016/j.tim.2005.08.003

Keating, G. M. (2006a). Rotavirus vaccine (RotaTeq). *Paediatr. Drugs* 8, 197–202. doi: 10.2165/00148581-200608030-00008

Keating, G. M. (2006b). Rotavirus vaccine RIX4414 (Rotarix). *Paediatr. Drugs* 8, 389–395. doi: 10.2165/00148581-200608060-00006

Kilpatrick, A. M., Dupuis, A. P., Chang, G. J., and Kramer, L. D. (2010). DNA vaccination of American robins (*Turdus migratorius*) against West Nile virus. *Vector Borne Zoonotic Dis.* 10, 377–380. doi: 10.1089/vbz.2009.0029

Kline, J. N., Krieg, A. M., Waldschmidt, T. J., Ballas, Z. K., Jain, V., and Businga, T. R. (1999). CpG oligodeoxynucleotides do not require TH1 cytokines to prevent eosinophilic airway inflammation in a murine model of asthma. *J. Allergy Clin. Immunol.* 104, 1258–1264. doi: 10.1016/S0091-6749(99)70022-9

Kline, J. N., Waldschmidt, T. J., Businga, T. R., Lemish, J. E., Weinstock, J. V., Thorne, P. S., et al. (1998). Modulation of airway inflammation by CpG oligodeoxynucleotides in a murine model of asthma. *J. Immunol.* 160, 2555–2559.

Ko, S.-Y., Ko, H.-J., Chang, W.-S., Park, S.-H., Kweon, M.-N., and Kang, C.-Y. (2005). {alpha}-Galactosylceramide can act as a nasal vaccine adjuvant inducing protective immune responses against viral infection and tumor. *J. Immunol.* 175, 3309–3317. doi: 10.4049/jimmunol.175.5.3309

Kwon, S. S., Kim, N., and Yoo, T. J. (2001). The effect of vaccination with DNA encoding murine T-cell epitopes on the Der p 1 and 2 induced immunoglobulin E synthesis. *Allergy* 56, 741–748. doi: 10.1034/j.1398-9995.2001.056008741.x

Ladhani, S., Slack, M. P., Heys, M., White, J., and Ramsay, M. E. (2008). Fall in *Haemophilus influenzae* serotype b (Hib) disease following implementation of a booster campaign. *Arch. Dis. Child.* 93, 665–669. doi: 10.1136/adc.2007.126888

Larson, H. J., Jarrett, C., Eckersberger, E., Smith, D. M. D., and Paterson, P. (2014). Understanding vaccine hesitancy around vaccines and vaccination from a global perspective: a systematic review of published literature, 2007–2012. *Vaccine* 32, 2150–2159. doi: 10.1016/j.vaccine.2014.01.081

Lee, D. J., Tighe, H., Corr, M., Roman, M., Carson, D. A., Spiegelberg, H. L., et al. (1997). Inhibition of IgE antibody formation by plasmid DNA immunization is mediated by both $CD4^+$ and $CD8^+$ T cells. *Int. Arch. Allergy Immunol.* 113, 227–230. doi: 10.1159/000237554

Lee, H., Choi, E. H., and Lee, H. J. (2014). Efficacy and effectiveness of extended-valency pneumococcal conjugate vaccines. *Korean J. Pediatr.* 57, 55–66. doi: 10.3345/kjp.2014.57.2.55

Li, X. M., Srivastava, K., Grishin, A., Huang, C. K., Schofield, B., Burks, W., et al. (2003a). Persistent protective effect of heat-killed Escherichia coli producing "engineered," recombinant peanut proteins in a murine model of peanut allergy. *J. Allergy Clin. Immunol.* 112, 159–167. doi: 10.1067/mai.2003.1622

Li, X. M., Srivastava, K., Huleatt, J. W., Bottomly, K., Burks, A. W., and Sampson, H. A. (2003b). Engineered recombinant peanut protein and heat-killed *Listeria monocytogenes* coadministration protects against peanut-induced anaphylaxis in a murine model. *J. Immunol.* 170, 3289–3295. doi: 10.4049/jimmunol.170.6.3289

Lin, S. W., Cun, A. S., Harris-Mccoy, K., and Ertl, H. C. (2007). Intramuscular rather than oral administration of replication-defective adenoviral vaccine vector induces specific CD8+ T cell responses in the gut. *Vaccine* 25, 2187–2193. doi: 10.1016/j.vaccine.2006.11.044

Lindblad, E. B. (2004). Aluminium adjuvants – in retrospect and prospect. *Vaccine* 22, 3658–3668. doi: 10.1016/j.vaccine.2004.03.032

Linhart, B., Hartl, A., Jahn-Schmid, B., Verdino, P., Keller, W., Krauth, M. T., et al. (2005). A hybrid molecule resembling the epitope spectrum of grass pollen for allergy vaccination. *J. Allergy Clin. Immunol.* 115, 1010–1016. doi: 10.1016/j.jaci.2004.12.1142

Linhart, B., Mothes-Luksch, N., Vrtala, S., Kneidinger, M., Valent, P., and Valenta, R. (2008). A hypoallergenic hybrid molecule with increased immunogenicity consisting of derivatives of the major grass pollen allergens, Phl p 2 and Phl p 6. *Biol. Chem.* 389, 925–933. doi: 10.1515/BC.2008.105

Litwin, A., Flanagan, M., Entis, G., Gottschlich, G., Esch, R., Gartside, P., et al. (1996). Immunologic effects of encapsulated short ragweed extract: a potent new agent for oral immunotherapy. *Ann. Allergy Asthma Immunol.* 77, 132–138. doi: 10.1016/S1081-1206(10)63499-0

Loessner, H., Endmann, A., Leschner, S., Bauer, H., Zelmer, A., Zur Lage, S., et al. (2008). Improving live attenuated bacterial carriers for vaccination and therapy. *Int. J. Med. Microbiol.* 298, 21–26. doi: 10.1016/j.ijmm.2007.07.005

Lotter, H., Russmann, H., Heesemann, J., and Tannich, E. (2008). Attenuated recombinant Yersinia as live oral vaccine carrier to protect against amoebiasis. *Int. J. Med. Microbiol.* 298, 79–86. doi: 10.1016/j.ijmm.2007.07.001

Ludwig-Portugall, I., Montermann, E., Kremer, A., Reske-Kunz, A. B., and Sudowe, S. (2004). Prevention of long-term IgE antibody production by gene gun-mediated DNA vaccination. *J. Allergy Clin. Immunol.* 114, 951–957. doi: 10.1016/j.jaci.2004.06.013

Maecker, H. T., Hansen, G., Walter, D. M., Dekruyff, R. H., Levy, S., and Umetsu, D. T. (2001). Vaccination with allergen-IL-18 fusion DNA protects against, and reverses established, airway hyperreactivity in a murine asthma model. *J. Immunol.* 166, 959–965. doi: 10.4049/jimmunol.166.2.959

Magone, M. T., Chan, C. C., Beck, L., Whitcup, S. M., and Raz, E. (2000). Systemic or mucosal administration of immunostimulatory DNA inhibits early and late phases of murine allergic conjunctivitis. *Eur. J. Immunol.* 30, 1841–1850. doi: 10.1002/1521-4141(200007)30:7<1841::AID-IMMU1841>3.0.CO;2-E

Marciani, D. J. (2003). Vaccine adjuvants: role and mechanisms of action in vaccine immunogenicity. *Drug Discov. Today* 8, 934–943. doi: 10.1016/S1359-6446(03)02864-2

Marshall, J. D., Abtahi, S., Eiden, J. J., Tuck, S., Milley, R., Haycock, F., et al. (2001). Immunostimulatory sequence DNA linked to the Amb a 1 allergen promotes T(H)1 cytokine expression while downregulating T(H)2 cytokine expression in PBMCs from human patients with ragweed allergy. *J. Allergy Clin. Immunol.* 108, 191–197. doi: 10.1067/mai.2001.116984

Mast, T. C., Kierstead, L., Gupta, S. B., Nikas, A. A., Kallas, E. G., Novitsky, V., et al. (2010). International epidemiology of human pre-existing adenovirus (Ad) type-5, type-6, type-26 and type-36 neutralizing antibodies: correlates of high Ad5 titers and implications for potential HIV vaccine trials. *Vaccine* 28, 950–957. doi: 10.1016/j.vaccine.2009.10.145

McElrath, M. J., De Rosa, S. C., Moodie, Z., Dubey, S., Kierstead, L., Janes, H., et al. (2008). HIV-1 vaccine-induced immunity in the test-of-concept step study: a case-cohort analysis. *Lancet* 372, 1894–1905. doi: 10.1016/S0140-6736(08)61592-5

McPhillips, H. A., Davis, R. L., Marcuse, E. K., and Taylor, J. A. (2001). The rotavirus vaccine's withdrawal and physicians' trust in vaccine safety mechanisms. *Arch. Pediatr. Adolesc. Med.* 155, 1051–1056. doi: 10.1001/archpedi.155.9.1051

Mohan, T., Verma, P., and Rao, D. N. (2013). Novel adjuvants & delivery vehicles for vaccines development: a road ahead. *Indian J. Med. Res.* 138, 779–795.

Mora, M., Veggi, D., Santini, L., Pizza, M., and Rappuoli, R. (2003). Reverse vaccinology. *Drug Discov. Today* 8, 459–464. doi: 10.1016/S1359-6446(03)02689-8

Morrow, W. J. W., Yang, Y.-W., and Sheikh, N. A. (2004). Immunobiology of the Tomatine adjuvant. *Vaccine* 22, 2380–2384. doi: 10.1016/j.vaccine.2004.03.022

Moylett, E. H., and Hanson, I. C. (2003). 29. Immunization. *J. Allergy Clin. Immunol.* 111, 754–765. doi: 10.1067/mai.2003.83

Muganga, N., Uwimana, J., Fidele, N., Gahimbare, L., Gessner, B. D., Mueller, J. E., et al. (2007). *Haemophilus influenzae* type b conjugate vaccine impact against purulent meningitis in Rwanda. *Vaccine* 25, 7001–7005. doi: 10.1016/j.vaccine.2007.06.032

Murele, B., Vaz, R., Gasasira, A., Mkanda, P., Erbeto, T., and Okeibunor, J. (2014). Vaccine perception among acceptors and non-acceptors in Sokoto State, Nigeria. *Vaccine* 32, 3323–3327. doi: 10.1016/j.vaccine.2014.03.050

Mutschlechner, S., Deifl, S., and Bohle, B. (2009). Genetic allergen modification in the development of novel approaches to specific immunotherapy. *Clin. Exp. Allergy* 39, 1635–1642. doi: 10.1111/j.1365-2222.2009.03317.x

Nagai, Y., Shiraishi, D., Tanaka, Y., Nagasawa, Y., Ohwada, S., Shimauchi, H., et al. (2014). Transportation of sublingual antigens across sublingual ductal epithelial cells to the ductal antigen-presenting cells in mice. *Clin. Exp. Allergy* doi: 10.1111/cea.12329 [Epub ahead of print].

Nathanson, N., and Langmuir, A. D. (1995). The Cutter incident. Poliomyelitis following formaldehyde-inactivated poliovirus vaccination in the United States during the Spring of 1955. II. Relationship of poliomyelitis to Cutter vaccine. 1963. *Am. J. Epidemiol.* 142, 109–140.

Nieuwenhuizen, N. E., and Lopata, A. L. (2005). Fighting food allergy – current approaches. *Nat. Prod. Mol. Ther.* 1056, 30–45.

Noma, T., Yoshizawa, I., Aoki, K., Yamaguchi, K., and Baba, M. (1996). Cytokine production in children outgrowing hen egg allergy. *Clin. Exp. Allergy* 26, 1298–1307. doi: 10.1111/j.1365-2222.1996.tb00527.x

Offit, P. A. (2005). The Cutter incident, 50 years later. *N. Engl. J. Med.* 352, 1411–1412. doi: 10.1056/NEJMp048180

Palese, P. (2006). Making better influenza virus vaccines? *Emerg. Infect. Dis.* 12, 61–65. doi: 10.3201/eid1201.051043

Parashar, U. D., Hummelman, E. G., Bresee, J. S., Miller, M. A., and Glass, R. I. (2003). Global illness and deaths caused by rotavirus disease in children. *Emerg. Infect. Dis.* 9, 565–572. doi: 10.3201/eid0905.020562

Paterson, B. J., and Durrheim, D. N. (2013). Review of Australia's polio surveillance. *Commun. Dis. Intell. Q Rep.* 37, E149–E155.

Pead, P. J. (2003). Benjamin Jesty: new light in the dawn of vaccination. *Lancet* 362, 2104–2109. doi: 10.1016/S0140-6736(03)15111-2

Pead, P. J. (2006). Benjamin Jesty: the first vaccinator revealed. *Lancet* 368, 2202. doi: 10.1016/S0140-6736(06)69878-4

Peng, H. J., Su, S. N., Chang, Z. N., Chao, P. L., Kuo, S. W., and Tsai, L. C. (2002). Induction of specific Th1 responses and suppression of IgE antibody formation by vaccination with plasmid DNA encoding Der f 11. *Vaccine* 20, 1761–1768. doi: 10.1016/S0264-410X(02)00029-4

Pilishvili, T., Lexau, C., Farley, M. M., Hadler, J., Harrison, L. H., Bennett, N. M., et al. (2010). Sustained reductions in invasive pneumococcal disease in the era of conjugate vaccine. *J. Infect. Dis.* 201, 32–41. doi: 10.1086/648593

Plotkin, S. A. (2005). Why certain vaccines have been delayed or not developed at all. *Health Aff.* 24, 631–634. doi: 10.1377/hlthaff.24.3.631

Quinn, H. E., Wood, N. J., Cannings, K. L., Dey, A., Wang, H., Menzies, R. I., et al. (2014). Intussusception following monovalent human rotavirus vaccine in Australia: severity and comparison of using healthcare database records versus case-confirmation to assess risk. *Pediatr. Infect. Dis. J.* doi: 10.1097/INF.0000000000000362 [Epub ahead of print].

Raz, E., Tighe, H., Sato, Y., Corr, M., Dudler, J. A., Roman, M., et al. (1996). Preferential induction of a Th1 immune response and inhibition of specific IgE antibody formation by plasmid DNA immunization. *Proc. Natl. Acad. Sci. U.S.A.* 93, 5141–5145. doi: 10.1073/pnas.93.10.5141

Reardon, S. (2014). Infectious diseases: smallpox watch. *Nature* 509, 22–24. doi: 10.1038/509022a

Riaz, H., and Rehman, A. (2013). Polio vaccination workers gunned down in Pakistan. *Lancet Infect. Dis.* 13, 120. doi: 10.1016/S1473-3099(12)70344-4

Ribeiro-Muller, L., and Muller, M. (2014). Prophylactic papillomavirus vaccines. *Clin. Dermatol.* 32, 235–247. doi: 10.1016/j.clindermatol.2013.08.008

Ribeiro, G. S., Lima, J. B. T., Reis, J. N., Gouveia, E. L., Cordeiro, S. M., Lobo, T. S., et al. (2007). *Haemophilus influenzae* meningitis 5 years after introduction of the *Haemophilus influenzae* type b conjugate vaccine in Brazil. *Vaccine* 25, 4420–4428. doi: 10.1016/j.vaccine.2007.03.024

Roy, K., Mao, H. Q., Huang, S. K., and Leong, K. W. (1999). Oral gene delivery with chitosan – DNA nanoparticles generates immunologic protection in a murine model of peanut allergy. *Nat. Med.* 5, 387–391. doi: 10.1038/7385

Sabato, V., Faber, M., Van Gasse, A., Hagendorens, M. M., Leysen, J., Bridts, C. H., et al. (2014). State of the art and perspectives in food allergy (part II): therapy. *Curr. Pharm. Des.* 20, 964–972. doi: 10.2174/13816128113199990045

Sabin, A. B. (1957). Properties and behavior of orally administered attenuated poliovirus vaccine. *J. Am. Med. Assoc.* 164, 1216–1223. doi: 10.1001/jama.1957.62980110008008

Salk, J. E., Krech, U., Youngner, J. S., Bennett, B. L., Lewis, L. J., and Bazeley, P. L. (1954). Formaldehyde treatment and safety testing of experimental poliomyelitis vaccines. *Am. J. Public Health Nations Health* 44, 563–570. doi: 10.2105/AJPH.44.5.563

Sampson, H. A. (2003). 9. Food allergy. *J. Allergy Clin. Immunol.* 111, S540–S547. doi: 10.1067/mai.2003.134

Sampson, H. A. (2004). Update on food allergy. *J. Allergy Clin. Immunol.* 113, 805–819. doi: 10.1016/j.jaci.2004.03.014

Santeliz, J. V., Van Nest, G., Traquina, P., Larsen, E., and Wills-Karp, M. (2002). Amb a 1-linked CpG oligodeoxynucleotides reverse established airway hyperresponsiveness in a murine model of asthma. *J. Allergy Clin. Immunol.* 109, 455–462. doi: 10.1067/mai.2002.122156

Sato, Y., Roman, M., Tighe, H., Lee, D., Corr, M., Nguyen, M. D., et al. (1996). Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science* 273, 352–354. doi: 10.1126/science.273.5273.352

Saxena, M., Van, T. T., Baird, F. J., Coloe, P. J., and Smooker, P. M. (2013). Pre-existing immunity against vaccine vectors – friend or foe? *Microbiology* 159, 1–11. doi: 10.1099/mic.0.049601-0

Schade, R. P., Tiemessen, M. M., Knol, E. F., Bruijnzeel-Koomen, C. A., and Van Hoffen, E. (2003). The cow's milk protein-specific T cell response in infancy and childhood. *Clin. Exp. Allergy* 33, 725–730. doi: 10.1046/j.1365-2222.2003.01655.x

Schmitt, H.-J., Steul, K. S., Borkowski, A., Ceddia, F., Ypma, E., and Knuf, M. (2008). Two versus three doses of a meningococcal C conjugate vaccine concomitantly administered with a hexavalent DTaP-IPV-HBV/Hib vaccine in healthy infants. *Vaccine* 26, 2242–2252. doi: 10.1016/j.vaccine.2008.02.041

Schoen, C., Loeffler, D. I. M., Frentzen, A., Pilgrim, S., Goebel, W., and Stritzker, J. (2008). Listeria monocytogenes as novel carrier system for the development of live vaccines. *Int. J. Med. Microbiol.* 298, 45–58. doi: 10.1016/j.ijmm.2007.09.002

Serebrisky, D., Teper, A. A., Huang, C. K., Lee, S. Y., Zhang, T. F., Schofield, B. H., et al. (2000). CpG oligodeoxynucleotides can reverse Th2-associated allergic airway responses and alter the B7.1/B7.2 expression in a murine model of asthma. *J. Immunol.* 165, 5906–5912. doi: 10.4049/jimmunol.165.10.5906

Sicherer, S. H., and Sampson, H. A. (2014). Food allergy: epidemiology, pathogenesis, diagnosis, and treatment. *J. Allergy Clin. Immunol.* 133, 291–307; quiz 308. doi: 10.1016/j.jaci.2013.11.020

Simons, F. E., Shikishima, Y., Van Nest, G., Eiden, J. J., and Hayglass, K. T. (2004). Selective immune redirection in humans with ragweed allergy by injecting Amb a 1 linked to immunostimulatory DNA. *J. Allergy Clin. Immunol.* 113, 1144–1151. doi: 10.1016/j.jaci.2004.03.003

Singh, N., Hong, S., Scherer, D. C., Serizawa, I., Burdin, N., Kronenberg, M., et al. (1999). Activation of NK T cells by CD1d and {alpha}-galactosylceramide directs conventional T cells to the acquisition of a Th2 phenotype. *J. Immunol.* 163, 2373–2377.

Slater, J. E., Paupore, E., Zhang, Y. T., and Colberg-Poley, A. M. (1998). The latex allergen Hev b 5 transcript is widely distributed after subcutaneous injection in BALB/c mice of its DNA vaccine. *J. Allergy Clin. Immunol.* 102, 469–475. doi: 10.1016/S0091-6749(98)70137-X

Soares-Weiser, K., Maclehose, H., Bergman, H., Ben-Aharon, I., Nagpal, S., Goldberg, E., et al. (2012). Vaccines for preventing rotavirus diarrhoea: vaccines in use. *Cochrane Database Syst. Rev.* 11, CD008521. doi: 10.1002/14651858.CD008521

Spijkerman, J., Veenhoven, R. H., Wijmenga-Monsuur, A. J., Elberse, K. E., Van Gageldonk, P. G., Knol, M. J., et al. (2013). Immunogenicity of 13-valent pneumococcal conjugate vaccine administered according to 4 different primary immunization schedules in infants: a randomized clinical trial. *J. Am. Med. Assoc.* 310, 930–937. doi: 10.1001/jama.2013.228052

Stacey, K. J., Sester, D. P., Sweet, M. J., and Hume, D. A. (2000). Macrophage activation by immunostimulatory DNA. *Curr. Top. Microbiol. Immunol.* 247, 41–58. doi: 10.1007/978-3-642-59672-8_3

Stahl, P. D., and Ezekowitz, R. A. B. (1998). The mannose receptor is a pattern recognition receptor involved in host defense. *Curr. Opin. Immunol.* 10, 50–55. doi: 10.1016/S0952-7915(98)80031-9

Stevenson, D. D. (2000). Approach to the patient with a history of adverse reactions to aspirin or NSAIDs: diagnosis and treatment. *Allergy Asthma Proc.* 21, 25–31. doi: 10.2500/108854100778248944

Stevenson, D. D. (2003). Aspirin desensitization in patients with AERD. *Clin. Rev. Allergy Immunol.* 24, 159–168. doi: 10.1385/CRIAI:24:2:159

Stewart, A. J., and Devlin, P. M. (2006). The history of the smallpox vaccine. *J. Infect.* 52, 329–334. doi: 10.1016/j.jinf.2005.07.021

Szostak, M. P., Hensel, A., Eko, F. O., Klein, R., Auer, T., Mader, H., et al. (1996). Bacterial ghosts: non-living candidate vaccines. *J. Biotechnol.* 44, 161–170. doi: 10.1016/0168-1656(95)00123-129

Tang, D. C., De Vit, M., and Johnston, S. A. (1992). Genetic immunization is a simple method for eliciting an immune response. *Nature* 356, 152–154. doi: 10.1038/356152a0

Tano, Y., Shimizu, H., Martin, J., Nishimura, Y., Simizu, B., and Miyamura, T. (2007). Antigenic characterization of a formalin-inactivated poliovirus vaccine derived from live-attenuated Sabin strains. *Vaccine* 25, 7041–7046. doi: 10.1016/j.vaccine.2007.07.060

Taylor, L. E., Swerdfeger, A. L., and Eslick, G. D. (2014). Vaccines are not associated with autism: an evidence-based meta-analysis of case–control and cohort studies. *Vaccine* 32, 3623–3629. doi: 10.1016/j.vaccine.2014.04.085

TePas, E. C., Hoyte, E. G., Mcintire, J. J., and Umetsu, D. T. (2004). Clinical efficacy of microencapsulated timothy grass pollen extract in grass-allergic individuals. *Ann. Allergy Asthma Immunol.* 92, 25–31. doi: 10.1016/S1081-1206(10)61706-1

Theves, C., Biagini, P., and Crubezy, E. (2014). The rediscovery of smallpox. *Clin. Microbiol. Infect* 20, 210–218. doi: 10.1111/1469-0691.12536

Tiemessen, M. M., Van Ieperen-Van Dijk, A. G., Bruijnzeel-Koomen, C. A., Garssen, J., Knol, E. F., and Van Hoffen, E. (2004). Cow's milk-specific T-cell reactivity of children with and without persistent cow's milk allergy: key role for IL-10. *J. Allergy Clin. Immunol.* 113, 932–939. doi: 10.1016/j.jaci.2003.12.016

Tighe, H., Takabayashi, K., Schwartz, D., Van Nest, G., Tuck, S., Eiden, J. J., et al. (2000). Conjugation of immunostimulatory DNA to the short ragweed allergen amb a 1 enhances its immunogenicity and reduces its allergenicity. *J. Allergy Clin. Immunol.* 106, 124–134. doi: 10.1067/mai.2000.107927

Toda, M., Sato, H., Takebe, Y., Taniguchi, Y., Saito, S., Inouye, S., et al. (2000). Inhibition of immunoglobulin E response to Japanese cedar pollen allergen (Cry j 1) in mice by DNA immunization: different outcomes dependent on the plasmid DNA inoculation method. *Immunology* 99, 179–186. doi: 10.1046/j.1365-2567.2000.00935.x

Tulic, M. K., Fiset, P. O., Christodoulopoulos, P., Vaillancourt, P., Desrosiers, M., Lavigne, F., et al. (2004). Amb a 1-immunostimulatory oligodeoxynucleotide conjugate immunotherapy decreases the nasal inflammatory response. *J. Allergy Clin. Immunol.* 113, 235–241. doi: 10.1016/j.jaci.2003.11.001

Turcanu, V., Maleki, S. J., and Lack, G. (2003). Characterization of lymphocyte responses to peanuts in normal children, peanut-allergic children, and allergic children who acquired tolerance to peanuts. *J. Clin. Invest.* 111, 1065–1072. doi: 10.1172/JCI200316142

Turell, M. J., Bunning, M., Ludwig, G. V., Ortman, B., Chang, J., Speaker, T., et al. (2003). DNA vaccine for West Nile virus infection in fish crows (*Corvus ossifragus*). *Emerg. Infect. Dis.* 9, 1077–1081. doi: 10.3201/eid0909.030025

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

U.S. Food and Drug Administration (2014). *Common Ingredients in U.S. Lincensed Vaccines.* Available at: http://www.fda.gov/BiologicsBloodVaccines/SafetyAvailability/VaccineSafety/ucm187810.htm [accessed June 26, 2014].

Valenta, R., Ferreira, F., Focke-Tejkl, M., Linhart, B., Niederberger, V., Swoboda, I., et al. (2010). From allergen genes to allergy vaccines. *Annu. Rev. Immunol.* 28, 211–241. doi: 10.1146/annurev-immunol-030409-101218

Vandermeulen, C., Mathieu, R., Geert, L.-R., Pierre, V. D., and Karel, H. (2007). Long-term persistence of antibodies after one or two doses of MMR-vaccine. *Vaccine* 25, 6672–6676. doi: 10.1016/j.vaccine.2007.07.008

Vesikari, T., Karvonen, A., Korhonen, T., Espo, M., Lebacq, E., Forster, J., et al. (2004). Safety and immunogenicity of RIX4414 live attenuated human rotavirus vaccine in adults, toddlers and previously uninfected infants. *Vaccine* 22, 2836–2842. doi: 10.1016/j.vaccine.2004.01.044

Wallner, M., Himly, M., Neubauer, A., Erler, A., Hauser, M., Asam, C., et al. (2009). The influence of recombinant production on the immunologic behavior of birch pollen isoallergens. *PLoS ONE* 4:e8457. doi: 10.1371/journal.pone.0008457

Weiss, R., Scheiblhofer, S., and Thalhamer, J. (2013). Allergens are not pathogens: why immunization against allergy differs from vaccination against infectious diseases. *Hum. Vaccin. Immunother.* 10, 703–707.

Whitney, C. G., Goldblatt, D., and O'brien, K. L. (2014). Dosing schedules for pneumococcal conjugate vaccine: considerations for policy makers. *Pediatr. Infect. Dis. J.* 33(Suppl. 2), S172–S181. doi: 10.1097/INF.0000000000000076

Willyard, C. (2014). Polio: the eradication endgame. *Nature* 507, S14–S15. doi: 10.1038/507S14a

World Health Organization (WHO). (1980). "The global eradication of smallpox: Final report of the Global Commission for the Certification of Smallpox Eradication," in *Global Commission for the Certification of Smallpox Eradication.* Geneva: WHO.

World Health Organization (WHO). (2013). Rotavirus vaccines. WHO position paper – January (2013). *Wkly Epidemiol. Rec.* 88, 49–64.

World Health Organization (WHO). (2014a). Polio vaccines: WHO position paper, January 2014. *Wkly Epidemiol. Rec.* 89, 73–92.

World Health Organization (WHO). (2014b). Global Advisory Committee on Vaccine Safety, 11–12 December 2013. *Wkly Epidemiol. Rec.* 89, 53–60.

World Health Organization (WHO). (2014c). *HIV Programme: Achieving Our Goals Operational Plan 2014–2015.* Lyon: World Health Organization.

Yoshida, A., Nagata, T., Uchijima, M., Higashi, T., and Koide, Y. (2000). Advantage of gene gun-mediated over intramuscular inoculation of plasmid DNA vaccine in reproducible induction of specific immune responses. *Vaccine* 18, 1725–1729. doi: 10.1016/S0264-410X(99)00432-6

Zhou, J., Sun, X. Y., Stenzel, D. J., and Frazer, I. H. (1991). Expression of vaccinia recombinant HPV 16 L1 and L2 ORF proteins in epithelial cells is sufficient for assembly of HPV virion-like particles. *Virology* 185, 251–257. doi: 10.1016/0042-6822(91)90772-4

Zuercher, A. W., Coffin, S. E., Thurnheer, M. C., Fundova, P., and Cebra, J. J. (2002). Nasal-associated lymphoid tissue is a mucosal inductive site for virus-specific humoral and cellular immune responses. *J. Immunol.* 168, 1796–1803. doi: 10.4049/jimmunol.168.4.1796

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.

Received: 02 June 2014; accepted: 30 June 2014; published online: 16 July 2014.
Citation: Baird FJ and Lopata AL (2014) The dichotomy of pathogens and allergens in vaccination approaches. Front. Microbiol. ***5****:365. doi: 10.3389/fmicb.2014.00365*
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Microbiology.
Copyright © 2014 Baird and Lopata. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read, for greatest visibility

COLLABORATIVE PEER-REVIEW
Designed to be rigorous – yet also collaborative, fair and constructive

FAST PUBLICATION
Average 85 days from submission to publication (across all journals)

COPYRIGHT TO AUTHORS
No limit to article distribution and re-use

TRANSPARENT
Editors and reviewers acknowledged by name on published articles

SUPPORT
By our Swiss-based editorial team

IMPACT METRICS
Advanced metrics track your article's impact

GLOBAL SPREAD
5'100'000+ monthly article views and downloads

LOOP RESEARCH NETWORK
Our network increases readership for your article

Frontiers
EPFL Innovation Park, Building I • 1015 Lausanne • Switzerland
Tel +41 21 510 17 00 • Fax +41 21 510 17 01 • info@frontiersin.org
www.frontiersin.org

Find us on