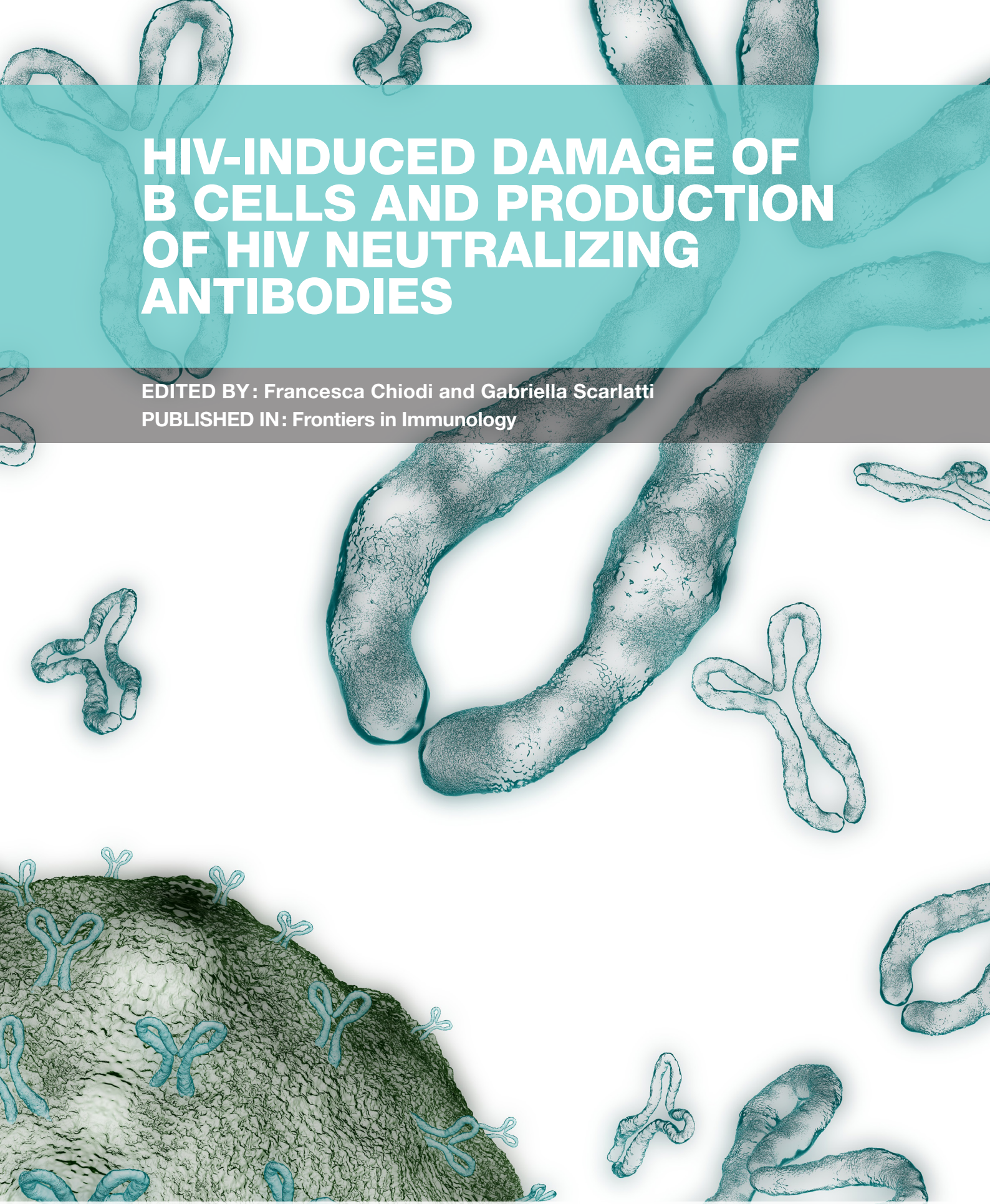

HIV-INDUCED DAMAGE OF B CELLS AND PRODUCTION OF HIV NEUTRALIZING ANTIBODIES

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HIV-INDUCED DAMAGE OF B CELLS AND PRODUCTION OF HIV NEUTRALIZING ANTIBODIES

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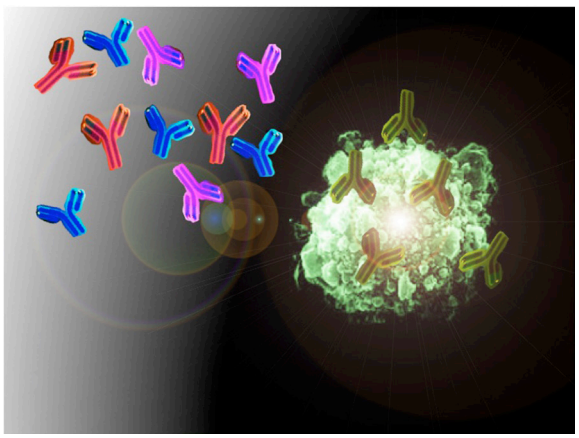

Neutralizing antibodies are needed to block HIV-1 infection of target cells. During HIV-1 infection B cells produce antibodies to several components of the virus. Only a small portion of these antibodies (in green) will neutralize HIV-1 (in green) by binding to the virus envelope spikes. An effective HIV-1 vaccine should induce neutralizing antibodies.

Image: Monica Tolazzi and Gabriella Scarlatti. The image was created for the EAVI2020 Photo Exhibition and permission was obtained from EAVI2020 to reproduce the picture.

Cover image: "Antibodies and plasma cell, artwork." Tim Vernon/Science Photo Library/IBL Bildbyrå.

Multiple dysfunctions take place in the B cell compartment during HIV-1 infection, comprising depletion of resting memory B cells carrying serological memory to vaccines and previously met pathogens. In addition, population of B cells characterized by the expression of exhaustion markers are enlarged during HIV-1 infection.

Antibodies with the capacity to neutralize a broad range of HIV-1 isolates can be detected only in a minority of infected patients, after a year or more from acute infection. An open question is whether the inability of producing neutralizing HIV-1 antibodies is somehow linked to the B cell immunopathology observed in patients.

In this Research Topic, we invited scientists to summarize the current state of knowledge on regulation and development of B cells and antibody responses during HIV-1 infection; fifteen contributions were received comprising both reviews and original articles. The articles are related to B cell dysfunctions identified in HIV-1 infected individuals, production of different types of antibodies (neutralizing versus non neutralizing, and of different isotypes) in vivo during HIV-1 infection and the biological factors which may impact on this process, clinical potential and applications of

anti-HIV antibodies and how to achieve neutralizing antibody responses to HIV-1 epitopes upon vaccination.

The topic has gathered articles on front-line research undertaken in the field of B cells and antibodies in HIV-1 infection. It is our hope that the collection of articles presented in this book may be useful for new and experienced scholars in the field and add a piece to the complex puzzle of knowledge needed for the development of an HIV-1 vaccine.

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EDITORIAL
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Editorial: HIV-Induced Damage of B Cells and Production of HIV Neutralizing Antibodies

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Keywords: HIV, B cells, neutralizing antibodies, vaccination strategies, IgA

Editorial on the Research Topic

HIV-Induced Damage of B Cells and Production of HIV Neutralizing Antibodies

Memory B cells and long-lived plasma cells are pivotal for maintenance of serological memories to vaccines and infections. Studies in HIV-1-infected children and adults have shown that blood resting memory (RM) cells are reduced in number during HIV-1 infection and that their decline correlates with reduction of antibody (Ab) titers against childhood vaccinations [reviewed in Ref. (1, 2)]. Initiation of antiretroviral therapy (ART) shortly after infection restored RM cells to physiological levels in HIV-1-infected children and adults (3, 4) although very few studies have been conducted on this specific topic. One additional interesting feature of HIV-1 immunopathology is that exhausted memory B cells are expanded in circulation during HIV-1 infection; these exhausted cells comprise activated memory B (AM) and tissue-like memory (TLM) B cells, the latter displaying phenotypic similarities with tonsillar B cells (5, 6). The mechanism driving B cell abnormalities during HIV-1 infection remains poorly characterized. One possibility is that expression of inhibitory receptors on the surface of TLM B cells during HIV-1 infection, including the inhibitory receptor Fc receptor-like-4 (FCRL4), may engage specific pathway leading to inhibition of B cell proliferation and Ab production. Reverting *in vivo* the damage which HIV-1 exerts on B cells could possibly result in the production of adequate and persistent levels of HIV-1 neutralizing antibodies (NAbs) able to neutralize a broad range of HIV-1 isolates.

During the course of natural HIV-1 infection, humoral immune responses take place to HIV-1 epitopes resulting in specific Abs with non-neutralizing and neutralizing capacity. Only in a minority of infected individuals, Abs with the capacity to neutralize a broad range of HIV-1 isolates, called broad NAbs (bNAbs), can be detected after more than one year from primary infection. During the last decades a large number of potent HIV-1 bNAbs have been isolated from infected patients, which target the CD4 binding site, determinants within the V2 envelope (env) region, the V3 region or the gp120-gp41 interface region. These bNAbs have been tested in HIV-1 animal models, and phase I and II clinical studies have demonstrated safety in adults and children. Although some Fc-modifications are needed to increase the half-life of bNAbs, there is no doubt that they represent valuable tools in the contexts of HIV-1 prevention and treatment.

The clinical trials conducted with candidate HIV-1 vaccines targeting env showed that it is difficult to elicit high titers of HIV-1 bNAbs in humans. Accordingly, highly innovative approaches need to be applied to this field; integrated knowledge from vaccine design for other pathogens may accelerate the design of preventive or therapeutic HIV-1 vaccines with the property of inducing bNAbs.

In this research topic, we invited scientists to summarize the current state of knowledge on regulation and development of B cells and Abs responses during HIV-1 infection; 15 contributions were received comprising both reviews and original articles. A short introduction of these contributions follows.

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Serological responses to vaccines and establishment of B cell memory is mediated through the interactions between Tfh cells and B cells in germinal centers (GCs). Pallikkuth et al. reviewed current knowledge on Tfh cells and B cells dysfunction in aging and HIV-1 infection. Chronic immune activation during HIV-1 infection affects the expression of molecules important for the function of Tfh cells and other T cell subsets including PD-1 and ICOS; T cell exhaustion could also take place as a result of protracted expression of inhibitory receptors. The number and function of circulating Tfh cells declined during HIV-1 infection but ART treatment resulted in increased frequencies of Tfh cells; however, upon these conditions the frequencies of RM cells remained low.

The expression of FcRL4 and IL-6 is increased in B cells during HIV-1 infection. Increased IL-6 expression leads to aberrant B cell differentiation and FcRL4 acts by dampening B cell receptor (BCR) signaling. Siewe et al. report that the expression of FcRL4 in viremic HIV-1-infected patients identifies an IL-6 producing pro-inflammatory B cell subset. In viremic patients AM and TLM cells expressed the highest levels of FcRL4 and IL-6; in addition, AM cells, followed by TLM cells, comprised the highest frequency of $FcRL4^{hi}IL\text{-}6^{hi}$ cells among B cell subpopulations. The authors present interesting mechanisms linking expression and signaling of FcLR4 with B cell damage and expression of inflammatory cytokines.

It is recommended that children born HIV-1-infected receive ART from birth and further studies should be conducted to analyze whether the damage to RM B cells is prevented by early ART introduction. Cotugno et al. reported that the frequencies of B cell subpopulations did not differ between controls and ART treated HIV-1-infected children who responded to treatment. Gene expression arrays performed on isolated B cells from selected HIV-1-infected patients revealed few differentially expressed genes in purified RM B cells when comparing controls and HIV-1-infected children. It is interesting however that 25 genes were differentially expressed in RM cells at baseline prior to influenza vaccination in the RM cells of vaccine non-responders as compared to vaccine responders. Gene profiles were also derived for AM cells in HIV-1-infected children and controls providing novel findings in the field of B cell damage.

B cells are involved in bone biology in health and disease. In her review, Titanji discusses the contribution of two cytokines produced by B cells, OPG, and RANKL, to HIV-1-induced bone loss. The members of the OPG/RANKL pathway are produced by a large number of cells present in several tissues of three major organ systems: skeletal, vascular, and immune systems. A strong link between joint destruction in rheumatoid arthritis (RA) and pathogenic RANKL producing B cells was found when patients with RA were treated with anti-CD20 Ab Rituximab. This treatment eliminated B cells and reduced also RANKL levels in synovium. Increased longevity in HIV-1-infected individuals receiving ART has been associated with higher prevalence of non-AIDS end-organ comorbidities including osteoporosis and cardiovascular diseases. During HIV-1 infection, the subset of TLM B cells, expanded as result of inflammation, has been linked to increased RANKL production. Both in HIV-1 transgenic rats and in untreated HIV-1-infected individuals an increased RANKL/OPG ratio was described, suggesting a link between the OPG/RANKL pathway and skeletal damage in HIV-1 infection. The exciting possibility of RANKL blockade by already available medicines during HIV-1 infection is discussed to reduce the impact of osteoporosis in aging patients.

Approximately 90%, of new HIV-1 acquisitions take place through mucosal contact. Kulkarni et al. describe how loss of B cells and plasma cells during HIV-1 infection results in a declined production of anti-HIV IgA responses at the mucosal sites. IgA present in mucosal secretions is produced at the mucosal site by plasma cells in the lamina propria and has a critical role for defense against pathogens. HIV-1 infection results in loss of integrity of mucosal barriers which are ultimately devoided of protective IgA and IgG; this scenario may contribute to superinfection with new HIV-1 strains and possibly give rise to the generation of new circulating recombinant HIV-1 forms. Passive immunization with either IgA or IgG is a potent tool to protect macaques from SIV infection at the mucosal level. When combining passive immunization of IgA and IgG, 100% protection was achieved although the mechanism of interactions between these two classes of Abs has yet not been elucidated. Vaccine strategies aimed at the induction of mucosal antibody responses needs to be further developed as preventive and therapeutic tool for HIV-1 infection.

Several HIV-1 bNAbs, especially the ones directed to the CD4 binding site and the gp120-gp41 interface region, also demonstrate specificity for self-antigens. Borhis et al. studied the interaction of B-cell-activating factor (BAFF) with its receptors BAFF-R and TACI. BAFF is a pivotal cytokine for B cell development, which, present at high levels during some autoimmune diseases, leads to increased rescue of self-reacting B cells. BAFF is also overproduced, in membrane-bound and soluble forms, during HIV-1 and SIV infections, where it may contribute to survival of immature transitional B cells, a population of cells which is enlarged during these infections. Based on these findings, the authors aim at understanding whether the interactions between BAFF and its receptors may be useful to enlarge pool of auto-reactive B cells producing bNAbs. These interesting findings point to the possibility that soluble TACI and BAFF-R may act as decoy receptors and that interactions between BAFF and its receptors may have a regulatory role in GC reaction acting on both B and Tfh cells.

Circulating biomarkers could be important to pin-point mechanisms which influence humoral immune responses and the development of HIV-1 bNAbs. Mabuka et al. examined whether dysfunctions taking place in B cell subpopulations during acute HIV-1 infection and the production of cytokines involved in B cell development (BAFF and CXCL13) can be linked to bNAbs development. Pathological changes in the composition of B cell subsets during acute HIV-1 infection were not predictive of the development of bNAbs. Interestingly, early high levels of CXCL13, but not BAFF, correlated with detectable bNAbs at 1-year postinfection. This finding calls for further studies to elucidate how elevated levels of the chemoattractant CXCL13, important for homing of Tfh and B cells to the GCs, may imprint the production of bNAbs.

Further intervention strategies, in addition to ART, may be needed to put an end to mother to child transmission (MTCT)

of HIV-1. Douglas et al. reviewed the possibility that additional therapy opportunities for preventing HIV-1 MTCT may be provided by mapping the detailed specificity of protective maternal HIV-1 NAbs and characterizing the mechanisms through which maternal circulating viruses escape recognition from autologous NAbs. In the context of MTCT, vaccine strategies aimed at eliminating HIV-1 infection in children may only need to elicit Ab responses able to neutralize the virus pool from the mother to which the newborn is exposed. As shown in some of the reviewed studies, passively acquired ADCC mediating Abs from the HIV-1-infected mother may prolong survival in the infected infant; whether ADCC HIV-1 Abs need to be elicited by vaccines to protect children from HIV-1 MTCT should be further investigated.

Departing from the finding of naturally occurring Abs to the CC chemokine receptor 5 (CCR5) in healthy individuals and HIV-1-infected patients, Venuti et al. review the mechanism mediated by these Abs and suggest the use of anti-CCR5 Abs in therapeutic and vaccination strategies to combat viral infections. It is unclear why auto-Abs to CCR5 are produced in absence of autoimmune diseases, but a role for CCR5-Abs in homeostatic control is envisaged. Interestingly, CCR5 auto-Abs modulate CCR5 expression through a long-lasting internalization of this receptor and thus, may block HIV-1 transmission through CCR5, one of the two major chemokine receptors used by HIV-1 in attachment and penetration of target cells. Indeed, several novel immunization approaches have been used to induce anti-CCR5 Abs.

Soldemo et al. compared the induction of NAbs in chronically HIV-1-infected and immunized subjects. The HIV-1 bNAbs isolated from infected patients are generated through an extensive somatic hypermutation process as consequence of prolonged antigenic exposure upon chronic inflammation. Conventional immunization regimens of primates have so far failed to induce HIV-1 bNabs; the reasons for this failure is not known but the complex interplay between HIV-1 antigenic variability and B cell selection occurring *in vivo* may not be easy to mimic upon vaccination. Further studies in different animal models may define similarities and differences in germline antibody genes and expressed repertoires, thus paving the way to the design of effective HIV-1 vaccines.

The review by Molinos-Albert et al. focuses on the opportunities and challenges of utilizing the conserved membrane proximal external region (MPER) region within the Env gp41 protein to evoke bNAbs in HIV-1 immunization protocols. The MPER region, together with the gp41 fusion peptide, is involved in membrane destabilization. Structural and physical properties, including steric hindrance by gp120, do not render this region an easily accessible site to immunological responses. However, the isolation of some potent bNAbs against the MPER conserved region from HIV-1-infected subjects shows that, *in vivo*, this region can be a target of bNAbs. The authors present novel biochemical and immunological strategies on how to render the MPER site more accessible to B cell responses.

Non-neutralizing inhibitory Abs (nNAbs) may play an important role in decreasing HIV-1 load and may be useful in the context of HIV-1 protection. Mayr et al. present challenges and opportunities associated with HIV-1 nNAbs. These nNAbs can bind and capture infectious virus and form immune complexes and aggregates with the virus. Their inhibitory function is mediated through the binding of its Fc-domain to specific FcRs present at the surface of immune cells. Polymorphism of FcRs may pose a limitation to the development of HIV-1 vaccines aimed at inducing nNAbs. An interesting picture is emerging depicting the role that Fc-mediated phagocytosis of immune complexes may have in inducing immune activation and promoting adaptive antiviral responses.

In the review by Hua et al. the authors present the different scenarios where bNAbs may be of clinical utility ranging from preventing viral infection, enhancing therapeutic potential in acute infection and chronic infection. The pharmacological modalities of bNAbs action are multiple and vary from the capacity to enhance adaptive immune responses to potential reduction of virus reservoirs. There are however limitations to be dealt with before bNAbs can be introduced in clinical HIV-1 contexts; for example, selection of resistant viral populations, development of Ab responses directed to the administered bNAbs and risk of eliminating HIV-1 reservoirs in regeneration limited compartments. In this review engineering and biological approaches are widely discussed to overcome limitations to the use of bNAbs.

Modification of the structure of the immunogen is a front-line research topic to increase its capacity to induce and stimulate bNAb responses. Soldemo et al. present in their article how cross-linking of HIV-1 env trimers with glutaraldehyde (GLA) affects thermo-stability and exposure of nNAbs epitopes *in vitro* and env-specific IgG Ab responses *in vivo*. GLA fixation improved the stability of the env-trimers, however at the expense of a lower Ab response to the trimers upon repeated immunizations. Mice inoculated with GLA fixed trimers displayed a more Th2-skewed subclass profile as compared to animals inoculated with native trimers. Coadministration of adjuvants known to balance Th1/Th2 responses were not able to redirect this Th2-skewed profile.

Forsell et al. investigated a mechanism for epitope-specific regulation and maturation of B cell responses. The experimental set-up aimed at pin-pointing the profiles of GC B cell responses evoked by one injection with an env protein in a murine system and at understanding if injection with env-Abs could exert regulation of GC B cell responses in an epitope-specific manner. The results suggest that env-specific B cell responses are negatively regulated through epitope masking by high affinity Abs. Ab-mediated feedback to GC B cells may be effective only when GC B cells share the same specificity with an injected or circulating Ab. This proposed mechanism of Ab-mediated feedback, in addition to unraveling basic aspects of regulation of GC B cell responses, will be important in efforts aimed at developing effective HIV-1 vaccine.

It is our hope that the collection of articles presented in this research topic may be useful for new and experienced scholars in the field and add a piece to the complex puzzle of knowledge needed for the development of an HIV-1 vaccine.

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FC and GS are responsible for the research topic: HIV-induced damage of B Cells and Production of HIV Neutralizing Antibodies (5357).

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MINI REVIEW
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T Follicular Helper Cells and B Cell Dysfunction in Aging and HIV-1 Infection

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T follicular helper (Tfh) cells are a subset of CD4 T cells that provide critical signals to antigen-primed B cells in germinal centers to undergo proliferation, isotype switching, and somatic hypermutation to generate long-lived plasma cells and memory B cells during an immune response. The quantity and quality of Tfh cells therefore must be tightly controlled to prevent immune dysfunction in the form of autoimmunity and, on the other hand, immune deficiency. Both Tfh and B cell perturbations appear during HIV infection resulting in impaired antibody responses to vaccines such as seasonal trivalent influenza vaccine, also seen in biologic aging. Although many of the HIV-associated defects improve with antiretroviral therapy (ART), excess immune activation and antigen-specific B and T cell responses including Tfh function are still impaired in virologically controlled HIV-infected persons on ART. Interestingly, HIV infected individuals experience increased risk of age-associated pathologies. This review will discuss Tfh and B cell dysfunction in HIV infection and highlight the impact of chronic HIV infection and aging on Tfh–B cell interactions.

Keywords: T follicular helper cells and HIV, T follicular helper cells and immunity, HIV and aging, T follicular helper cells and influenza vaccine, T follicular helper cells in aging and HIV

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INTRODUCTION

Chronic infectious diseases, such as HIV infection, and the biological process of aging are known to impact humoral immune responses to vaccination and infection (1–5). The issue of aging during HIV infection has gained importance due to the success of antiretroviral therapy (ART) that can lead to near normal life expectancy and is resulting in increasing the numbers of aging HIV-infected people (3, 6, 7). Older HIV-uninfected individuals in the general population, especially those >80 years develop immune senescence, a term signifying immune defects affecting multiple cell types, characterized by quantitative reduction in hematopoietic stem cells, thymic involution with reduced naive cells and accumulation of effector and memory cell subsets with narrow TCR repertoires with low clonality, and reduced CD4:CD8 T cell ratio (8–11). Memory T cells tend to lose expression of CD28 and their antigen-specific responses are impaired (12). In addition, profound B cell alterations occur in biologic aging characterized by a reduction of the naive B cell pool and qualitative impairment of their function along with reduced vaccine induced immune responses (13–22). Concurrently, increased inflammation coined by the term inflamm-aging (21, 23) occurs with increased C-reactive protein (CRP), D-dimer, IL-6, and TNFα that correlate with occurance of age-associated diseases.

Immunologic changes similar to biologic aging have been described in HIV infection, including accelerated immune senescence and inflammation, with increased IL-6, CRP, and D-dimer (24–26)

despite virologic suppression with ART and have been attributed to persistent immune activation (25, 27–29). Cellular markers of immune senescence, including low CD4:CD8 ratio and higher frequencies of CD57 + CD28− CD4 and CD8 T cells are prominent especially in those who initiate ART at lower CD4 counts. Based upon epigenetic changes, age of HIV inflected people is approximately 5 years greater (and more without viral suppression) than uninfected people (30, 31) of the same chronologic age. They manifest increased risk for non-AIDS morbidity and mortality, including neurocognitive decline, cardiovascular disease, kidney disease, and cancer (32). Because of the associated immune deficiency in both biologic aging and HIV infection, and the aging of HIV-infected population, it is important to determine how the immune systems in HIV-infected and -uninfected differ and to delineate the underlying mechanisms which could lead to therapeutic interventions. This review will focus on cellular basis of vaccine responses in the context of T follicular helper (Tfh) cells and their interaction with B cells, how these cells are affected by HIV infection and finally discuss recent findings on the impact of aging in HIV-infected and -uninfected persons using response to influenza vaccine as a readout of immune competence.

Tfh Cells in Lymph Node (LN) and Periphery

T follicular helper cells are a specialized subset of CD4 T cells in lymphoid organs that express the transcription factor B cell CLL/lymphoma 6 (Bcl-6), with high surface expression of programed death receptor 1 (PD-1) and CXC chemokine receptor 5 (CXCR5) [reviewed in Refs. (33–37)]. During an immune response Tfh cells provide critical signals to antigen-experienced B cells in germinal centers (GCs) to undergo proliferation, isotype switching, and somatic hypermutation (SHM) in order to generate long-lived plasma cells and memory B cells through cellular interaction and cross-signaling for antibody production [reviewed in Refs. (37–39)]. Tfh cell differentiation requires dendritic cell (DC) priming of naive antigen-specific CD4 T cells followed by the interaction with B cells resulting in upregulation of costimulatory molecules such as inducible costimulator (ICOS) and CD40 ligand (CD40L) and secretion of cytokines IL-21 and IL-4 that play a critical role for the ensuing B cell response [reviewed in Refs. (33, 34, 39)].

Because of the difficulties in studying lymphoid tissue in humans, the field has increasingly relied on a circulating subset of memory CD4 T cells that partially resemble LN Tfh cells and have been designated as peripheral Tfh (pTfh) (40–47). The pTfh cells display a memory phenotype and are characterized by expression of CXCR5, the B cell follicle homing molecule, and by secretion of IL-21 during interactions with B cells (42, 48). Unlike LN Tfh cells, pTfh cells express only moderate levels of PD-1 and Bcl-6 but are similar in their ability to upregulate costimulatory molecules such as ICOS and CD40L upon antigen stimulation (42, 49–52). More recently, based on the surface expression of CXCR3, CCR6 and CXCR4 Tfh cells have been further characterized as Th1 (CXCR3 + CCR4 − CCR6−), Th2 (CXCR3 − CCR4 + CCR6−), and Th17 (CXCR3 − CCR4 − CCR6+) memory CD4 T helper subtypes (42, 53, 54), indicative of reveals the heterogeneous nature of pTfh cells with respect to phenotypic, functional and transcription factor profiles (42, 54). It is now widely considered that a balance of pTfh subsets is important for maintaining healthy immune function.

Tfh, B cells, and HIV infection

T follicular helper cells are highly permissive to HIV becoming readily infected by follicular DC that transport infectious virions into lymphoid organs. Tfh cells are now considered as major reservoirs of transcriptionally silent integrated HIV genomes (55–58). In non-human primates, chronic infection with simian immunodeficiency virus (SIV) is associated with an expansion of Tfh cells within GC (59, 60), along with increase in numbers of B cells in LN, spleen, and gut tissues of rhesus macaques (60–63). Early initiation of ART can rapidly control the virus replication but not the early lymphoid activation, thereby increasing the risk of infection of Tfh and magnitude of viral reservoir (64). Contrary to the expansion of GC Tfh cells seen in chronic HIV/SIV infection (59, 60), we and others have reported a significant loss of circulating pTfh cells in chronic viremic HIV-infected subjects compared to HIV-uninfected persons (65, 66); 12 months of ART incorporating Raltegravir resulted in increased frequencies of pTfh cells (66). However, pTfh cells from HIV$^+$ virologically suppressed patients on ART exhibit functional impairment in their ability to provide adequate B cell help in a number of systems (41, 67–69).

In chronic HIV infection, B cells exhibit immune dysfunction and altered B cell subset distribution, with a shift in resting memory (RM) B cells to an activated state with expression of activation markers such as CD71, CD80, and CD86 (70, 71). There is also an increase in inflammatory B cell subsets referred to as double negative (DN: CD27 − IgD − B cells) and tissue-like memory B cells (15, 72–75). ART-mediated viral suppression restores many of the B cell defects, especially when initiated during the acute phase of infection (76). However, reduced frequencies of RM B cells, elevated DN B cells, as well as chronic immune activation persist (31, 71, 77–79).

Vaccine-Induced Antibody Responses During HIV Infection

In healthy states, antibody responses to T-dependent antigens are generated in GCs within lymphoid tissue when antigen-primed B and T cells engage in interactions to promote B cell differentiation, SHM, and class switch recombination to develop into memory B cells and plasma cells (80–83). Studies in humans and animal models indicate that HIV infection affects the GC reaction, increases immune activation/exhaustion of lymphocytes, and results qualitative deficiency of Tfh and B cell function (57, 59–61, 69). These defects altogether lead to increased susceptibility to vaccine-preventable diseases (84, 85). Studies focusing on pTfh cells have been informative for understanding the phenotypic complexity within the Tfh subset and for determining the relationship between Tfh and B cells in immunological outcomes [reviewed in Ref. (86)].

Influenza vaccine studies have provided a valuable model system to analyze the immune system in vaccine induced antibody

responses (87). We initiated such studies in virally suppressed HIV+ adults on ART during the 2009/H1N1 pandemic influenza outbreak (43, 88, 89). Following monovalent H1N1 vaccination, vaccinees were classified as vaccine responders (VRs) if postvaccination hemagglutination inhibition (HAI) serum H1N1 Ab titer was 1:40 or more and exhibited a 4-fold increase, from baseline titer, and those who did not meet these criteria were classified as vaccine non-responders (VNRs). In study participants, administration of the vaccine resulted in VR status only in 50% HIV+, compared to all age matched healthy controls. In the HIV + VR and VNR, prevaccination CD4 and CD8 T cell counts, B cell frequencies, and plasma HIV RNA were similar, but phenotypic and qualitative immunological differences were identified. In VR, there was upregulation of IL-21R in B cells that correlated with plasmablasts and memory B cell responses post-vaccination (89), together with an expansion of pTfh cells with secretion of IL-21 and CXCL-13 in H1N1-stimulated PBMC culture supernatants. In coculture experiments, pTfh supported HIN1-stimulated IgG production by autologous B cells (43). More recent findings point to the ability to perform qualitative assessment of pTfh/CD4 T cells and B cells prior to immunization in previously vaccinated HIV+ children and young adults (90, 91). Examples of such assessments include (i) *ex vivo* stimulation with H1N1 resulting in induction of CXCR5 mRNA and protein in CD4 T cells and (ii) induction of *IL21* gene in pTfh cells. These antigen-specific prevaccination measures strongly associated with H1N1-specific B cell responses by ELISPOT at postvaccination (91). Interestingly, CD4 T cells from VNR exhibit increased expression of *IL2* and *STAT5* genes, which are known to antagonize pTfh function (92). Our main findings of pTfh and B cells in relation to vaccine responses are summarized in **Table 1**. Other vaccine studies have shown associations between pTfh expansion and phenotype with vaccine response. Expansion of HIV-specific PD-1 + ICOS + pTfh correlated with vaccine-specific serum IgG after booster immunization in three different human HIV vaccine trials (93). Expression of ICOS, PD-1, CD38, and IL-21 in pTfh subsets have been useful for evaluating the influenza vaccine response in HIV-infected and -uninfected adults in other studies as well (50, 87, 93–95). Studies with Ebola vaccine (rVSV-ZEB OV) demonstrated that CXCR5 + PD-1 + pTfh correlated with expansion of plasmablasts (96). Taken together, these studies support the concept that both quality and quantity of pTfh cells are important determinants for the outcome of vaccine response in HIV infection.

TABLE 1 | Signature immunological changes in pTfh and B cells in vaccine responders (VRs) following influenza vaccine at TO (baseline), T1 (7 days), and T2 (4 weeks).

Changes in pTfh cell compartment in vaccine responders
Antigen induced IL-21 gene expression at TO
Expansion of pTfh at T1, T2
Ag-stimulated intracellular IL-21 production in pTfh at T2
"Help" to autologous B cells for H1N1-specific IgG production and B cell differentiation in pTfh plus B cell cocultures at T2
B cell changes in vaccine responders
Increase in frequencies of plasmablasts at T1
Increase in spontaneous H1N1-specific ASC at T1
Increase in memory B cells and switch memory at T2
Upregulation of IL-21R on total B and memory B cells at T2
Increase in TACI expression on total B and memory B cells at T2
Downregulation of BAFT-R expression on total B and memory B cells at T2
PBMC culture sups/plasma findings in vaccine responders
Production of IL-21 and CXCL13 in H1N1-stimulated culture sups with increases in plasma IL-21
Increase in plasma BAFF and APRIL levels

pTfh, peripheral T follicular helper; PBMCs, peripheral blood mononuclear cells; Ab, antibody; BAPF-R, B cell activating factor receptor; APRIL, a proliferation inducing ligand; CXCL13, C-X-C motif chemokine ligand 13; ASCs, antibody secreting cells.

Tfh Cells and B Cells in HIV and Aging

Our group has been interested in the question of immune function of aging HIV+ individuals who are well controlled on ART, the extent to which it resembles biologic aging of HIV− individuals, and implications of aging with HIV infection. Earlier pilot studies in virologically suppressed postmenopausal women as representative of an aging population established the persistence of inflammation and gut microbial translocation and detrimental role of underlying immune activation on influenza vaccine responses that were associated with quantitative and qualitative deficiencies of pTfh cells (45, 97, 98). Our studies showed lower H1N1 influenza antibody titers in HIV-infected women compared to HIV-uninfected women at prevaccination. Following vaccination, magnitude of antibody responses and frequency of study participants achieving seroprotective titers were lower in HIV+ than in HIV− women. Frequencies of pTfh cells at postvaccination correlated with memory B cell function and H1N1 antibody titers. Antibody responses postvaccination were inversely correlated with inflammatory cytokine TNFα in plasma and with markers of cellular immune activation (CD38 and HLA-DR) on CD4 T cells, including pTfh subset, indicating an adverse influence of baseline immune activation and inflammation on vaccine induced antibody response in older age.

To examine the role of age and HIV infection further, we are engaged in a large ongoing study (99, 100) in virologically suppressed HIV+ and HIV− adults grouped by age as young (<40 years), middle aged (40–59 years), and old (≥60 years). Following seasonal trivalent influenza vaccine (TIV), magnitude of Ab titers against each vaccine strain were found to be lower in old age compared to others, regardless of HIV status. Baseline titers in seroprotective range were higher in HIV+ but the frequency of VR was lower in HIV+ than HIV−. Interestingly the young HIV+ showed maximum variance from HIV− and more rapid decay in titer after peak at 28 days postvaccination. In statistical analysis somewhat surprisingly effect of age rather than HIV dominated the impaired immune response observed in old persons (age > 60 years), whereas HIV clearly had a strong effect on immunity at younger ages (99, 100).

We examined phenotypic characteristics of T and B cells in this group of participants prior to vaccination. T cell phenotypic analysis revealed a core signature of aging comprised of decreasing naive T cells and a loss of CD38 expression on CD4 and CD8 T cells. Frequencies of activated CD4 T cells (and not CD8 T cells) identified by coexpression of HLA-DR and CD38,

as well as expression of PD-1, ICOS, and Ki-67 were higher in HIV$^+$ participants compared to HIV$^-$ participants. Increases in activation markers previously associated with aging such as ICOS (87) were already evident in young HIV$^+$ compared to young HIV$^-$, indicative of HIV causing a state of premature immune senescence. Predictive modeling to determine the key T cell variables most closely associated with vaccine response revealed pTfh as an important biomarker. In HIV$^-$, baseline pTfh frequency was positively associated with vaccine response, while in HIV$^+$ expression of multiple activation markers on pTfh (including PD-1) was negatively associated with vaccine response (99).

Prevaccination status of B cells also revealed perturbations as evidenced by alteration in markers of activation, exhaustion and immune regulation and were more prevalent in young HIV$^+$ than in young HIV$^-$ (100). HIV infection in younger adults exhibited similarities with biological aging resulting in alterations in B cell phenotypic and functional characteristics similar to those observed in older HIV$^-$ individuals but underlying mechanisms appear to be distinct from that associated with biological aging (100). For example, the interaction between T and B cells through the PD-1:PD-L1 signaling pathway is involved only in HIV induced impairment of B cell function (101). These results provide the basis for immune correlates of premature aging in HIV$^+$, even with prolonged ART-induced virological suppression (**Figure 1**). Additional mechanistic studies to understand the cellular basis of immunological impairments in pTfh and B cells in aging and HIV infection are currently ongoing in our laboratory.

Other factors that could influence the influenza vaccine response in aging also need consideration. Data from literature suggest that vaccine-induced immune responses are considerably influenced by demographic variables such as age, sex, ethnicity, and race (102–105). Many studies indicate that aged females consistently have higher antibody responses and increased vaccine efficacy to influenza vaccines than males [reviewed in Refs. (106, 107)].

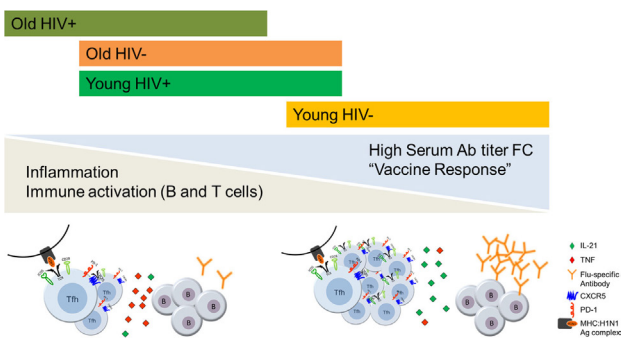

FIGURE 1 | The effects of aging and HIV infection on T follicular helper (Tfh):B cell responses to influenza vaccination. Persistent inflammation and immune activation of CD4 T cells and B cells negatively influence the outcome of influenza vaccine response in antiretroviral therapy (ART)-treated HIV-infected virologically suppressed individuals through impairing the Tfh and B cell functions. HIV induced premature Immunosenescence further advanced immune dysfunction which is more evident in the young HIV$^+$ individuals.

Sex differences in HAI antibody titers to either the standard-dose or high-dose influenza vaccine are apparent, in which antibody responses are higher in older females than in males (108, 109). A role played by male hormone testosterone in lowering the immune response has been proposed (109, 110). There is growing interest in how latent cytomegalovirus (CMV) infections impact the outcome of vaccination [reviewed in Ref. (111)]. In young adults, CMV infection is associated with elevated antibody responses to influenza vaccines. In aged individuals, CMV seropositivity is associated with chronic inflammation and lower antibody responses to influenza vaccines (112, 113). However, lack of association between CMV status and influenza response in elderly population has also been reported (114). Thus the overall impact of CMV infection on influenza vaccine responsiveness remains controversial. A direct link between CMV seropositivity with increased risk of influenza illness in vaccinated older adults has not been reported in either HIV-infected or healthy individuals. Moreover, the influence of gender and CMV infection status on the cellular basis of immune impairment involving pTfh and B cell compartments are not been studied in aging and HIV infection. In aged mice, CD4$^+$ and CD8$^+$ T cells express several inhibitory receptor molecules, including PD-1, LAG-3, CTLA-4, and KLRG1 (115, 116) that could interfere with the immune response to vaccination. Prolonged expression of inhibitory molecules is a well-known feature of T cell exhaustion in chronic viral infections and exhausted T cells have also been identified in different viral infections, such as HIV and hepatitis A and B virus in humans [reviewed in Refs. (117–120)]. However, further studies are warranted to elucidate the significance of T cell exhaustion in HIV infection in the context of aging and its influence on vaccine induced immune response through regulation of pTfh and B cell function.

CONCLUSIONS AND FUTURE PERSPECTIVES

Development of a protective antibody response to vaccine or infection is important for the control or eradication of many pathogenic infections. Efficient Tfh–B cell interactions are required for regulating B cell differentiation toward the development of high affinity antibodies. Immune mechanisms underlying the regulation of Tfh–B cell interactions at the inductive sites of the immune response are an active area of immunology research. Several studies have highlighted the qualitative and quantitative impairment of Tfh compartment and their subsequent impact on humoral arm of immune response in treated HIV infection (43, 45, 67, 87, 94, 98). Since HIV-infected people are aging, research on the cumulative impact of premature and physiological immune senescence on immune function in HIV infection is of great importance. Our work underscores the adverse effect of inflammation, a cardinal feature associated with biologic aging and chronic HIV infection, on immune response to vaccination and functional impairment of Tfh and B cells as a consequence of persistent immune activation.

Recent advances in the field of immune checkpoint inhibitor-based immunotherapeutic approaches in cancer immunology

have highlighted the importance of cell to cell interactions on immune function. Many aspects of checkpoint molecule-based regulation of humoral immune response on Tfh and B cell interactions at the GC are not known. Trials employing checkpoint inhibitors in HIV infection will need to ensure that improved Tfh–B cell interactions not associated with autoimmunity. Immune checkpoints are negative regulators of T cell activation, T cell proliferation and effector functions and inhibiting immune checkpoints could influence and disrupt the resting status of latently infected cells and reverse latency with increase in HIV replication within GC (121). Future studies are needed to explore combination approaches targeting immune checkpoint molecules and costimulatory signaling pathways during an immune response to understand the coregulation of immunity by these molecules in the GC reaction. The ultimate goal should be to establish strategies to improve the immune function at inductive sites. Interventions aimed at reducing chronic inflammation and immune activation along with immunomodulatory approaches may improve response to vaccines in aging HIV$^+$ individuals.

AUTHOR CONTRIBUTIONS

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

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FcRL4 Expression Identifies a Pro-inflammatory B Cell Subset in Viremic HIV-Infected Subjects

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In autoimmune diseases, toll-like receptor (TLR)-stimulated pro-inflammatory IL-6-secreting B cells exert pathogenic roles. Similarly, B cell Fc receptor-like 4 (FcRL4) expression amplifies TLR stimulation, and in rheumatoid arthritis patients, FcRL4 expression identifies a pro-inflammatory B cell subset. B cells from HIV-infected subjects also express heightened levels of FcRL4 and secrete high levels of IL-6: a critical mediator of HIV disease progression. In this study, we sought to determine if FcRL4 identifies a pro-inflammatory B cell subset in HIV-infected subjects and further elucidate the mechanisms underlying FcRL4 amplification of TLR stimulation. We determine that tissue-like memory B cells express the highest endogenous levels of FcRL4 positively correlating with IL-6 expression ($p = 0.0022$, $r = 0.8667$), but activated memory B cells exhibit the highest frequency of FcRL4^{hi}IL-6^{hi} cells. FcRL4^{hi} B cells exhibit an activated TLR-signaling pathway identified by elevated phosphorylation levels of: pERK ($p = 0.0373$), p38 ($p = 0.0337$), p65 ($p = 0.1097$), and cJUN ($p = 0.0239$), concomitant with significantly elevated expression of the TLR-signaling modulator hematopoietic cell kinase (HcK, $p = 0.0414$). Compared to FcRL4^{neg} B cells from healthy controls, TLR9-stimulated FcRL4^{pos} B cells express significantly higher levels of IL-6 ($p = 0.0179$). Further, TLR9-stimulated B cells also upregulate the expression of FcRL4 ($p = 0.0415$) and HcK ($p = 0.0386$). In B-cell lines, siRNA-mediated HcK knockdown downmodulates TLR9-induced FcRL4-mediated activation quantified by CD23 upregulation ($p = 0.0553$). We present data suggesting that, in viremic HIV-infected individuals, FcRL4 expression identifies unique IL-6 producing pro-inflammatory B-cell subsets. Further, TLR stimulation likely modulates FcRL4 expression and FcRL4 expression is associated with Hck, potentially enhancing the activation of TLR-signaling associated transcription factors. Pathogenic B-cells have been identified in other disease settings, and this study represents a novel report describing a pro-inflammatory B cell subset in HIV-infected patients.

Keywords: Fc receptor-like 4, pro-inflammatory cytokine, HIV, B cells, IL-6, viremic

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INTRODUCTION

The elevated serum level of the pro-inflammatory cytokine IL-6 is an indicator of chronic immune activation and a driver of HIV disease progression (1, 2). During HIV infection, IL-6 overexpression drives B-cell proliferation, enhances secretion of antibodies, and leads to aberrant B cell terminal differentiation (3, 4). Further, *in vitro*, IL-6 has been shown to drive HIV replication and, in HIV-infected individuals, the observed high levels of IL-6 are associated with increased mortality and morbidity (5, 6). Due to these factors, it is critical to determine the sources of IL-6 as well as the mechanisms underlying IL-6 overexpression during HIV infection. HIV infection is characterized by heightened microbial translocation and the presence of microbial products encoding toll-like receptor ligands (TLR-L) (7–9). TLR-stimulated monocytes have been identified to be a significant contributor to the HIV-induced inflammatory state (10–12). However, published data also suggest that B cells from HIV-infected individuals express high levels of IL-6 possibly due to TLR-stimulation (3, 9, 13). Additionally, in autoimmune diseases, TLR-stimulated B-cells are critical mediators of inflammation (14, 15). Further, data from a study in rheumatoid arthritis identified a pro-inflammatory B-cell subset expressing high levels of Fc receptor-like 4 (FcRL4) (16). FcRL4 acts as a molecular switch, dampening B cell receptor (BCR) signaling while simultaneously enhancing TLR-signaling through association of SHP-1 and SHP-2 with its cytoplasmic tail (17). Finally, B cells from HIV-infected viremic subjects exhibit heightened FcRL4 expression associated with an "exhausted" phenotype, with impaired antibody expressing functions (18–20).

In this study, we investigated: (1) if in untreated HIV infection, FcRL4^{hi} B-cells represent a pro-inflammatory B cell subset and (2) the mechanisms underlying FcRL4 expression and amplification of TLR-signaling. Our data indicate that FcRL4^{hi} B-cell subsets are high producers of IL-6, and TLR-signaling modulates FcRL4 expression. Finally, FcRL4 mediates amplification of TLR-signaling likely by recruiting Src Kinase proteins.

MATERIALS AND METHODS

Study Participants

All studies were performed after signed, informed written research consent by each study subject. The study was reviewed and approved by the Institutional Review Board of the Rush University Medical Center, and the University of Iowa City VAMC and University of Iowa. All work was performed in adherence with appropriate laboratory safety protocols such as use of personal protective equipment. HIV-infected viremic (HIV_{VIR}), naïve subjects had a median CD4 count of 466 cells/μl (range, 144–566), and median viral load of 20,000 copies/ml (range, 2,000–117,000) (**Table 1**).

TABLE 1 | HIV viremic cohort description.

Participant	Age (years)	Gender	CD4 count (cells/μL)	Viral load (copies/mL)
1	40	M	566	10,000
2	27	F	443	80,000
3	45	F	515	117,000
4	57	M	592	14,000
5	46	M	337	38,000
6	39	M	550	3,000
7	46	F	217	34,000
8	36	M	144	2,000
9	23	M	489	5,000
10	56	F	288	26,000

M, male; F, female.

Cell Lines

Ramos (a human Burkitt lymphoma cell line) FcRL4 stable transfectants were a generous gift from Dr. Susan Pierce (NIH) and previously described (17). The FcRL4.FFF mutant carries mutations (tyrosine to phenylalanine) in the cytoplasmic ITIM tail at positions 451, 463, and 493. The cells were maintained in RPMI medium supplemented with 10% FBS, Pen/Strep, 2mM L-glutamine, 10 mM HEPES, and 55 μM β-mercaptoethanol (Invitrogen).

Antibodies

Cells were stained with the following antibodies: FcRL4-APC (Biolegend), IL-6-PE, CD23-PE-Cy7, CD19-PE-Texas Red, CD10-Pe-Cy5, CD21-V450, CD27-AF700, phospho-p38-PE, phospho-Erk-AF647, phospho-p65-PE, phospho-C-Jun-FITC, (BD Biosciences), Sheep anti-rabbit IgG-DyLight 488 (Biolegend), purified hematopoietic cell kinase (Hck), and phospho-Hck (Abcam).

Isolation, Purification, and TLR Stimulation of PBMCs

PBMCs were isolated from whole blood using Ficoll (Lymphocyte® Cell Separation Media, Mediatech) gradient centrifugation. Cryopreserved PBMC from HIV-infected subjects were used in the immunophenotyping experiments. The cells were stained with FcRL4-APC and CD19-PE-Texas Red and CD19^{+}FcRL4^{pos} and CD19^{+}FcRL4^{neg} B cells were FACS purified and cultured overnight in the presence of 10 μg/ml CpG-B ODN2006 (TLR9L), 2 μg/ml PAM3CSK4 (TLR2L), or 2 μg/ml Imiquimod (InvivoGen). B cells (CD19^{+}) from healthy controls were purified from PBMC using the B Cell Isolation Kit II (Miltenyi Biotec) and AUTOmacs (Miltenyi Biotec). After 4H, the cultures of CD19^{+} B cells were supplemented with Brefeldin A (1:1,000, BD). After overnight incubation, the cells were surface stained (CD23-PE-Cy7, BD Biosciences), fixed/permeabilized (Fix/Perm Kit BD Biosciences), and stained for intracellular IL-6 (IL-6-PE, BD Biosciences). All samples were acquired on an LRSII (BD Biosciences) flow cytometer and the data analyzed using FlowJo software (Tree Star Inc.). Florescence parameters were normalized using Rainbow Calibration Particles (Spherotech) and antibody bound CompBead (BD Biosciences). Gating was determined by unstained controls.

Inhibition Assays

Chemical inhibition of Hck was achieved using PP2 (Millipore). Cells were incubated overnight with indicated concentrations of

the inhibitor, supplemented with TLR9-L, and further cultured overnight. Only events corresponding to living cells (determined by Live/Dead® Fixable Aqua staining, Life Technologies) were acquired on an LRSII (BD Biosciences) flow cytometer and the data analyzed using FlowJo software (Tree Star Inc.).

Real-time RT-PCR

RNA was extracted using the RNeasy Kit (QIAGEN) according to the manufacturer's instructions. The extracted RNA was measured by spectrophotometer and equimolar concentrations used for cDNA synthesis according to the manufacturer's instructions (iScript cDNA syntesis Kit, Bio-Rad). The following primers were used for the qPCR reaction: HcK-Forward 5′-CGGATCCCACATCCACCATCA-3′, Reverse 5′-ACCACGATGATGTCCTCAGAGC-3′, FcRL4-Forward 5′-TCAGCTGGGAGAAGAAGAGGAA-3′, Reverse 5′-GAGTTATCTGGGTGTTGTGTCTTTACC-3′, GAPDH-Forward 5′-CTTCAACGACCACTTTGT-3′ and reverse 5′-TGGTCCAGGGGTCTTACT-3′. Real-time RT-PCR was performed using a Quantitect SYBR Green PCR kit (Qiagen) in a 7900HT Fast Real-Time PCR system (Applied Biosystems). Melting curve analysis was performed to ensure that the primers amplified the desired amplicon and that primer-dimers were absent. Fold change in mRNA expression was calculated by relative quantification using the comparative cycle threshold method. *GAPDH* expression was used as an endogenous control.

siRNA-Mediated Knockdown

siRNA targeting HcK were purchased from Santa Cruz Biotechnology and Dharmacon, and cells were transfected using the Lipofectamine RNAiMax kit (Life Technologies) according to the manufacturer's instructions. Knockdown was confirmed by qPCR 48H post-transfection.

Statistical Analysis

Results are expressed as mean ± SEM or as indicated. GraphPad Prism software, version 5.03 was used for all statistical analysis. The statistical significance *p*-value between group parameters was determined using either unpaired or paired Student's *t*-test (with a confidence level of 95%). The statistical dependence between variables was calculated using the Spearman rank correlation analysis. *p*-Values of <0.05 were considered statistically significant. Pair and multiple comparisons were done using the Wilcoxon-matched-pairs signed rank test.

RESULTS

FcRL4^{hi} B-Cell Subsets from HIV-Infected Viremic Subjects Spontaneously Express High Levels of IL-6

In rheumatoid arthritis patients, FcRL4 expression identifies a pro-inflammatory B-cell subset (16). Differential FcRL4 expression among B cell subsets has been reported in HIV viremic (HIV_{VIR}) subjects (18); however, the relationship between FcRL4 expression and production of pro-inflammatory cytokines has not been fully elucidated. Our prior data indicate that B cells from HIV-infected individuals express primarily IL-6 and not TNF-α (9). We investigated if FcRL4 expression on B cell subsets from HIV_{VIR} subjects associated with heightened endogenous levels of IL-6 expression. Tissue-like memory B cells (TLM, $CD19^+CD20^+CD10^-CD21^{lo}CD27^-$) expressed the highest levels of FcRL4 among different B cell subsets (**Figures 1A,B**), comparable to activated memory B cells (AM, $CD19^+CD20^+CD10^-CD21^-CD27^+$), but significantly higher than naïve B cells (N, $CD19^+CD20^+CD10^-CD27^-CD21^+$, $p<0.0001$) and resting memory B cells (RM, $CD19^+CD20^+CD10^-CD21^+CD27^+$, $p < 0.0001$). TLM B cells also expressed the highest endogenous levels of IL-6 (**Figure 1B**) compared to naïve ($p = 0.01081$) and RM B cells ($p = 0.0204$). Likewise, in AM B cells (**Figure 1B**); the level of IL-6 was much higher as compared to naïve ($p = 0.0041$) and RM B cells ($p = 0.0241$). Moreover, AM cells expressed the highest frequency of $FcRL4^+IL\text{-}6^+$ cells (**Figure 1B**): significantly higher than TLM ($p = 0.005$), N ($p < 0.0001$) and RM ($p < 0.0001$) B cells. Taken together, TLM and AM B cells express the highest levels of FcRL4 and IL-6 as well as the frequency of $FcRL4^+IL\text{-}6^+$ cells. Finally, in the TLM B cells, we observed a significant positive correlation between the FcRL4 and IL-6 expression (**Figure 1C**, $p = 0.0022$, $r = 0.8667$) as well as FcRL4 and HIV viral load (**Figure 1C**, $p = 0.0390$, $r = 0.6727$).

FcRL4^{pos} B Cells from HIV-Infected Viremic (HIV_{VIR}) Subjects Constitutively Exhibit an Activated TLR-Signaling Cascade

HIV-infection is associated with an increase in serum concentration of several TLR ligands (7–9), and B cells from HIV_{VIR} individuals exhibit enhanced FcRL4 expression (18). As FcRL4 enhances B-cell responsiveness to TLR stimulation (17), we next investigated if, in HIV_{VIR} subjects, constitutive FcRL4 expression is associated with an activated TLR-signaling pathway. We determined that FcRL4^{pos} B cells of HIV_{VIR} subjects exhibit a constitutively activated TLR-signaling pathway phenotype characterized by significantly elevated levels of phosphorylated ERK, p38, and c-JUN (**Figures 2A,B**, $p = 0.0373$, $p = 0.0337$, and $p = 0.0239$, respectively). Although the level of phosphorylated p65 was higher in FcRL4^{pos} B cells than FcRL4^{neg} B cells, the difference did not attain statistical significance (**Figure 2B**, $p = 0.1097$).

FcRL4^{pos} B Cells from HIV-Uninfected Subjects Are Highly Responsive to TLR Stimulation

We previously demonstrated that TLR stimulated B cells from healthy controls (HIV_{NEG}) subjects upregulate expression of the pro-inflammatory cytokine IL-6 (9). We, therefore, examined if FcRL4 modulates the expression of IL-6 upon TLR stimulation. We found that compared to FcRL4-negative (FcRL4^{neg}) B cells, TLR stimulation of purified FcRL4^{pos} B cells significantly upregulated IL-6 expression (**Figure 3**: TLR2, $p = 0.0022$, TLR7, $p = 0.0286$, TLR9, $p = 0.0179$).

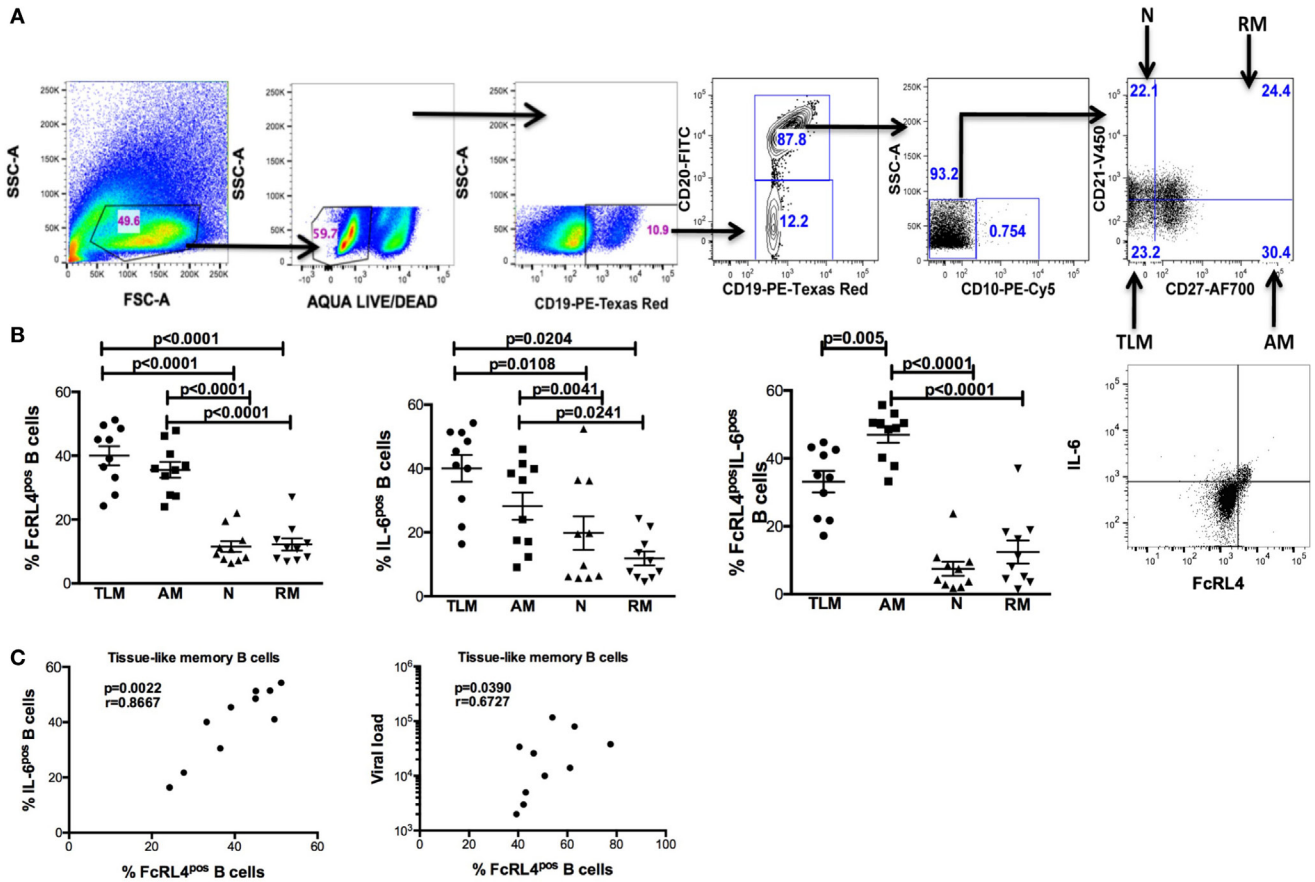

FIGURE 1 | FcRL4^{hi} blood B cell subsets from HIV viremic subjects express high endogenous levels of IL-6. In blood B cell subsets [**(A)**, gating strategy] from HIV viremic subjects (n = 10), the endogenous levels of **(C)** FcRL4, IL-6, and FcRL4^{+}IL-6^{+} [representative plots depicted in **(B)**] as well as **(C)** the relationship between FcRL4 expression and IL-6 (left) and viral load (right) on tissue-like memory B cells were determined; N, naïve; RM, resting memory; AM, activated memory; TLM, tissue-like memory. p-Values as determined by Mann–Whitney test are indicated, in **(C)** association was calculated using the spearman correlation.

B Cells Exposed to TLR-9 Ligand Upregulate Expression of FcRL4 and HcK Concomitantly

Elevated FcRL4 expression on blood B cells has been identified in malaria and HIV-infected viremic patients (18, 19), conditions associated with heightened serum levels of TLR ligands (7, 8, 21). Additionally, it has been previously demonstrated that TLR stimulation modulates FcRL expression in mice (22). We determined that exposure of PBMC from HIV_{NEG} subjects to TLR9 stimulation led to a significant upregulation in FcRL4 expression (**Figure 4A**, $p = 0.0415$). We confirmed that while TLR stimulation induces FcRL4 upregulation, the anti-FcRL4 flow cytometry antibody did not lead to FcRL4 upregulation. In human secondary lymphoid tissue, elevated FcRL4 expression is associated with heightened levels of the Src kinase family member HcK (23), which in macrophages, promotes TLR-induced expression of pro-inflammatory cytokines (24). We, therefore, investigated if in TLR-stimulated blood B cells, the observed FcRL4 upregulation (**Figure 4A**) is associated with heightened HcK expression contributing to the amplification of the TLR-signaling. We determined that TLR9-stimulation of purified B cells from HIV_{NEG} resulted in the upregulation of *HcK* levels (**Figure 4B**, $p = 0.0386$) (gating Figure S2 in Supplementary Material). Finally, in HIV_{VIR} subjects, FcRL4^{pos} B cells, expressed significantly higher endogenous levels of total (**Figure 4C**, $p = 0.0414$) and phosphorylated HcK (**Figure 4C**, $p = 0.0398$).

HcK Is Required for FcRL4-Mediated Amplification of TLR Signaling

The effect of HcK on TLR-signaling in B cells was further investigated using a B cell line stably expressing FcRL4 (FcRL4.WT) and a loss-of-function FcRL4 mutant cell line, incapable of amplifying TLR-signaling (FcRL4.FFF) (17). We determined that after TLR stimulation, HcK upregulation was evident only in the FcRL4.WT cells (**Figure 5A**). *HcK* expression in FcRL4.WT transfectants was reduced using siRNA and confirmed by qPCR (**Figure 5B**, $p = 0.0079$, compared to control). Finally, TLR9 activation was quantified by change in CD23 expression, a

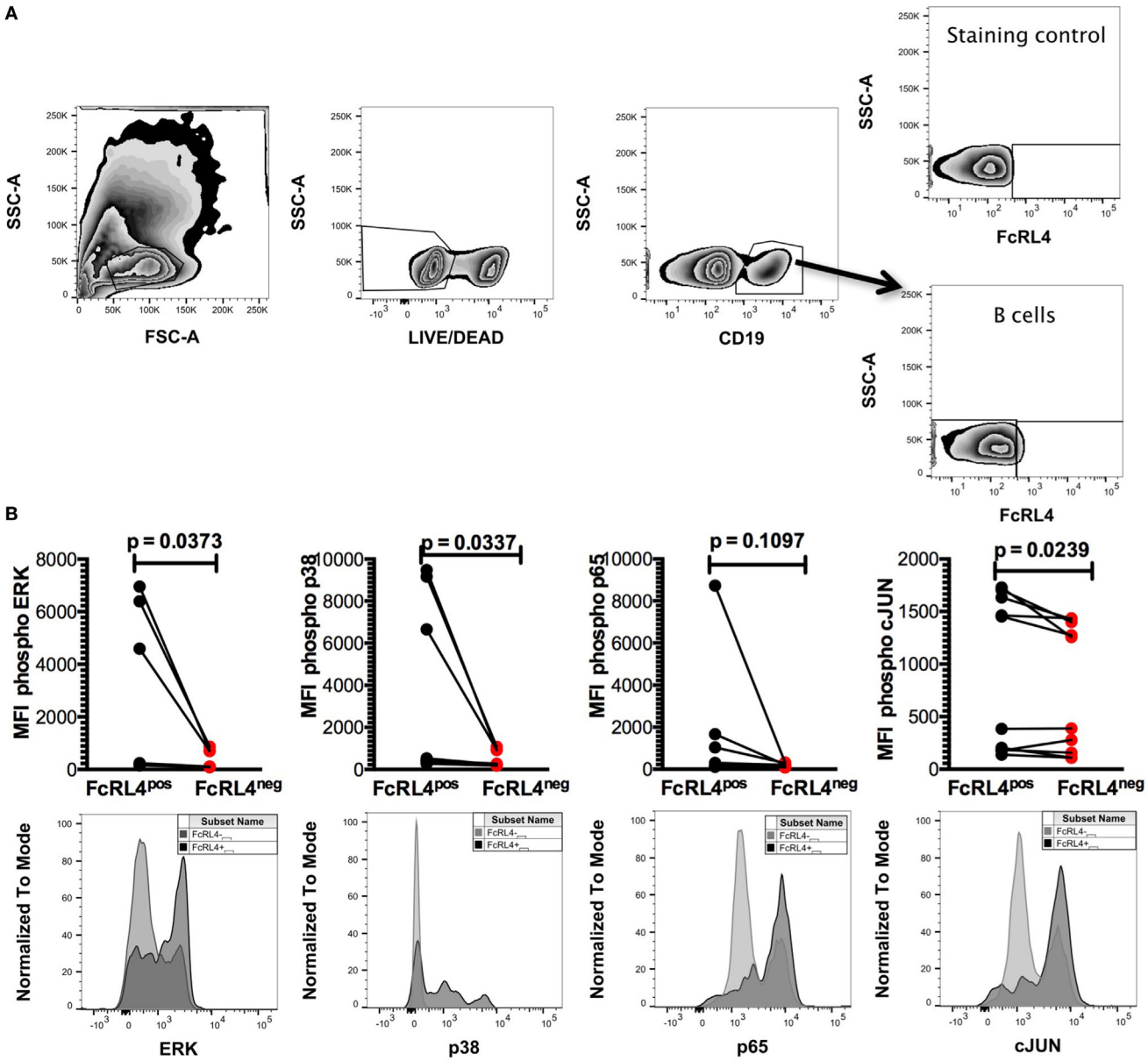

FIGURE 2 | FcRL4^{pos} B cells from HIV viremic subjects are Hck^{hi} and exhibit activated TLR-signaling signature. In FcRL4^{pos}, B cells from HIV viremic subjects (n = 10) **(A)** gating strategy for FcRL4, using flow cytometry, **(B)** the endogenous activation levels of MAPK pathway members Erk and p38 as well as activation of NF-κB (p65) and AP-1 (c-Jun) were determined: Representative overlays of FcRL4$^-$ and FcRL4$^+$ populations shown. p-Values as determined by Mann–Whitney test are indicated.

readout of TLR9 activity (17). HcK knockdown led to a reduction in CD23 expression (**Figure 5B**, $p = 0.0553$) following TLR9 stimulation. To confirm these results, we chemically inhibited HcK using 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d] pyrimidine (PP2) as described elsewhere (25, 26). HcK chemical inhibition reduced TLR9-induced CD23 expression significantly in FcRL4.WT compared to FcRL4.FFF in a dose-dependent manner (Figure S1 in Supplementary Material, 1 mM and 10 mM PP2, $p = 0.0059$ and $p = 0.0052$, respectively).

DISCUSSION

In this study, we demonstrate that during viremic HIV infection, FcRL4^{hi} TLM and AM blood B cells express high endogenous levels of IL-6, strongly indicating that high FcRL4 expression identifies pro-inflammatory B cells. We demonstrate that frequency of FcRL4$^+$ B-cells correlates strongly with IL-6$^+$ B-cell frequency in the TLM subset. However, AM B cells exhibit the highest frequency of FcRL4$^+$IL-6$^+$ double-positive

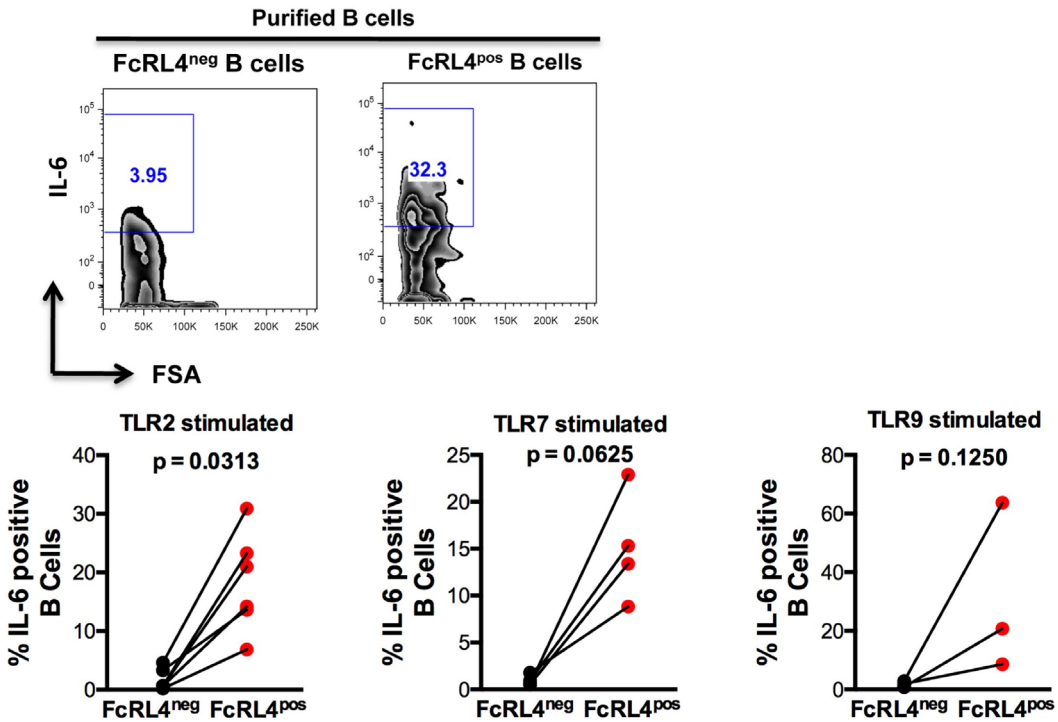

FIGURE 3 | FcRL4^{pos} B cells from HIV-uninfected subjects are highly responsive to TLR stimulation. FcRL4^{hi} and FcRL4^{lo} B cells were enriched from primary B cells of HIV$_{neg}$ subjects, exposed to TLR2-Ligand (TLR-2 stimulated, 2 μg/ml, Pam_3Csk_4), TLR7L (TLR-7 stimulated 2 μg/ml, Imiquimod), or TLR9-ligand (TLR-9 stimulated 10 μg/ml CpGODN2006). After overnight incubation, IL-6 expression was determined by intracellular cytokine staining. Representative plots are shown in the top panels, in lower panels, each dot represents a subject (n = 6, 4, 3 from left to right), p-values as determined by paired t-test are indicated.

cells suggesting the possibility that divergent mechanisms drive IL-6 production in AM and TLM B cells. This concept of divergent mechanisms is further supported by the distinct characteristics of these subsets, with TLM displaying elevated expression of inhibitory receptors and increased frequency of HIV-specific B cells, while the AM subset show greater specificity for other pathogens (20). Taken together, our report identifies pro-inflammatory functions of FcRL4^{+} TLM B cells in viremic HIV-infected subjects, corroborating findings, which identify FcRL4^{hi} B cells as a marker of pro-inflammatory B cells in rheumatoid arthritis patients (16).

Though FcRL4 has previously been identified on exhausted B-cell subsets (20), weak proliferation following BCR stimulation may be indicative of a shift in function rather than a general failure to respond. FcRL4 has been identified as a molecular switch, dampening BCR signaling while enhancing B-cell responsiveness to TLR-stimulation (17). HIV-infected viremic (HIV$_{VIR}$) subjects exhibit elevated serum levels of TLR-ligands (7–9) concomitant with high expression of FcRL4 on B cells (18, 20). It is, therefore, tempting to suggest that in HIV$_{VIR}$ subjects, TLM and AM B cells are stimulated by TLR-ligands resulting in upregulated FcRL4 expression. This increases sensitivity to TLR stimulation, leading to a positive feedback loop culminating in high expression of IL-6, inflammation, and HIV disease progression. Though we cannot exclude the possibility that FcRL4-expressing B cells coincidently express IL-6, our data provide further evidence supporting a role for FcRL4 in mediating *in vivo* TLR-signaling-dependent hyperstimulation during HIV infection. We also determined that *ex vivo*, FcRL4^{hi} B cells from HIV$_{VIR}$ subjects exhibit a TLR-signaling signature, characterized by heightened activation of NF-κB and AP1 pathways, transcription factors critical for the expression of pro-inflammatory genes (27–29).

While FcRL4 expression has been well documented in HIV, its function remains only partly elucidated. During HIV-1 infection, FcRL4 is elevated on TLM of non-treated individuals, but expression is greatly reduced following treatment (30); this suggests a unique role for FcRL4 during HIV infection. Jelicic et al. report that HIV gp120 induces FcRL4 expression on B cells (31), suggesting another mechanism inducing FcRL4 expression, which enhances susceptibility to TLR stimulation in HIV infection. Previous studies also suggest that another FcRL family protein, FcRL3, is upregulated in response to TLR stimulation (32); however, a role for TLR stimulation in regulating FcRL4 expression in HIV infection has not been explored. We provide data suggesting that TLR-signaling augments B-cell FcRL4 expression, corroborating reports of TLR-regulation of FcRL3 (32). Though we present data indicating B cells exposed to TLR9-ligand CpG-ODN2006 upregulate FcRL4 expression, we also observed comparable effects when B cells are exposed to either TLR7 (Imiquimod) or TLR2 (Pam_3Csk_4) ligands (not shown). Sohn et al. elegantly demonstrated that FcRL4 expression

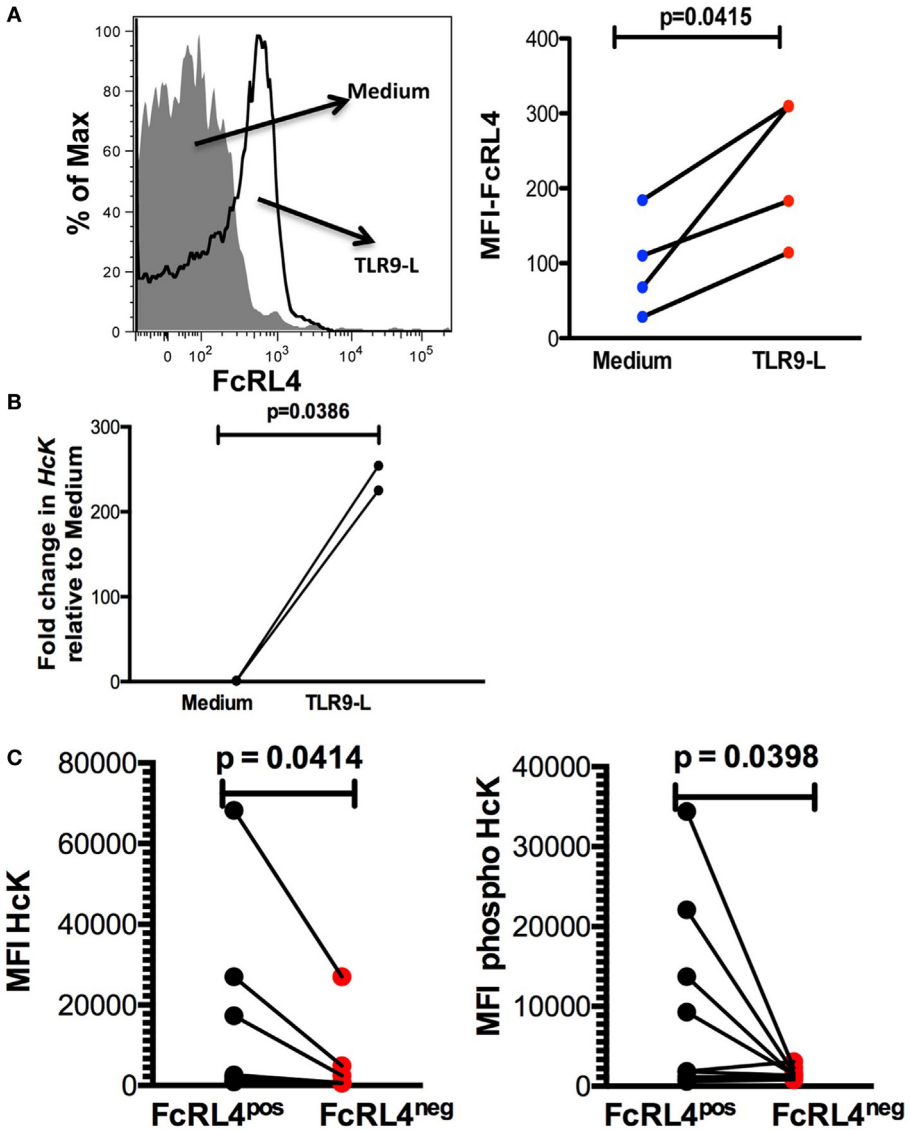

FIGURE 4 | TLR-stimulated B cells upregulate FcRL4 and hematopoietic cell kinase (Hck) expression. Purified B cells from HIV_{neg} subjects were stimulated overnight with 10 μg/ml CpGODN 2006 and the expression of **(A)** FcRL4 and **(B)** *Hck* determined by flow-cytometry and RT-qPCR, respectively. Each dot represents a subject. **(C)** In $FcRL4^{pos}$, B cells from HIV viremic subjects (n = 10, **Figure 2A**, gating strategy for FcRL4), the endogenous level of total HcK (left) and phosphorylated HcK (right) expression were determined. p-Values as determined by paired t-test are indicated.

switches B-cell responsiveness from adaptive to innate stimulus (17); however, the underlying mechanism is still undefined. Our data present a potential mechanism underlying FcRL4-mediated amplification of TLR-signaling in B cells. Ehrhardt et al. (23) reported that human tissue $FcRL4^{hi}$ B cells concurrently express high levels of the Src-kinase family member HcK, and Smolinska et al. (24) determined that Hck recruitment amplifies TLR4 signaling in macrophages. Our data confirm these findings, as we show that TLR9-stimulated B cells from HIV_{NEG} subjects upregulate HcK and $FcRL4^{hi}$ B cells from HIV_{VIR} subjects exhibit elevated endogenous levels of HcK. Further, HcK downmodulation resulted in a reduction of TLR-signaling in FcRL4 B-cell transfectants. These data confirm recent reports by Liu et al. (33) and suggest that FcRL4 in human B cells likely recruits the Src-kinase family member HcK, resulting in amplification of TLR-signaling. However, further studies are needed to determine the precise association between FcRL4 and HcK. Our finding that TLR9-stimulation was impervious to HcK chemical inhibition in the FcRL4.FFF loss-of-function mutant suggests a role for the ITIM in HcK recruitment following TLR-signaling, as FcRL4 of this mutant is incapable of specific ITIM phosphorylation events.

B cells have been well established as a critical source of pro-inflammatory IL-6 in autoimmune diseases (14), and some reports also suggest that during HIV infection B-cells express IL-6, thus

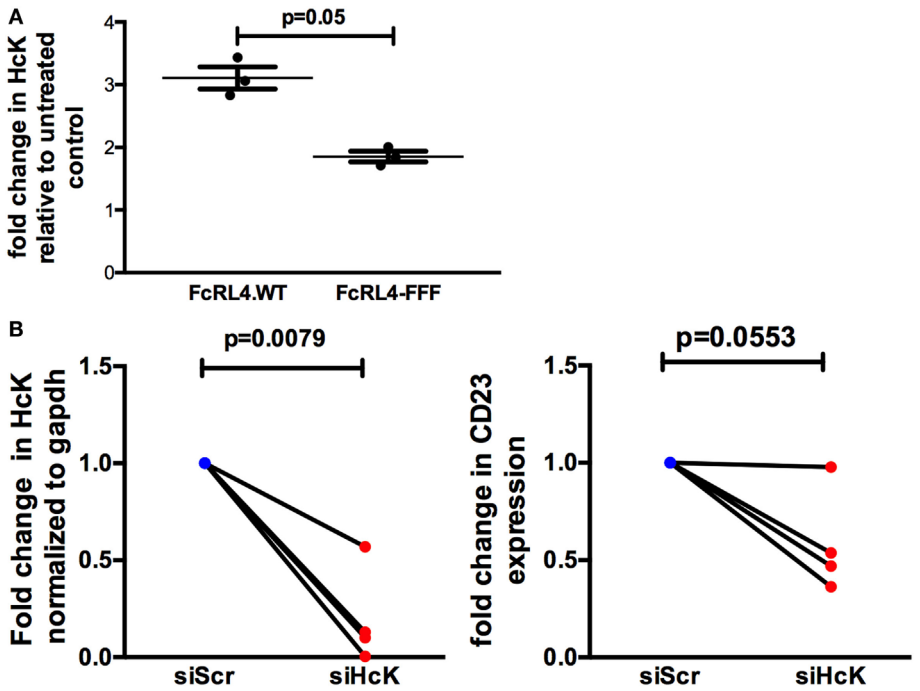

FIGURE 5 | Hematopoietic cell kinase (HcK) is necessary for FcLR4-mediated amplification of TLR signaling. In FcRL4 transfectants, **(A)** HcK expression was determined after TLR9 stimulation by flow cytometry, $n = 3$. **(B)** *Hck* expression was determined by RT-qPCR post RNAi (left); siHCK and control RNAi treated cells were incubated overnight with TLR9-L and CD23 expression detected by flow-cytometry (right) shown as fold change. siScr = scrambled control; $n = 4$. *p*-Values as determined by paired *t*-test are indicated.

likely exerting a pathogenic role (3, 9, 13). Our data present FcRL4 as a marker identifying potential pro-inflammatory B cells during viremic HIV infection.

CONCLUSION

The data from this study indicate that in viremic HIV infected subjects, high expression of FcRL4 identifies pro-inflammatory B cell subsets. In autoimmune conditions, B cells have been established as critical IL-6 expressing cells (16). Our data demonstrate a pro-inflammatory function of FcRL4$^+$ B cells, a population of B cells previously identified as exhausted, in viremic HIV infection. Finally, we present data elucidating the mechanisms of FcRL4-mediated amplification of TLR-signaling in B cells. We provide data indicating that increased expression of FcRL4 coincides with upregulation of the Src kinase HcK, and HcK is necessary for FcRL4's amplification of TLR signaling.

ETHICS STATEMENT

All studies were performed after signed informed written research consent by each study subject. The study was reviewed and approved by the Institutional Review Board of the Rush University Medical Center, and the University of Iowa City VAMC and University of Iowa.

AUTHOR CONTRIBUTIONS

BS and AL conceived/designed study and wrote manuscript. BS and AN performed experiments. BS, AN, AL, HS, and JS analyzed data and edited manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/article/10.3389/fimmu.2017.01339/full#supplementary-material.

FIGURE S1 | CD23 expression following overnight treatment with HcK inhibitor PP2. FcRL4.WT and FcRL4.FFF cells were treated with TLR9-L and HcK inhibitor. Expression of CD23 reported as fold change between TLR9-L alone and in the presence of inhibitor n=3.

FIGURE S2 | Representative gating of HcK and phospho HcK.

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Perturbation of B Cell Gene Expression Persists in HIV-Infected Children Despite Effective Antiretroviral Therapy and Predicts H1N1 Response

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Despite effective antiretroviral therapy (ART), HIV-infected individuals with apparently similar clinical and immunological characteristics can vary in responsiveness to vaccinations. However, molecular mechanisms responsible for such impairment, as well as biomarkers able to predict vaccine responsiveness in HIV-infected children, remain unknown. Following the hypothesis that a B cell qualitative impairment persists in HIV-infected children (HIV) despite effective ART and phenotypic B cell immune reconstitution, the aim of the current study was to investigate B cell gene expression of HIV compared to age-matched healthy controls (HCs) and to determine whether distinct gene expression patterns could predict the ability to respond to influenza vaccine. To do so, we analyzed prevaccination transcriptional levels of a 96-gene panel in equal numbers of sort-purified B cell subsets (SPBS) isolated from peripheral blood mononuclear cells using multiplexed RT-PCR. Immune responses to H1N1 antigen were determined by hemaglutination inhibition and memory B cell ELISpot assays following trivalent-inactivated influenza vaccination (TIV) for all study participants. Although there were no differences in terms of cell frequencies of SPBS between HIV and HC, the groups were distinguishable based upon gene expression analyses. Indeed, a 28-gene signature, characterized by higher expression of genes involved in the inflammatory response and immune activation was observed in activated memory B cells ($CD27^+CD21^-$) from HIV when compared to HC despite long-term viral control (>24 months). Further analysis, taking into account H1N1 responses after TIV in HIV participants, revealed that a 25-gene signature in resting memory (RM) B cells ($CD27^+CD21^+$) was able to distinguish vaccine responders from non-responders (NR). In fact, prevaccination RM B cells of responders showed a higher expression of gene sets involved in B cell adaptive immune responses (*APRIL, BTK, BLIMP1*) and BCR signaling (*MTOR, FYN, CD86*) when compared to NR. Overall, these data suggest that a perturbation at a transcriptional level in

the B cell compartment persists despite stable virus control achieved through ART in HIV-infected children. Additionally, the present study demonstrates the potential utility of transcriptional evaluation of RM B cells before vaccination for identifying predictive correlates of vaccine responses in this population.

Keywords: vaccinomics, systems biology, B cells, pediatric HIV, transcriptomics, H1N1, B cell receptor, influenza vaccination

INTRODUCTION

HIV-infected patients have a lower ability to induce and maintain an effective response to routine vaccinations due to the depletion of central memory CD4 T cells, particularly T follicular helper cells, and perturbation of the B cell compartment with reduced resting memory (RM) B cells (1–4). Antiretroviral therapy (ART) can restore the quantitative loss of RM B cells in HIV-infected children (5, 6). However, a suboptimal antibody response against infection and vaccination may persist, suggesting a qualitative impairment of B cells. Indeed, a sizeable proportion of HIV-infected children require booster immunizations to provide adequate protection usually achieved by routine vaccinations in healthy children (7–9). Additionally, children with apparently similar clinical and immunological characteristics can vary in adequacy of responsiveness to infection and/or vaccination bringing into question host factors that are critical for mounting an immune response (10, 11). The molecular correlates governing effective and long lasting immune responses are still unknown (4, 12–14). In recent years, systems biology and vaccinomics approaches have attempted to dissect vaccine-induced responses in humans (15–19). For influenza, gene expression and robustness of response have been found to differ upon vaccination with trivalent-inactivated influenza vaccination (TIV) as compared to live attenuated influenza vaccine (20). In addition, advanced "omics" and systems biology approaches have led to increased knowledge regarding molecular mechanisms underlying adaptive immune responses to different types of vaccines (21, 22). In most instances however, these data have been derived from RNA extracted from whole blood or from the heterogeneous pool of peripheral blood mononuclear cells (PBMCs) of healthy volunteers (18, 23), thereby limiting interpretation due to dilution of gene transcripts derived from individual cell subsets or single cells which may be crucial for adaptive immune responses. To mitigate this drawback, analysis of purified cell subsets of interest is preferred, especially in the context of diseases that alter the distribution of specific cell subsets such as HIV infection (24, 25).

In the present study, we have applied basic principles of vaccinomics and systems biology, with the aim to dissect gene expression differences evident before vaccine administration between HIV-infected children under ART with stable virus control and their age matched healthy peers. Our analysis of B cell gene expression among HIV-infected children differentially responding to H1N1 revealed biologically meaningful predictive signatures of response to vaccination.

Abbreviations: ART, antiretroviral therapy; BCR, B cell receptor; TIV, trivalent-inactivated influenza vaccination; LAIV, live attenuated influenza vaccination; PBMCs, peripheral blood mononuclear cells; RM, resting memory; AM, activated memory; DN, double negative; DEGs, differentially expressed genes; ANOVA, analysis of variance; HCs, healthy controls; HAI, hemagglutination inhibition; Ct, cycle threshold; Et, expression threshold; PCR, polymerase chain reaction; SPBS, sort-purified B cell subsets.

MATERIALS AND METHODS

Study Subjects and Design

Twenty-three ART-treated HIV-1 vertically infected patients (HIV) and 10 healthy age-matched controls [healthy controls (HCs)] were enrolled at Bambino Gesù Children's hospital. Participant characteristics are shown in **Table 1**. Written informed consent was obtained from all subjects or parents/guardians of all minors for participation in a prospective, open label influenza vaccine study (Figure S1A in Supplementary Material). Bambino Gesù Children's hospital ethics committee approved the study. Participants were immunized with a single dose of Inactivated Influenza Vaccine Trivalent Types A and B (Split Virion) VAXIGRIP® (sanofi pasteur). The strains for the 2012–2013 season were A/California/7/2009 (H1N1) pdm09-like strain (abbreviated as H1N1), A/Victoria/361/2011 (H3N2)-like strain (abbreviated as H3N2), and B/Wisconsin/1/2010-like strain (abbreviated as B). Study design is outlined in Figure S1A in Supplementary Material. PBMCs, sera, and plasma were collected pre (T0) and 21 days postvaccination (T1) as previously described (26, 27). Among HIV, only patients with good adherence to ART and with history of long-term viral control (at least 24 months) were considered eligible for the study. No significant differences for ART type nor

TABLE 1 | Characteristics of study population.

Baseline characteristics	HIV NR	HIV R	HC
Age (years), mean (SEM)	15.16 (2.1)	13.72 (2.3)	14.3 (3.3)
n (female)	12 (7)	11 (5)	10 (5)
%CD4$^+$ T cells, mean (SEM)	37.97 (4.9)	32.49 (6.0)	29.79 (6.2)
HIV RNA <50 cp/mL, *n*	11	10	N/A
IgG (mg/dL) (mean)	1,387.4	1,356	1,054.7
IgM (mg/dL) (mean)	135.1	118.9	106.8
IgA (mg/dL) (mean)	210.7	225.1	150
CDC (A/B/C) (1/2/3)	(3/4/5) (3/4/5)	(2/5/4) (4/3/4)	N/A
Lymphocytes/mm^3, mean (SEM)	2,494 (278.9)	3,109 (363.1)	3,063 (427.8)
WBC (10^3/μL), mean (SEM)	7.6 (1.5)	7.3 (0.7)	7.9 (0.5)
ART regimen (2 NRTI + PI/2 NRTI + nNRTI/2 NRTI + ii)	(5/5/2)	(5/4/2)	N/A

CDC, Center for Disease Control classification of AIDS; WBC, white blood cells; ART, antiretroviral treatment; NRTI, nucleoside and nucleotide analog reverse transcriptase inhibitors; PI, protease inhibitors; nNRTI, non-nucleoside analog reverse transcriptase inhibitors; ii, integrase inhibitors.

treatment duration were found between HIV Responders and HIV non-responders (NR, **Table 1**).

Hemagglutination Inhibition (HAI) Assay

The HAI assay was performed and analyzed as previously described (28) (http://www.gmp-compliance.org/guidemgr/files/021496EN.PDF). The HAI antibody titers were expressed as the reciprocal of the highest serum dilution at which hemagglutination was prevented. Study participants were classified as vaccine responders (R) and vaccine NR according to the criteria established by Food and Drug Administration Guidance for Industry (fda.gov). R were characterized by HAI titer to H1N1 at T1 of ≥1:40 *and* ≥4-fold increase compared to baseline.

ELISpot

Peripheral blood mononuclear cells collected at T0 and T1 from HIV and HC were thawed and polyclonally activated *in vitro* in complete RPMI medium (Invitrogen) supplemented with 2.5 µg/mL CpG type B (Hycult biotech), 20 ng/mL IL-4 (Peprotech), and 20 ng/mL IL-21 (ProSpec). Cells were harvested after 5 days of culture at 37°C. ELISpot 96-well filtration plates (Millipore) were coated with purified H1N1 inactivated virus particles and subsequently loaded with 2×10^5 cells/well. Plates were then processed as previously described (2). Response to H1N1 Ag was determined using the criteria ≥ or <80 spots/10^6 PBMCs in R and NR, respectively.

Cell Sorting, RNA Extraction and FACS Analysis

Cryopreserved PBMC from T0 and T1 were thawed, stained for the following previously titrated surface antibodies: CD10 (PECy7), CD20 (PE), CD27 (APC), IgD (FITC), CD21 (PECy5), and sorted by FACSAriaII (BD Biosciences). Vivid (Pacific Blue) was used to determine viability of cells. The gating strategy to identify B cell subsets, comprising total B cells (live, singlets, $CD20^+$), total naive (IgD^+CD27^-), double negative (DN) (IgD^-CD27^-), RM ($IgD^-CD27^+CD21^+$), and activated memory (AM) ($IgD^-CD27^+CD21^-$) that were gated on the IgD^-CD27^+ class switched memory are shown in **Figure 1** and Figure S1B in Supplementary Material. IL-21 receptor on B cells has been analyzed as previously described (11). The purity of sorted cell populations was >99%. Five hundred live cells per B cell subset

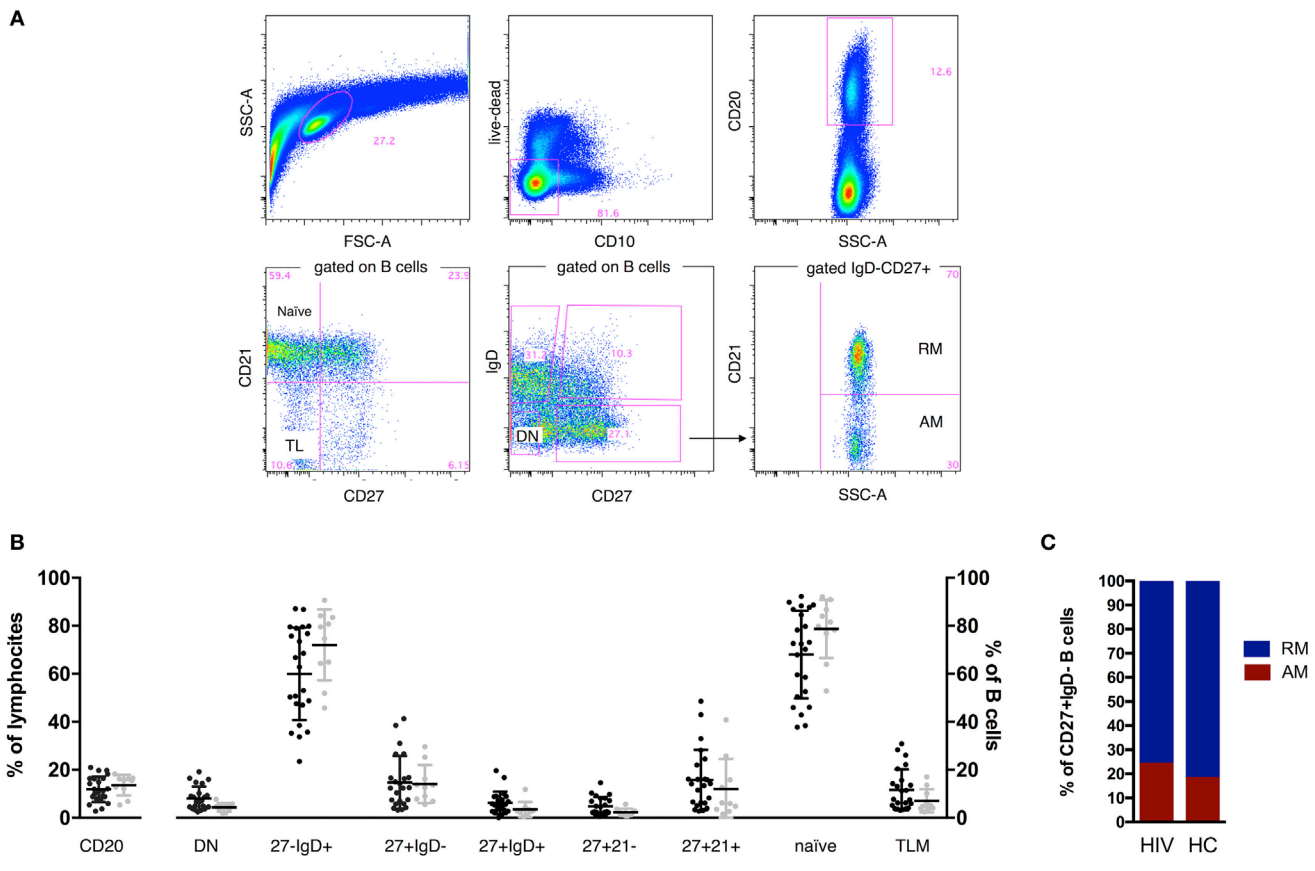


FIGURE 1 | B cell phenotype in HIV and age-matched healthy control (HC). Representative gates **(A)** and comparisons of B cell percentages **(B,C)**. Two tailed Mann–Whitney was used for comparisons. $CD20^+$ cells established the B cell population, and expression of IgD, CD27, CD21, and CD10 was used to define total naive ($CD27^-IgD^+$), class switched $CD27^+$ memory B cells ($CD27^+IgD^-$), double-negative (DN; $CD27^-IgD^-$), resting memory (RM), tissue-like (TL), activated memory (AM), and naive. FSC, forward scatter; SSC, side scatter. Contingency plot in **(C)** represents frequency of AM and RM in HIV and HC.

were sorted with the sorting strategy depicted in Figure S1B in Supplementary Material in tubes previously loaded with 9 µL of CellsDirect one-step polymerase chain reaction (PCR) buffer and pooled TaqMan gene expression assays (2× CellsDirect Reaction mix 5 µL, Superscript III + Taq polymerase 0.5 µL, 0.2× TaqMan primer pool 2.5 µL, Resuspension Buffer 1 µL). After sorting, samples were transferred to PCR tubes and reverse transcription and target-specific preamplification was performed on a C1000 Thermal Cycler (BioRad) with the following scheme (50°C for 20 min, 95°C for 2 min, 95°C for 15 s, 60°C for 4 min, last two steps repeated for 18 cycles). Resulting cDNA was stored at −20°C until further analysis.

Multiplexed RT-PCR

Previously amplified samples were loaded on a Fluidigm 96.96 standard chip following manufacturer's instructions. All primers/probes used for the gene mix are TaqMan gene expression assays (Table S1 in Supplementary Material) and have been qualified on Human PBMCs and lymphocyte subsets following the method previously described (29). Gene selection was made according to previous analysis on Microarray of HIV-infected children (data not shown), literature, online gene banks, and biological queries. The sorting experiments and BioMark experiments were randomized to include a mix of HC, HIV, R, and NR patient samples so as not to bias the data toward one group by batch effects. Analysis was performed using Fluidigm Real-Time PCR Analysis software and "Multiple Chip Run" analysis mode. Cycle threshold values (Ct) were corrected according to the number of cells sorted if less than 500 according to the following calculation: $Y/X = 67.5/500$ (where $X =$ actual number of cells sorted and $Y =$ cDNA equivalent loaded onto BioMark chip). The dilution factor (n) was then calculated as $n = 67.5/Y$ and $\log2(n)$ was subtracted from the Ct value to obtain Corrected Ct (cCt). Expression threshold (Et) values were calculated using the formula: $Et = 40 - cCT$, and Et was used for all downstream analysis. To verify consistency between individual BioMark runs, Et variance in B cells was calculated on the full set of genes. Housekeeping genes (*GAPDH, CD74*) included in our panel showed a low variation (<0.1 score) across all samples in both PBMCs and sorted B cell subsets (not shown).

Enzyme-Linked Immunosorbent Assay (ELISA)

Plasma BAFF titers were measured as previously described (30). Briefly, plasma samples were diluted 1:1 and run in duplicate with 50 µL/well added to ELISA plates for human BAFF (R&D Systems).

Bioinformatics and Statistical Analysis

Data were analyzed using Fluidigm SingulaR (SingulaR analysis toolset 3.0) package loaded on R (software R 3.0.2 GUI 1.62). We performed outlier identification analysis following manufacturer's instructions (Singular Analysis Toolset User Guide) on the whole dataset by cell subset and removed outliers from subsequent analysis. ANOVA was used to identify differentially expressed genes (DEGs), and interplay between cell subsets or patient groups was assessed through fold increase of the averages. Inter-individual differences and outliers were analyzed by SingulaR. The "mixOmics" package (Omics data integration project) for R was used as previously described (31). Pearson or Spearman correlation plots were generated with Prism 6.0 (GraphPad) after performing kolmogorov-smirnov normality test to determine distribution of the data. Statistical differences between postvaccination (T1) and prevaccination (T0) gene expression were determined by Wilcoxon matched paired test, and volcano plot was generated in Prism 6.0.

RESULTS

Perturbed Gene Expression in Memory B Cells Persists in HIV-Infected Children Despite Effective ART and Normal B Cell Frequency

In order to characterize the B cell compartment of vertically HIV-infected children under ART and stable viral control, we assessed frequencies of total B cells and B cell subsets by flow cytometry. No differences in frequencies were found between HIV-infected and HC groups (**Figures 1B,C**).

To evaluate the B cell compartment at the transcriptional level, we performed multiplexed RT-PCR of a panel of 96 genes (Table S1 in Supplementary Material) by Fluidigm Biomark™ in purified B cells from prevaccination samples. Principal component analysis (PCA) and hierarchical cluster analysis confirmed expected heterogeneity between memory subsets (AM and RM), and IgD^+CD27^- (total naive) and DN subsets in HC and HIV (Figures S2A and S3 in Supplementary Material). The greatest transcriptional variation was found between RM and the other three subsets in both HIV and HC participants, especially between RM and AM (77 DEGs in HIV and 23 DEGs in HC) marked by overall lower gene expression in RM (Figure S2B in Supplementary Material).

As shown in Figure S2B in Supplementary Material, all 23 DEGs identified by the comparison of RM to AM transcripts in HC are also present in HIV-infected individuals. To better understand the biological context of genes identified by differential expression analysis between RM and AM, we performed gene set enrichment analysis (GSEA) of preranked gene lists using published blood transcription modules as gene sets (32). These genes are mainly involved in regulation of lymphocyte activation and leukocyte proliferation (*CD28, PILRB, FOXO3, CD38, STAT5A, ABCB1, CD40L*), suggesting common intrinsic gene expression patterns characterizing AM in both HIV and HC. However, 54 additional DEGs were identified in the HIV-infected group when comparing gene transcripts present in RM and AM. These genes include additional immune activation and lymphocyte proliferation genes (*CD86, CAV1, CAMK4, TNFSF13, BTLA, MTOR*) as well as genes involved in the inflammatory response (*CYBB, NOD2, MYD88, IL10, CCR2*), type I Interferon signaling (*IFIT2, MX1, STAT1*) and response to virus (*APOBEC3G, BST2, TRIM 5*), all with significantly higher expression in AM compared to RM. Overall, the lower gene expression found in RM compared to AM may suggest that they are in a quiescent phase.

Next, we compared gene expression of each sorted B cell subset between HIV and HC to evaluate persistent defects in HIV infection despite viral control. Our results show that AM B cells clearly contrasted with 28 DEGs between HIV and HC (**Figure 2A**). Indeed, in this specific subset, already shown to dominate the HIV specific immune response in chronically infected adults (24), the DEGs showed higher expression in HIV compared to HC. Interestingly, this was not the case in PBMC and other sorted B cell subsets where few DEGs were identified in comparisons between HIV and HC: PBMC (5 DEGs), total B cells (0 DEGs), DN (2 DEGs), RM (2 DEGs), and total naive B cells (3 DEGs) (**Figure 2A**). GSEA analysis showed that genes expressed at higher levels in HIV compared to HC were mainly involved in inflammatory response and immune activation (*NOD2*, *IL2RA*, *SOCS1*, *IKBKG, CD69, CYBB, MYD88*) (**Figure 2B**; Figure S4 in Supplementary Material). Enrichment of *NOD2* (fivefold) and *IL2RA* (fourfold) was found in AM from HIV compared to HC. *NOD2* is mainly involved in signal transduction and activation of nuclear factor kappa-B during inflammatory responses, and the *IL2RA* is part of the IL-2 receptor complex and is involved in activation and proliferation of the cell after an external stimulus. Other genes involved in response to HIV entry (*APOBEC3G, TRIM5*) and positive regulation of B cell-mediated immunity (*BTK, TNSF13*) were also higher in AM of HIV compared to HC, suggesting that underlying activation in this cell subset persists despite effective ART and long-term viral control.

B Cell Gene Expression Profiles in HIV-Infected Children with Differing Response to H1N1 Vaccine Antigen

To determine how phenotype and transcriptional data associated with the ability of enrolled participants to respond to TIV, we applied two selection criteria (serology and Elispot) for separating study participants into responders (R) and NR (**Figure 3A–C**). The HIV-infected group contained approximately equal numbers of participants identified as R and NR, while all participants in the HC group were characterized as R. In agreement with our previous report (4), we found higher frequencies of $IgD^{-}CD27^{-}$ (DN) in NR compared to HC (Figure S5A in Supplementary Material). We also observed similar frequencies of class switched $CD27^{+}$ memory B cells ($CD19^{+}CD27^{+}IgD^{-}$) among the groups (HC, NR, and R) (Figure S5A in Supplementary Material); however, AM were significantly higher in NR compared to both R and HC (Figure S5B in Supplementary Material).

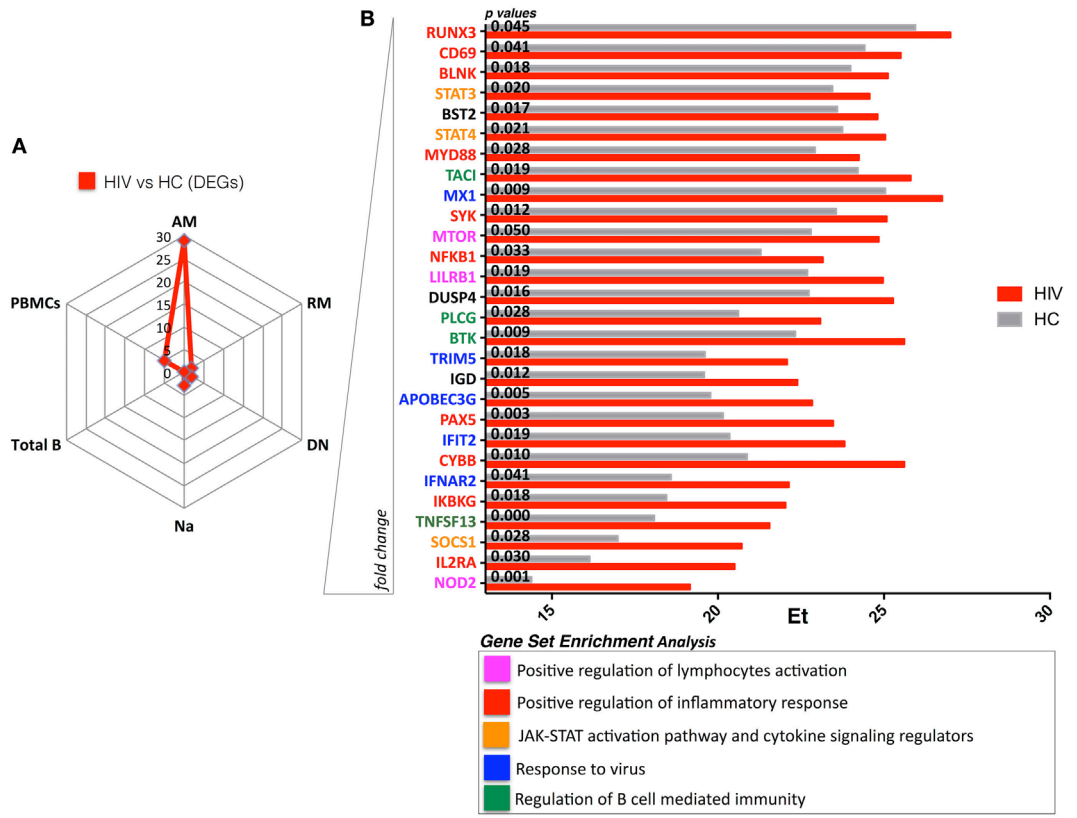


FIGURE 2 | HIV present higher expression of genes involved in immuneactivation and inflammation in activated memory (AM) B cells despite effective antiretroviral therapy (ART) and long-term viral suppression. Graphs in panels **(A,B)** show comparisons in gene expression between healthy control (HC) and HIV. **(A)** Spider plot shows number of differentially expressed genes (DEGs) for all the subsets and total peripheral blood mononuclear cells (PBMCs). Box plots in panel **(B)** show gene expression averages from DEGS resulting in AM between HIV and HC (gene ranking defined by fold change). In this figure, *p*-values resulting from ANOVA analysis are shown. Color-labeled genes are defined according gene set enrichment analysis (performed by genemania.org) as described in the legend.

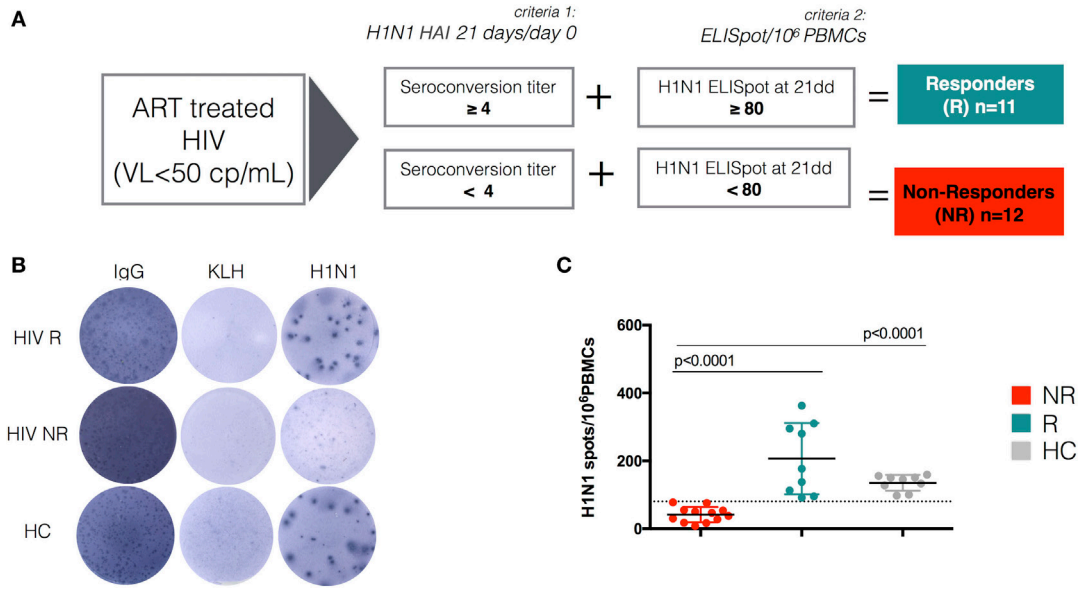


FIGURE 3 | H1N1 response after trivalent inactivated influenza in HIV and perturbation of the memory compartment among non-responders. **(A)** The flow chart describes the criteria of selection used to define responders and non-responders to trivalent-inactivated influenza vaccination (TIV) among HIV-infected children. As a first criteria of selection, all patients were selected according to the fold increase of H1N1 hemagglutination inhibition (HAI) titer. Patients responding or not responding to the first selection criteria were further selected according to H1N1 ELISpot response. Selected healthy donors responded to the vaccination and met both criteria of selection. Representative ELISpot assay **(B)** and scatter dot plot **(C)** show ELISpot data for H1N1.

At a transcriptional level, intersubset analysis comparing AM and RM revealed fewer DEGs in R than NR due to overall higher gene expression in the RM subset from R (Figure S6A in Supplementary Material). We further noted that although most of the DEGs in the total HIV group were present in the comparison between NR and HC (47 DEGs) (Figure S6A and Table S2 in Supplementary Material), only 20 additional DEGs were identified between HIV R and HC (AM vs. RM).

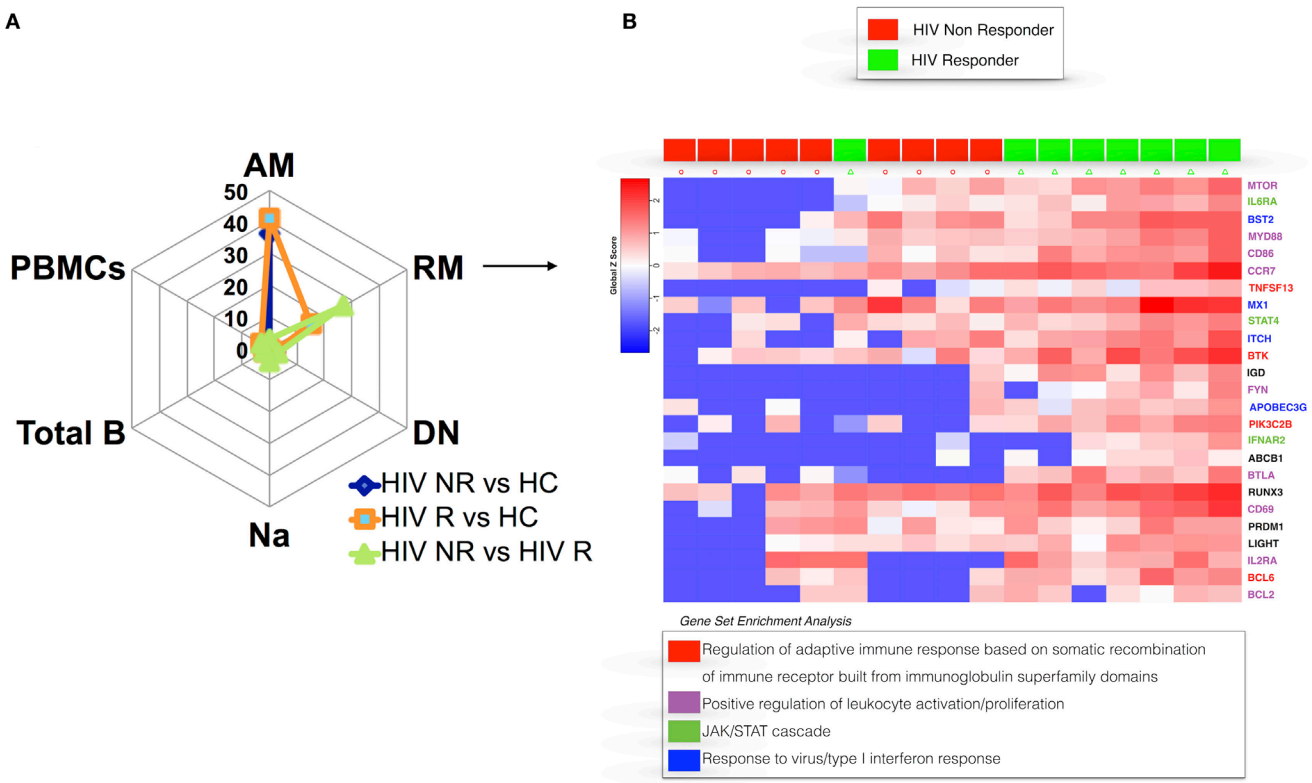
Next, we performed GSEA on DEGs within AM from comparisons between HIV-infected participants (both R and NR separately) and HC. This analysis showed enriched pathways in positive regulation of apoptotic process (*FAS, BAX, PILRB*), B cell activation, and Fc receptor signaling (*BATF, FYN, PLCG1, CD27, CD28*) in HIV (Figures S6B,C and Table S3 in Supplementary Material). Collectively, gene expression data from AM B cells demonstrate that this subset, which has been shown to accumulate in individuals with HIV infection (14, 33), displays a distinct transcription profile compared to HC independent of TIV response.

Distinct Prevaccination Gene Expression Patterns in RM from HIV-Infected Children Responding to H1N1

Our analysis of RM identified 25 genes that were differentially expressed between NR and R in HIV participants prior to vaccination with TIV (**Figure 4A**). RM from NR exhibited overall lower gene expression compared to HC and R. DEGs from this analysis, which were expressed higher in R, are directly involved in regulation of the adaptive immune response through somatic recombination from the immunoglobulin superfamily domain [*TNFSF13*(APRIL), *BTK*], leukocyte activation and BCR signaling pathways (*MTOR, FYN, CD86*). As shown in **Figure 4B**, genes involved in the *JAK/STAT* signaling cascade (*STAT4, IL6R, IFNAR*) and the closely related type I interferon response (*IFNAR2, MX1*) were higher in R. In addition, *PRDM1* (BLIMP1), able to induce B cell differentiation into plasma cells after encountering Ag (34), was higher in RM of R compared to NR. Collectively, these results show that the RM B cell subset, crucial for potent and specific adaptive immune responses, exhibits a distinct prevaccination transcriptional profile in HIV-infected participants who will mount an effective response to H1N1.

The gene set found to be different between NR and R was further analyzed for differences between pre vaccination (T0) and post vaccination (T1) gene expression in RM. Paired analysis revealed that *PRDM1* (BLIMP1) was significantly reduced at T1 when compared to T0 in HIV ($p = 0.0039$, median difference $= -7.52$) (Figure S7A in Supplementary Material). This longitudinal reduction was strongly confirmed in R with all R showing a reduction of *PRDM1* at T1 ($p = 0.0001$, median difference $= -8.9$), whereas significance was lost when only NR were taken into account (Figure S7B in Supplementary Material).

To further dissect the relationship of clinical (i.e., serological) markers of response to H1N1 and gene expression patterns, we performed pairwise correlation analysis using the two datasets. We confirmed the findings from differential gene expression analysis of prevaccination RM and found a positive correlation of *BTK* expression in RM at T0 and H1N1 seroconversion (HAI H1N1 Titer T1/T0) (**Figure 4C**) and H1N1 ELIspot at T1 (**Figure 4D**). Additional genes actively involved in proliferation and lymphocyte



C Correlation analysis

D

H1N1 HIA seroconversion rate (T1/T0) vs gene expression in RM

	ABCB1	STAT4	CD69	CD86	BTK	IGD
P value (two-tailed)	0.0169	0.0274	0.0337	0.0278	0.0162	0.0291
Spearman r	0.5402	0.5052	0.4887	0.5041	0.5432	0.5006

H1N1 spots at T1 vs gene expression in RM

	LIGHT	ABCB1	CD69	BCL2	IL2RA	PRDM1
P value (two-tailed)	0.0408	0.0365	0.0243	0.0259	0.0472	0.0409
Spearman r	0.4735	0.4824	0.5143	0.5095	0.4606	0.4728

FIGURE 4 | Prevaccination gene signatures in RM B cell subset discriminate HIV-infected R and non-responder (NR). **(A)** Spider plot shows number of differentially expressed genes (DEGs) for all the subsets and total peripheral blood mononuclear cells (PBMCs). **(B)** Heatmap shows gene expression in R and NR. Colored genes' names refer to gene set enrichment analysis (GSEA) legend. In panels **(C,D)**, correlation between gene expression in resting memory and H1N1-seroconversion **(C)** and ELISpot at T1 **(D)** are shown. *p* and *r* values show results from correlation analyses (Pearson or Spearman tests for parametric and non-parametric data, respectively).

activation correlated with vaccine response in terms of H1N1 seroconversion (*CD69, CD86*) and H1N1 ELISpot at T1 (*CD69*). Interestingly, genes involved in inhibition of the apoptotic process (*LIGHT, BCL2*) showed positive correlations with H1N1 ELISpot at T1. Overall, these results demonstrate that the memory B cell compartment is highly impacted by HIV infection and suggest that an activated profile of specific genes in RM is required to maintain a normal adaptive response in HIV-infected patients.

We further asked whether gene expression analysis was correlated to measurements of plasma biomarkers or cell surface molecule expression encoded by the corresponding genes. To do so, we correlated gene expression data with plasma levels of BAFF (B cell activating factor) and with IL-21R$^+$ B cell frequency, two molecules already shown to be involved in the immune response against H1N1 after vaccination (4, 30). Interestingly, *TNFSF13B* (BAFF) gene expression in RM was positively correlated with plasma BAFF levels at the time of vaccination (Figures S8A,C in Supplementary Material). Further, gene expression of *IL21R* in sorted RM was positively correlated with the expression of IL-21R as analyzed by flow cytometry (Figures S8B,D in Supplementary Material). These data show that transcriptional data may provide a functional correlate in specific molecules involved in the B cell memory response and maintenance over time.

DISCUSSION

This study represents the first evaluation of gene expression patterns in B cell subsets, total B cells and PBMCs in the field of

pediatric HIV infection and in the context of immune responses to H1N1 antigen post-TIV. In the field of vaccinomics, systems biology tools have lately generated exciting data revealing molecular mechanisms of immunity induced by vaccination and correlates of protection in order to predict the vaccine efficacy in healthy adults (35, 36). However, as recently shown, the influence of age on gene expression patterns should be taken into account when interpreting systems biology data (37). Additionally, gene signatures identified in healthy adults and in the heterogeneous pool of PBMCs (38, 39) are not directly applicable to pediatric studies (40), and most likely not even in patients affected by chronic conditions such as HIV infection (41). Therefore, we believe that gene expression patterns identified in specific cell subsets may be crucial to investigate the dynamic of vaccine response in HIV-infected children.

In the present study, the analysis of gene expression from purified B cell subsets showed that perturbations in memory B cells persist in HIV-infected children despite stable and long-term virological control. Our results suggest that in these patients, the recovery achieved in overall B cell frequencies is not accompanied by recovery of gene expression and B cell function. We identified clear-cut differences in gene signatures between AM B cells of HIV-infected children and their healthy peers. B cell subsets between study groups were skewed in AM of HIV-infected children toward hyperexpression of genes involved in immune exhaustion/inflammation (*CYBB, MYD88, NOD2, IL2RA*) and apoptosis (*SOCS1, RUNX3*). The immune activation and exhaustion pattern, hereby confirmed at a transcriptional level in this particular subset of B cells, may play a key role in the "inflamm-aging" process which leaves ART-treated HIV-infected patients vulnerable to increased risk of non-AIDS defining comorbidities such as malignancies and cardiovascular diseases (42, 43). Indeed, despite the advent of ART which has dramatically increased life expectancy, non-AIDS defining malignancies are still increasing in ART-treated and virologically controlled HIV-infected children (44–46). The AM B cell subset was previously described to be enriched in HIV (47), to be prone to functional "exhaustion," and to dominate HIV-specific responses (24). Furthermore, it has been recently reported in adults that signs of chronic inflammation persist over time even when treatment is started during acute infection (48). It is still unknown whether antiretroviral regimens may differentially impact B cell gene transcriptional patterns. Although in the present study, no differences in terms of ART regimen were found between the study groups (responders and not responders), these specific effects should be addressed in future investigations and in larger cohorts. Other differences between ART-treated HIV and uninfected children have emerged through comparison of B cell subsets within participant groups (see Figure S2B in Supplementary Material). However, we believe that transcriptional analysis of rare and still biologically ill-expanded cell subsets, such as DN and AM (47, 49), would benefit more from an unbiased whole transcriptome approach (e.g., RNA Seq) on sorted subsets and after *in vitro* or *in vivo* stimulation in order to provide more definitive results.

Despite this limitation, in line with our previous report (27) and together with findings reported herein, the perturbation of the AM subset may underlie mechanisms of premature aging of the immune system and impaired ability of HIV-infected patients to respond to vaccinations and to maintain a long-term immune response (50).

Although limited by the small sample size, gene expression data from RM B cells, revealed a 25 gene signature that distinguished responders and NR to H1N1. Interestingly, these data were derived from samples collected *before* vaccination. This observation may suggest that in the context of HIV infection, RM B cells, which provide secondary, potent and specific immune responses (51) need to present a specific gene expression pattern in order to provide an effective response to vaccination. Most of the genes involved in the signature are directly involved in the B cell receptor gene signaling cascade and in B cell development (*APRIL, BTK, PI3K, MTOR, BST2*), suggesting that a lower expression of these genes may contribute to a reduced Ab production upon Ag-recall responses. These results are in line with recent data suggesting that modules of genes related to B cell and plasmablasts may be crucial indicators and biomarkers of vaccine induced immunogenicity and protection (21). Although our study was mainly focused on prevaccination signature of response in HIV-infected patients, we performed longitudinal analysis to investigate differences in gene expression of RM from samples collected at 21 days after vaccination (T1) compared to baseline (T0). Expression of *PRDM1* (BLIMP1), a transcriptional repressor that drives terminal differentiation into plasma cells was found higher in RM of HIV responders at baseline and was significantly reduced at T1 when compared to T0 in HIV and particularly in R (Figure S7 in Supplementary Material). Reduction of *PRDM1* may represent the resting phase of Ag specific B cells after migration to the germinal centers, class switch recombination, and somatic hypermutation (34, 52). Another consideration is that earlier timepoints (24 h to 1 week) after immunization or after re-exposure to the Ag will need to be tested in order to confirm this hypothesis and define the genes' activation programs which orchestrate memory B cell responses in HIV-infected children. Indeed in recent studies early changes of genes enriched in B cells, plasmablasts and immunoglobulins after administration of the RTS,S/AS01 malaria vaccine in healthy malaria-naive adults, were found to be related to vaccine Ab production and cell-related immunogenicity (19).

Our data on RM transcriptional signatures revealed that H1N1 responders expressed higher JAK-STAT cascade genes (*MX1, IFNAR, STAT4*). These findings are consistent with previous reports showing that STAT genes are crucial in the differentiation of RM B cells induced by IL-21 (53). In this context and following a similar experimental approach as this, we recently reported that *IL21* gene expression from prevaccination purified peripheral T follicular helper cells (pTfh) after *in vitro* stimulation, is an indicator of vaccine response (54).

The present study identified predictive correlates of seroconversion following immunization using pair-wise correlation analysis between individual gene expression data and serological data. In RM, *BTK*, involved in B cell development, and *CD86*, a lymphocyte activation gene, showed significant positive correlations with H1N1 seroconversion after vaccination in HIV

supporting the hypothesis that gene signatures in purified RM B cells at the time of immunization may predict the ability of HIV-infected children to respond to vaccinations. Taken together these findings suggest that specific gene signatures in cell subsets directly involved in Ab production and response to Ag (pTfh and RM B cells) are needed to provide an efficient immune response and are altered in HIV infection.

This experimental approach, based on a targeted gene selection ($n = 96$) rather than unbiased whole transcriptome sequencing, illustrates the benefits of analysis of purified cell subsets. The increased specificity resulting from this approach is important, considering the observed phenotypic alterations in immune cells from HIV-infected patients. We believe that these data provide a strong rationale to warrant future larger studies that can expand and validate these findings.

ETHICS STATEMENT

Written informed consent was obtained from all subjects or parents/guardians and the local Institutional review board approved the study.

AUTHOR CONTRIBUTIONS

NC, LA, SuP, PP, and SP conceived the study and designed the experiments. NC and LA performed the experimental procedures. NC drafted the first version of the article. All authors participated in writing, review and editing of the article. NC, LA, BI, and SR performed statistical analysis and bioinformatics. Supervision and resources were provided by PR, PP, and SP.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.01083/full#supplementary-material.

FIGURE S1 | Experimental design. Cartoon in panel **(A)** depicts design of the study. Peripheral blood mononuclear cells (PBMCs) were collected at the time of vaccination (T0) and 21 days after vaccination (T1). At both timepoints, hemagglutination inhibition assay (HIA), and H1N1 ELISpot were performed. Briefly, in panel **(B)** PBMCs stored in liquid nitrogen were thawed and stained for surface molecules and analyzed by flow cytometry (ARIA II cs). Equal number (500 cells) of cells from 5 subsets and total PBMCs from unstimulated samples were sorted as depicted in the sorting strategy into tubes previously coated with specific polymerase chain reaction (PCR) buffer. Data, collected through Fluidigm Real Time PCR analysis software was then analyzed through Fluidigm SingulaR (SingulaR analysis toolset 3.0) package, loaded on R (software R 3.0.2 GUI 1.62). Data were later used for mixOmics on R (mixOmics package) and for gene set enrichment analysis (GSEA).

FIGURE S2 | Gene expression patterns of memory B cell subsets rather than B cell frequencies differentiates virally controlled HIV-infected children from their healthy peers. **(A)** Principal component analysis (PCA) shows segregation among the four different B cell subsets in healthy controls (HC) and HIV. JMP©, SAS® has been used to produce the PCA. **(B)** Venn diagram shows differentially expressed genes (DEGs) found when gene expression of activated memory (AM) and resting memory (RM) are compared between HIV and HC. Only ANOVA analyses with a p value < 0.05 are shown. Genes marked with the asterisk show p values ≤ 0.01. All 23 DEGs found in HC were overlapping in HIV (light gray box). 54 additional DEGs were found in HIV only (dark gray box).

FIGURE S3 | Differential inter subset analysis. Heatmap of intersubset analysis in healthy controls (HC) **(A)** HIV **(B)**, R **(C)**, and NR **(D)**. Heatmap analyses were generated by singular analysis toolset after identification and removal of outliers.

FIGURE S4 | Gene set enrichment analysis from differentially expressed genes resulting from activated memory in HIV vs. healthy controls (HC). Gene Set Enrichment Analysis in the graph was generated by genemania cytoscape app (genemania.org).

FIGURE S5 | Scatter dot plot in panel **(A)** shows frequencies of total B cells (live, $CD10^-$, $CD20^+$); and among B cells: double negative ($CD27^-$, IgD^-), total naive ($CD27^-$, IgD^+), class switched $CD27^+$ memory B cells ($CD27^+$, IgD^-), tissue like ($CD27^-$, IgD^-, $CD21^-$). In panel **(B)** activated memory ($CD27^+$, IgD^-, $CD21^-$) and resting memory ($CD27^+$, IgD^-, $CD21^+$) (two tailed Mann–Whitney test for comparisons) are shown.

FIGURE S6 | Activated memory (AM) vs. resting memory (RM) differentially expressed genes (DEGs) in healthy controls (HC), R and NR. Venn Diagram in panel **(A)** shows DEGs found when gene expression of AM and RM are compared between HIV R, HIV NR, and HC. Only ANOVA analysis with a p value < 0.05 are shown. **(B,C)** gene set enrichment analysis of DEGs derived from activated memory vs. resting memory of HIV NR vs. HC **(B)** and HIV R vs. HC **(C)** are shown.

FIGURE S7 | Post vaccination (T1) *PRDM1* (*BLIMP1*) gene expression is reduced in resting memory (RM) of HIV-infected children compared to baseline (T0). In panel **(A)**, volcano plot shows differences in gene expression between T1 and T0 in selected genes from sorted RM. In panel **(B)** paired analysis in R (green) and NR (red) of PRDM1 are shown. *P*-Values and median differences derive from Wilcoxon paired t test performed by graphpad (prism 6.0).

FIGURE S8 | IL21R and BAFF (*TNFSF13B*) gene expression in resting memory (RM) correlates with surface molecules and plasma levels. In panel **(A)** heatmap analysis shows r values resulting from pairwise Spearman correlations between the aforementioned observations. In **panels (B–D)**, correlation dot plots of the afore mentioned analysis are shown. In the graph gene expression resulting from RM is shown.

TABLE S1 | The full list of genes, alias names and assay codes used for multiplexed principal component analysis are shown in the table.

TABLE S2 | Differentially expressed genes (DEGs) between cell subsets in HIV, healthy controls (HC), R and NR. Table shows DEGs in all groups (HIV, HC, HIV NR, HIV R) when subsets were compared. In the table, cells are highlighted in orange when *p* values were <0.01 and not highlighted when *p* value <0.05. Outlined cells define an opposite relation compared to the one in the headline.

TABLE S3 | Differentially expressed genes between study groups. Note: *genemania.org gene set enrichment analysis (GSEA)* In every graph presented throughout the paper, circles with diagonal lines define genes included in the GSEA. Its function is described legend below the graph. The solid circles represent genes resulted to be involved in the pathways. Networks among the genes are defined by colored lines, further explained by legends.

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REVIEW
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Beyond Antibodies: B Cells and the OPG/RANK-RANKL Pathway in Health, Non-HIV Disease and HIV-Induced Bone Loss

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HIV infection leads to severe B cell dysfunction, which manifests as impaired humoral immune response to infection and vaccinations and is not completely reversed by otherwise effective antiretroviral therapy (ART). Despite its inability to correct HIV-induced B cell dysfunction, ART has led to significantly increased lifespans in people living with HIV/AIDS. This has in turn led to escalating prevalence of non-AIDS complications in aging HIV-infected individuals, including malignancies, cardiovascular disease, bone disease, and other end-organ damage. These complications, typically associated with aging, are a significant cause of morbidity and mortality and occur significantly earlier in HIV-infected individuals. Understanding the pathophysiology of these comorbidities and delineating clinical management strategies and potential cures is gaining in importance. Bone loss and osteoporosis, which lead to increase in fragility fracture prevalence, have in recent years emerged as important non-AIDS comorbidities in patients with chronic HIV infection. Interestingly, ART exacerbates bone loss, particularly within the first couple of years following initiation. The mechanisms underlying HIV-induced bone loss are multifactorial and complicated by the fact that HIV infection is linked to multiple risk factors for osteoporosis and fracture, but a very interesting role for B cells in HIV-induced bone loss has recently emerged. Although best known for their important antibody-producing capabilities, B cells also produce two cytokines critical for bone metabolism: the key osteoclastogenic cytokine receptor activator of NF-κB ligand (RANKL) and its physiological inhibitor osteoprotegerin (OPG). Dysregulated B cell production of OPG and RANKL was shown to be a major contributor to increased bone loss and fracture risk in animal models and HIV-infected humans. This review will summarize our current knowledge of the role of the OPG/RANK–RANKL pathway in B cells in health and disease, and the contribution of B cells to HIV-induced bone loss. Data from mouse studies indicate that RANKL and OPG may also play a role in B cell function and the implications of these findings for human B cell biology, as well as therapeutic strategies targeting the OPG/RANK–RANKL pathway, will be discussed.

Keywords: B cells, HIV, bone loss, comorbidities, cytokines, end-organ damage

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INTRODUCTION

Rising incidences of bone loss in the form of low bone mineral density (BMD), osteopenia, and osteoporosis, and the resulting increased risk of fracture have over the past decade emerged as important non-AIDS comorbidities affecting HIV-infected individuals (1–6). Successful antiretroviral therapy (ART) over the past couple of decades has been instrumental in significantly extending the life expectancies of HIV-infected individuals to levels comparable to those of the general population (7). A significant proportion of people currently living with HIV in Europe and North America are over the age of 50 (8–10), and it is estimated that by 2030 as many as >70% of HIV-positive individuals will fall within this demographic. Similar to cardiovascular, liver and chronic kidney disease, and other comorbidities, bone loss occurs earlier and at a higher prevalence in HIV-positive individuals than in the HIV negative population (1, 8, 11). This raises concerns of a potential impending epidemic of fragility fractures and other age-associated comorbidities in this population (8, 12).

The underlying mechanisms of HIV-associated bone loss are multifactorial, given that most of the traditional risk factors for bone loss including low body mass index (BMI), older age, tobacco use, metabolic diseases, alcohol, and substance abuse are more prevalent in the HIV-infected population (10, 13). HIV infection is now however clearly established as one of the independent risk factors for bone loss (11, 14, 15), driven by the prevalence of HIV-associated risk factors including chronic inflammation, co-infection with hepatitis B or C, and paradoxically, ART (8, 10, 13). More recently, osteoimmunology has revealed the prominent role the immune system plays in bone metabolism (16) and consequently revealed that HIV-induced immune dysfunction is one of the most important contributors to bone loss.

Osteoimmunology, a term originally coined to describe studies involving the interface between the immune and skeletal systems (17), has been instrumental in our understanding of the numerous ways both organ systems are intertwined. It is now known that in various inflammatory pathological conditions characterized by bone loss, including periodontal disease (PD) and rheumatoid arthritis (RA), both cellular and soluble immune effectors can contribute to bone loss (18, 19). T cells are major contributors to bone loss in RA (20) and PD (21, 22) but their role in HIV-induced bone loss has not been elucidated. Emerging evidence now shows that B cells play an important role in bone biology in health and disease (23–25) and HIV-induced B cell dysfunction significantly contributes to HIV-induced bone loss (26).

Bone homeostasis, which is essential for maintaining skeletal integrity and strength, is regulated by a balance of bone formation by osteoblasts and resorption by osteoclasts and disruption of this balance results in bone disease (18, 27, 28). Osteoclasts are generated in a process known as osteoclastogenesis, which is driven by the key osteoclastogenic cytokine receptor activator of NF-κB ligand (RANKL). Osteoclasts originate from cells of the myeloid lineage, which in the presence of M-CSF and RANKL differentiate into receptor activator of NF-κB (RANK)-expressing pre-osteoclasts which proliferate and fuse to form giant multinucleated osteoclasts capable of resorbing bone (15, 29).

Excessive osteoclast activity, as occurs in osteoporosis, results in loss of bone mass and increased susceptibility to fracture (12, 28). The effects of B and T cells on bone are mediated by several key cytokine regulators of bone metabolism (11, 18), including the inflammatory cytokines tumor necrosis factor-α (TNF-α) and interferon-γ, which have been implicated in bone loss in RA, periodontitis, postmenopausal osteoporosis, and HIV (30). Most importantly, RANKL and OPG (18) play important roles in both organ systems and perfectly illustrate the intersection of bone biology and immunity. The OPG/RANK–RANKL pathway also mediates physiological processes in the vascular system, thus intersecting with the skeletal and immune system at this axis (**Figure 1**).

This review will summarize our current knowledge of the role of the OPG/RANK–RANKL pathway in B cells in health and disease, and the contribution of B cells to HIV-induced bone loss. Data from mouse studies indicate that RANKL and OPG may also play a role in B cell function and the implications of these findings for human B cell biology as well as therapeutic strategies targeting the OPG/RANK–RANKL pathway will be discussed.

THE OPG/RANK–RANKL PATHWAY AND B CELLS IN HEALTH

B cells are inextricably linked to bone, from their development in the bone marrow to the homing of terminally differentiated

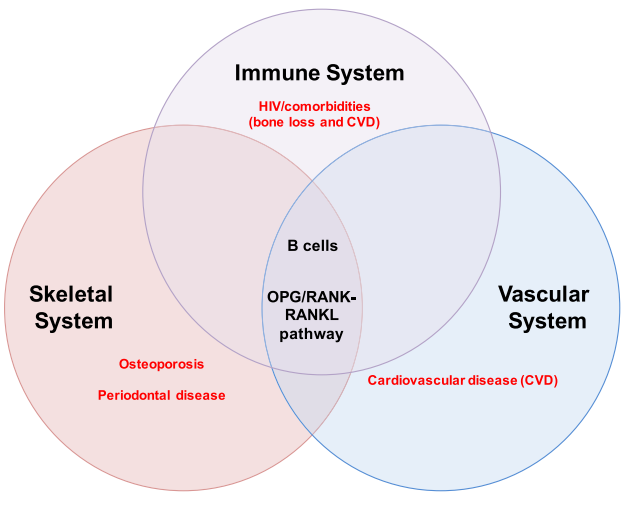

FIGURE 1 | B cells and the OPG/RANK-RANKL pathway at the intersection of the immune, skeletal, and vascular organ systems. B cells mediate biological processes in health and disease *via* the OPG/RANK–RANKL pathway in three major organ systems in humans: the immune, skeletal, and vascular systems. The extensive intertwining of the immune and skeletal systems has given rise to a whole new field of study called osteoimmunology; some major pathologies implicating B cells and the OPG/RANK–RANKL pathway are highlighted in red and include osteoporosis and periodontal disease in the skeletal system, cardiovascular disease (CVD) in the vascular system, and HIV/comorbidities (bone loss and CVD) in the immune system.

plasma cells back to the bone marrow (30, 31) and the bidirectional regulation of the skeletal system by B cells (23, 30, 32). Osteoblasts and bone marrow stromal cells regulate B lymphopoiesis through the production of IL-7, a critical cytokine for the differentiation of early-stage B cells in the bone marrow (33, 34). Another major interaction between the skeletal system and B cells revolves around the OPG/RANK–RANKL pathway.

B Cells and Osteoprotegerin (OPG)

The identification and characterization of OPG as a humoral regulator of bone resorption 20 years ago (35, 36) represents a major turning point in our understanding of the physiology of bone homeostasis (37, 38). OPG, named for its ability to protect bone by inhibiting osteoclast differentiation and activity, is a tumor necrosis factor receptor (TNFR) superfamily member which lacks transmembrane-spanning sequences and is secreted as a soluble protein (35, 36). OPG is the natural circulating inhibitor/decoy receptor of RANKL and can inhibit osteoclastogenesis by binding to RANKL, thus preventing bone resorption (35, 37). OPG mRNA is expressed by various tissues, including bone, brain, lung, heart, and kidney (35, 36). In the immune system, OPG is expressed in lymph nodes, B cells, and dendritic cells (DCs) and ligation of CD40 upregulates its expression (39).

Osteoblasts and their precursors were previously considered to be the primary source of OPG in the bone marrow (40, 41) but B lineage cells are now known to account for over 60% of total bone marrow OPG production (25). B cell knockout (KO) mice were osteoporotic and deficient in bone marrow OPG, confirming the critical role of B cells in the preservation of bone homeostasis and attainment of peak bone mass (25).

Unlike its role in bone homeostasis, the role of OPG in B cell function is less well documented. OPG KO mice develop severe osteoporosis due to unchecked osteoclastogenesis and bone resorption (42, 43). Interestingly, OPG-deficient mice also accumulated transitional/immature B cells in their spleens, and generated impaired antibody (Ab) responses to a T cell-dependent (DNP-KLH) antigen (Ag) challenge, suggesting that OPG may regulate B cell maturation and development of efficient Ab responses (44).

B Cells and RANKL

The ligand for OPG is identical to a TNFR family member called TNF-related activation-induced cytokine or RANKL (37, 45). Human RANKL exists in two forms: a cellular, membrane-bound form and a soluble form, and both forms were shown to be biologically capable of promoting osteoclast formation (46, 47). RANKL is also expressed in a variety of tissues, including bone marrow and lymphoid tissues (36, 47, 48). RANKL is best known for its indispensable role in the complete differentiation of mature osteoclasts (36, 37, 47). Unlike OPG, resting B cells have not been conclusively shown to produce significant amounts of RANKL, but activated B cells are an important source (23), particularly in inflammatory disease states.

B Cells and RANK

The receptor for RANKL, RANK, was initially identified on DCs (48) and later discovered to be expressed on preosteoclastic cells (37, 46, 49, 50) and B cells (39, 51). The binding of RANKL to RANK stimulates osteoclastogenesis, resulting in bone-resorbing osteoclasts (47).

Lack of functional RANK in both humans and mice results in osteopetrosis due to the absence of osteoclasts (19, 49, 52). Mice deficient in RANK had defects in B cell development which resulted in reduced numbers of mature B cells in the periphery (49). Humans with mutations in RANK also had B cell defects including hypogammaglobulinemia and impaired Ag-specific Ab responses (52).

THE OPG/RANK–RANKL PATHWAY AND B CELLS IN NON-HIV DISEASE

Osteoprotegerin, RANK, and RANKL are produced by a wide variety of cells and tissues in three major organ systems: the vascular, immune, and skeletal systems and are thus implicated in the pathogenesis of various diseases in these organs (15, 38) (**Figure 1**). Although best known for its involvement in the pathogenesis of osteoporosis and other bone diseases such as Paget's disease of bone (53–55) and PD (38, 56), the OPG/RANK–RANKL pathway has also been implicated in other diseases including RA (14, 38, 57) and CVD (58–60).

Rheumatoid Arthritis

The bone and joint destruction that occurs in the autoimmune disorder RA results from increased RANKL-induced osteoclastic bone resorption in the synovial joints (57, 61, 62). Several immune cells have been identified as the sources of RANKL in the arthritic synovium, including Th17 cells (63), macrophages, DCs (57), and activated B cells (64). Targeted B cell depletion therapy for RA using the anti-CD20 Ab rituximab suggests that B cells play a critical role in RA-associated joint damage (64–66). B cells were shown to contribute to RA pathogenesis through their Ag-presenting function, autoantibody production, and cytokine secretion (66, 67). A link between B cells and joint destruction in RA has been confirmed by studies demonstrating that Rituximab significantly reduces RANKL levels in the synovium (68, 69). This link has recently been confirmed by studies identifying pro-inflammatory B cells as major sources of RANKL in RA (64,66). These findings highlight the importance of Ab-independent (cytokine-producing) B cell functions in the pathogenesis of disease and make a case for the therapeutic potential of targeting the B cell OPG/RANK–RANKL pathway in RA and other diseases.

In contrast to RANKL, multiple studies have demonstrated that serum levels of OPG are elevated in RA, resulting in a decreased RANKL/OPG ratio (70, 71). Elevated OPG levels were independently associated with RA disease severity and CVD, and it has been suggested that OPG concentration could be used as a predictive marker for assessing RA-associated CVD risk (72, 73). Data on the role of B cell-produced OPG in the pathophysiology of RA are however lacking.

Cardiovascular Disease

A role for the OPG/RANK–RANKL pathway in the pathogenesis of vascular calcification and CVDs has been established for over a decade now. Both OPG and RANKL have been detected in

atherosclerotic plaques (74) and an increased RANKL/OPG ratio is associated with atherosclerosis (59). Transgenic expression of OPG in OPG KO mice prevented the development of arterial calcification but exogenous OPG administration did not reverse existing calcification, suggesting that similar to bone, OPG is a protective factor in the cardiovascular system (75, 76). Results in human studies however seem to conflict with the animal studies, with higher OPG levels consistently associated with CVD incidence (76, 77). The contribution of B cells to OPG/RANK–RANKL-linked CVD has however not been clearly elucidated. Low-density lipoprotein (LDL) receptor KO mice ($LDLR^{-/-}$) were B cell deficient and developed atherosclerosis, suggesting that B cells and/or antibodies are protective against atherosclerosis (78); it is conceivable that OPG produced by B cells mediates this protective effect.

Bone Diseases

Osteoporosis

Osteoporosis is characterized by loss of bone mass and mineral density resulting from an excess of bone resorption by osteoclasts relative to bone formation by osteoblasts (18, 27, 28). The role of the OPG/RANK–RANKL pathway in the pathogenesis of osteoporosis has been well documented and extensively reviewed (15, 37, 38, 62); the role of B cells is however still being elucidated.

Postmenopausal osteoporosis, the most common form of osteoporosis, arises from decreased estrogen levels (62) and was shown in both human patients and an animal model to be linked to increased RANKL expression by B cells (79). Mice subjected to ovariectomy, commonly used as an animal model of estrogen deficiency, have increased numbers of B cells, suggesting that B cells may play a role in estrogen-deficiency osteoporosis (79–81). Data on the contribution of B cells to ovariectomy-induced bone loss is however conflicting. Some studies have demonstrated that ovariectomy-induced bone loss occurs independently of mature B cells (82) and others show that ovariectomy-induced bone loss is linked to RANKL expression on immature B cells (79). Given the fact that B cells are able to express RANKL at various stages in their differentiation, this raises the possibility that the contribution of B lineage cells to estrogen-deficiency osteoporosis is dependent on the differentiation/maturation stage of the B cell. Beyond the differentiation stage however, the activation status of B cells seems to be a better indicator of their ability to produce bone-damaging RANKL (23). This is especially relevant in the context of inflammatory diseases like RA, PD, and HIV-induced bone loss.

Periodontal Disease

Periodontal diseases are inherited or acquired disorders affecting the supporting structures of the teeth and affect as many as 50–90% of the world's population (83). The underlying microbial infections were traditionally the focus of majority of the research on the pathogenesis of PDs but in recent years the focus has shifted to the role of the host response/factors in pathogenesis (83, 84). Host immune/inflammatory responses are critical for pathogenesis and inflammation (84) and the term PD generally refers to inflammation-induced disorders, ranging from the mildest form (gingivitis) to the more invasive severe periodontitis (83). Unlike gingivitis which is completely reversible by effective regular oral hygiene, periodontitis extends deeper into the tissue and can result in the permanent loss of the supporting structures of the teeth and alveolar bone (83).

One of the microorganisms most commonly implicated in PD pathogenesis is *Actinobacillus actinomycetemcomitans (Aa)*, which induces RANKL expression on a variety of cell types infiltrating in PD lesions (84). While the RANKL levels in PD lesions are consistently elevated in most clinical studies, some studies found lower (22) or unchanged (24) OPG levels in lesions, which both resulted in higher RANKL/OPG ratios in periodontitis compared to healthy controls (22, 24, 84). Activated B and T cells were shown to be the primary source of RANKL in gingival tissues from individuals with periodontitis (24, 85). B cell percentages in chronic PD lesions were associated with disease severity, suggesting that B cells promote PD (86) and interestingly, PD lesion-infiltrating B cells in humans were activated transitional $CD5^{+}$ cells (86, 87). Using a rat model, it was also demonstrated that B cells contributed to osteoclast formation and periodontal bone loss by secreting RANKL following activation by Aa in a T cell-independent manner (85).

B CELLS, THE OPG/RANK–RANKL PATHWAYS, AND HIV-INDUCED BONE LOSS

With the availability of ever-improving treatment regimens, ART is enabling HIV-infected individuals to live longer than ever before, but life expectancies of patients remain lower than those of the general population (7, 12, 15, 16, 59). Over 33% of people currently living with HIV in Europe are >50 years of age and this percentage is expected to increase to >70% by 2030 (88); in the US, the same demographic is estimated to constitute up to 50% of the HIV-positive population (89). This increased longevity is however accompanied by earlier occurrence and higher prevalence of several non-AIDS end-organ comorbidities including cardiovascular and bone diseases (90–92), which in turn imposes significant disease burdens on the patients, healthcare systems, and society.

As discussed above, under inflammatory conditions, B cells produce higher amounts of RANKL, leading to an increased RANKL/OPG ratio, which drives disease progression (26, 29) in inflammatory diseases such as RA. HIV infection is associated with persistent inflammation (93) and the success of B cell-targeted/depleting therapies in reducing inflammation in autoimmune disorders such as RA suggest that B cells may contribute to persistent inflammation (94, 95). Given the pivotal role this pathway plays in osteoclastogenesis and bone loss, its role in B cells and HIV is perfectly illustrated by its contribution to inflammation-driven HIV-induced bone loss.

A hallmark of chronic HIV infection is the altered distribution of subsets in the B cell compartment (93), notably the loss of resting memory B cells (26) and the expansion of exhausted/tissue-like memory B cells (26, 93, 96). Interestingly, OPG

expression was lowest in the HIV-expanded tissue-like memory B cell subset, which conversely showed higher RANKL expression (26) (**Figure 2**). This tissue-like memory B cell subset was also previously shown to express the inhibitory receptor FcRL4 (96), which in RA defined a pro-inflammatory RANKL-producing B cells subset (66). Taken together, this suggests that inflammation does drive B cell subset RANKL expression in HIV infection.

Low BMD increases the risk of fragility fractures and is widely prevalent in HIV-infected individuals, with as many as 67% presenting with osteopenia and ~15% with osteoporosis (91). Increased osteopenia and osteoporosis rates translate into significantly elevated fracture risk, and studies show that HIV-infected individuals do indeed suffer more fragility fractures, at younger ages, than the general population (1). The ubiquitous presence of traditional risk factors for low BMD such as increased smoking and low BMI in most HIV-infected cohorts complicates efforts to understand and elucidate the mechanisms underlying HIV-induced bone loss (11, 26, 97). HIV infection in itself is now recognized as a risk factor for bone loss (97).

HIV transgenic rats almost perfectly mimic the clinical hallmarks of human HIV-induced bone disease, including profound skeletal damage. Bone loss in this model was driven by increased B cell RANKL expression concurrent with decreased OPG expression, which in turn resulted in increased RANKL/OPG ratio and thus osteoclastogenesis and bone loss (98). This mechanism of HIV-induced B cell dysfunction-driven bone loss was later confirmed in a clinical study of untreated HIV-infected individuals where it was demonstrated that increased B cell RANKL/OPG was indeed associated with increased bone resorption (26). This demonstrated for the first time that the OPG/RANK–RANKL pathway is indeed a key pathway utilized by B cells to effect skeletal damage in HIV infection. This demonstrates clearly how HIV-induced B cell changes in the immune system translate directly into dysfunction and bone loss in the skeletal system (**Figure 2**).

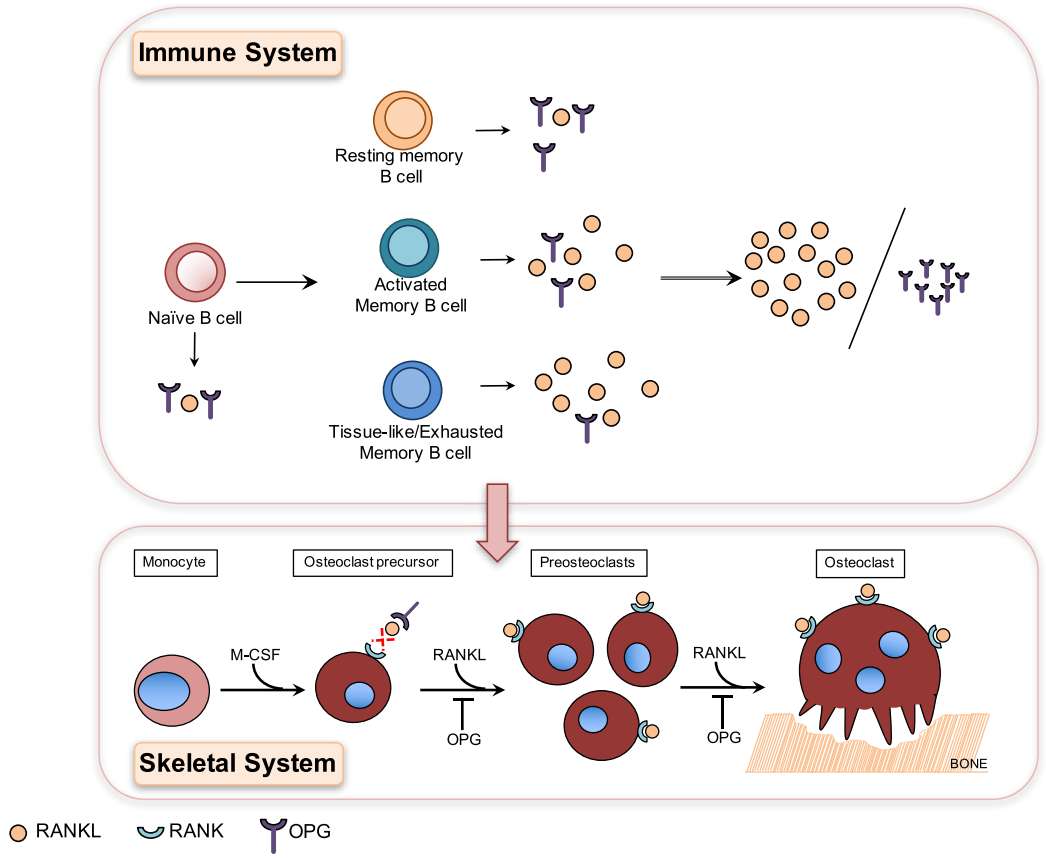

FIGURE 2 | Differential production of osteoprotegerin (OPG) and receptor-activator of NF-κB ligand (RANKL) by B cell subsets results in higher RANKL/OPG ratio, which contributes to HIV-induced osteoclastogenesis and bone loss. Osteoclasts are generated in a process known as osteoclastogenesis, which is driven by the key osteoclastogenic cytokine RANKL. Osteoclasts originate from cells of the myeloid lineage, which in the presence of M-CSF and RANKL differentiate into receptor-activator of NF-κB (RANK)-expressing pre-osteoclasts, which proliferate and fuse to form giant multinucleated osteoclasts capable of resorbing bone. HIV infection leads to the depletion of resting memory B cells and expansion of activated B cell subsets including activated memory and tissue-like memory B cells. Resting memory B cells produce the highest amounts of OPG and tissue-like memory B cells conversely the lowest amounts of OPG and the highest amounts of RANKL (26). HIV-induced B cell subset changes therefore translate into higher RANKL/OPG ratios, which contribute to increased osteoclastogenesis and bone loss in HIV-infected patients.

REGULATORY EFFECTS OF THE OPG/RANK–RANKL PATHWAY ON B CELLS AND HUMORAL IMMUNE RESPONSES

Due to the expression of OPG, RANK, and RANKL on a wide variety of immune cell types, the pathway is thought to play an important role in immune cell biology. Despite the involvement of B cell-expressed OPG and RANKL in the normal function of the immune, skeletal, and vascular systems and in the pathogenesis of multiple diseases, the effect of these molecules on B cell physiology has not been extensively described.

Receptor-activator of NF-κB ligand plays an important role in the development of secondary lymphoid organs. RANK- and RANKL-deficient mice had poorly developed or completely lacked secondary lymphoid tissues including lymph nodes, Peyer's patches, cryptopatches, and spleen (46, 49, 62).

The role of this pathway in B cell function has also been investigated in a few mouse studies. OPG-deficient mice accumulated transitional/immature B cells in their spleens and generated impaired Ab responses to a T cell-dependent (DNP-KLH) Ag challenge, suggesting that OPG may regulate B cell maturation and development of efficient Ab responses (44). Conversely, B cell development was impaired in RANKL-deficient mice, suggesting that OPG regulates B cell development.

In another study (99), OPG was used to treat mice induced to develop different types of cellular and humoral immune responses through: (1) infection with *Mycobacterium bovis* Bacillus Calmette and Guerin (BCG) followed by OPG-Fc treatment, (2) immunization with KLH in Freund's adjuvant or by i.p. injection of a Pneumococcal Vaccine Polyvalent (Pneumovax®23, Merck) (3) immunization with Keyhole Limpet Hemocyanin (KLH) *in vivo* followed by OPG-Fc treatment, and (4) In a bid to induce contact hypersensitivity, mice were also sensitized with the hapten oxazolone, followed by treatment with OPG-Fc. T and B cells were also exposed to OPG *in vitro.* OPG treatment did not affect cell-mediated responses including contact hypersensitivity but increased humoral immune responses to KLH and the pneumococcal vaccine. *In vitro,* OPG modestly stimulated T cells but not the proliferation of B cells. These results demonstrated that OPG has modest regulatory effects on humoral immune responses to certain Ags. The potential impact of the OPG/RANK–RANKL on the generation of human humoral immune responses is not clear and definitely merits further study.

THERAPEUTIC STRATEGIES TARGETING THE OPG/RANK–RANKL PATHWAY

Although initially described in the context of bone disease, the OPG/RANK–RANKL pathway is now known to influence normal physiology and pathology in the immune, skeletal, and vascular systems. This opens up the potential for a lot of cross application of potential therapeutic strategies targeting this pathway.

One such strategy involves RANKL inhibition; *E. coli*-derived Fc-OPG showed great promise in phase I trials, causing rapid decline in bone turnover markers in postmenopausal women (100), also serving as a proof of concept that RANKL blockade could meaningfully impact bone turnover in humans (46). Perhaps the best known RANKL inhibitor in clinical use to date is denosumab, a fully human IgG2 Ab which binds RANKL with high affinity and unlike Fc-OPG does not bind to mouse and rat RANKL and TRAIL (46). In clinical use, denosumab effectively reduces fracture risk by reducing bone resorption and was shown to be superior to bisphosphonates in its ability to increase BMD in postmenopausal women (46). When used to treat cancer-induced bone disease, denosumab effectively reduced levels of bone turnover markers in patients with solid tumor (breast, prostate, and lung) metastases to bone and prolonged bone metastasis-free survival and delayed the onset of first metastasis in certain prostate cancers (101). Denosumab was also well-tolerated and no significant changes in B cell numbers were noted (102, 103). The effect of denosumab on B cell function is not fully elucidated; in one study investigating its utility as a postmenopausal osteoporosis treatment (104), 2/412 women developed transient non-neutralizing anti-denosumab antibodies, which did not adversely affect the skeleton but did appear to alter the effectiveness of the drug (104). Due to the wide pattern of expression of RANKL, including on lymphocytes, and in the vascular and skeletal systems, RANKL inhibition using denosumab could potentially increase susceptibility to infections and neoplasias (105), particularly in immunocompromised patients. No significant alterations in inflammation and immunity have however been observed in preclinical and clinical studies of denosumab, although rare cases of severe skin infections of the lower extremities were reported (106). To date, no data are available on the use of denosumab in HIV infection and the effect of RANKL blockade on humoral immune responses in HIV-infected individuals remains to be elucidated.

CONCLUSION

In summary, B cells are intricately intertwined with the OPG/RANK–RANKL pathway, plays important roles in the immune, skeletal, and vascular systems, and much remains to be discovered about the influence of this pathway on human humoral immune responses.

AUTHOR CONTRIBUTIONS

KT designed, drafted, and revised the manuscript and is accountable for all aspects of the manuscript.

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REVIEW
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B-Cell-Activating Factor and the B-Cell Compartment in HIV/SIV Infection

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With the goal to design effective HIV vaccines, intensive studies focused on broadly neutralizing antibodies, which arise in a fraction of HIV-infected people. Apart from identifying new vulnerability sites in the viral envelope proteins, these studies have shown that a fraction of these antibodies are produced by self/poly-reactive B-cells. These findings prompted us to revisit the B-cell differentiation and selection process during HIV/SIV infection and to consider B-cells as active players possibly shaping the helper T-cell program within germinal centers (GCs). In this context, we paid a particular attention to B-cell-activating factor (BAFF), a key cytokine in B-cell development and immune response that is overproduced during HIV/SIV infection. As it does in autoimmune diseases, BAFF excess might contribute to the abnormal rescue of self-reactive B-cells at several checkpoints of the B-cell development and impair memory B-cell generation and functions. In this review, we first point out what is known about the functions of BAFF/a proliferation-inducing ligand and their receptors [B-cell maturation, transmembrane activator and CAML interactor (TACI), and BAFF-R], in physiological and pathophysiological settings, in mice and humans. In particular, we highlight recent results on the previously underappreciated regulatory functions of TACI and on the highly regulated production of soluble TACI and BAFF-R that act as decoy receptors. In light of recent data on BAFF, TACI, and BAFF-R, we then revisit the altered phenotypes and functions of B-cell subsets during the acute and chronic phase of HIV/SIV infection. Given the atypical phenotype and reduced functions of memory B-cells in HIV/SIV infection, we particularly discuss the GC reaction, a key checkpoint where self-reactive B-cells are eliminated and pathogen-specific memory B-cells and plasmablasts/cells are generated in physiological settings. Through its capacity to differentially bind and process BAFF-R and TACI on GC B-cells and possibly on follicular helper T-cells, BAFF appears as a key regulator of the physiological GC reaction. Its local excess during HIV/SIV infection could play a key role in B-cell dysregulations.

Keywords: B-cell-activating factor, B-cells, dendritic cells, germinal center, HIV, memory B-cells, follicular helper T-cells, SIV

INTRODUCTION

During pathogenic HIV/SIV infection, efficient antibody (Ab) protection hardly develops whereas immunoglobulin overproduction, germinal center (GC) hyperplasia (1), and increased recruitment of follicular helper T-cells (T_{FH}) into GC occur concurrently from the acute phase of infection (2–6). In addition to several reports showing increased proportions of atypical memory B-cells in lymphoid

organs and transitional B-cells in blood (7), recent molecular investigations established that a fraction of broadly neutralizing Abs (bNAbs) are produced by self/poly-reactive B-cells (8). In addition to direct B-cell activation by viral envelope proteins, inflammation is thought to play a major role in shaping these changes in B-cell phenotype and in virus-specific Ab responses (9–12). B-cell-activating factor (BAFF)/a proliferation-inducing ligand (APRIL) are instrumental cytokines for B-cell ontogeny and humoral responses in physiological settings (13), while their overproduction is detrimental in numerous autoimmune disorders (14, 15). During HIV/SIV or plasmodium infection, increased BAFF levels occur concurrently with expansion of atypical memory B-cells and inefficient Ab response (16–19). Thus, BAFF was thought to exert detrimental actions on pathogen-specific B-cells, and its overexpression has been associated with HIV/SIV disease progression (20–22). However, BAFF excess favors the expansion of immature-transitional B-cells and promotes self-Abs in mice and in patients with autoimmune diseases (23–25). Through a similar pathway, BAFF might be beneficial in expanding the pool of HIV cross-reactive B-cells, a potential source of bNAbs. Therefore, the role of BAFF excess in generating HIV/SIV-specific memory B-cells and neutralizing Abs needs to be further clarified. In this review, we first summarize what it is known about BAFF/APRIL and their receptors, with a special attention to transmembrane activator and CAML interactor (TACI), which might act as a key regulator of B-cell activation, BAFF-R shedding (26) and possibly self-reactivity. We then highlight data obtained in mice, humans, and macaques with the aim to better appreciate the role of BAFF and its receptors, BAFF-R and TACI, in HIV/SIV progression and in the expansion of HIV/SIV cross-reactive B-cells.

BAFF/APRIL AND THEIR RECEPTORS

The BAFF belonging to the tumor necrosis factor (TNF) superfamily (also called BLys) was first described as a key regulator of B-cell homeostasis and survival in mice and in humans (13). BAFF exerts its effects by binding to three different receptors: B-cell maturation (BCMA) (27, 28), TACI (29), and BAFF-R/BR3 (BLys receptor 3) (30). A highly similar homolog of BAFF (called APRIL) (31) also binds TACI and BCMA but not BAFF-R (32). APRIL only exists as a soluble form cleaved intracellularly, whereas BAFF can be found in both membrane-bound and soluble forms. In myeloid cells, BAFF is expressed on the cell surface as a membrane-bound form (mBAFF) and can then be released as a soluble form after cleavage by furin protease (33–35). Neutrophils directly release BAFF and APRIL as soluble cytokines (36, 37), whereas plasmacytoid dendritic cells (pDC) are unable to cleave mBAFF into its soluble form (19, 38, 39).

Through different expression and affinity for BAFF and APRIL, BAFF-R, TACI, and BCMA finely tune B-cell ontogeny and immune responses with species specificity (30, 40–44). Functional BAFF-R and TACI are expressed in B1 cells (45), and aging APRIL-transgenic mice develop B1 lymphoma (46, 47), whereas BAFF- and BAFF-R-deficient mice have normal proportions of B1 cells (48) (**Table 1**). This indicates that the TACI–APRIL pair likely plays a dominant role in murine B1 homeostasis. Absent from early transitional B-cells (T1, $CD10^{+}CD21^{lo}$), BAFF-R expression is acquired by transitional type-2 B-cells (T2, $CD10^{+}CD21^{+}$), and deficiency in BAFF-R inhibits B-cell ontogeny beyond the T1/T2 transition (49). However, this blockade is not absolute, and small proportions of mature B-cells are still present in BAFF-R-deficient mice and, to a lesser extent, in BAFF-deficient mice that mount residual responses to T-dependent (TD) antigens (50, 51). Consistently, BAFF- or BAFF-R-deficient mice form rudimentary GC in response to TD antigens (52, 53). Absent from naïve and memory B-cells, BCMA is dispensable for the survival of mature B-cells, spleen architecture, and GC development. Response to TD or T-independent (TI) antigens and isotype class switching are normal in BCMA-deficient mice (54–56). However, BCMA is important for long-term plasma cell biology (55, 57, 58) and antigen presentation (59). Upon binding to BCMA, APRIL and, to a lesser extent, BAFF promotes the survival of long-lived plasma cells in bone marrow (55). BCMA therefore constitutes one privileged target for the selective killing of malignant plasma cells, such as multiple myeloma cells (60, 61). Consistent with the recent description of constitutive BCMA shedding from the membrane of plasma cells by a γ-secretase (62), high serum BCMA level correlates with disease status and constitutes a valuable biomarker in multiple myeloma (63). Moreover, TACI expression distinguishes $TACI^{lo}$ from $TACI^{hi}$ myeloma, the latter with a signature of plasma cells, which are more dependent on

TABLE 1 | Phenotype of B-cell subsets and expression of B-cell-activating factor (BAFF)/a proliferation-inducing ligand (APRIL) receptors.

B-cell subset	Phenotype	BAFF/APRIL receptor expression	Reference
Mouse B1 cells	$CD19^{hi}SIgM^{hi}SIgD^{lo}CD43^{+}CD1d^{int}CD23^{-}CD5^{+}$ (B1a) or $CD5^{-}$ (B1b)	BAFF-$R^{+}TACI^{+}$	(45–48)
Early transitional B-cells (T1)	$CD19^{+}IgM^{hi}CD10^{+}CD24^{hi}CD38^{hi}CD21^{lo}$	BAFF-$R^{-/lo}TACI^{+/-}$	(25, 49, 51, 66–69)
Transitional type-2 B-cells (T2)	$CD19^{+}SIgM^{hi}SIgD^{lo}CD10^{+}CD24^{hi}CD38^{hi}CD21^{+}$	BAFF-$R^{+}TACI^{+/-}$	
Marginal zone B-cells	$CD19^{+}CD20^{+}IgM^{hi}CD21^{hi}SIgD^{+}CD23^{-}CD27^{+}$	BAFF-$R^{+}TACI^{hi}$ (short > long isoform)	(48, 54–56, 66, 70–75)
Naïve follicular B-cells	$CD19^{+}CD20^{+}SIgD^{hi}SIgM^{+}CD21^{+}CD23^{+}CD27^{-}CD95^{-}$	BAFF-$R^{hi}TACI^{-/lo}$ (long isoform)	(48, 52–55, 56, 70–74)
Germinal center (GC) B-cells centroblasts	$CD19^{+}CD20^{+}CD27^{int}Bcl6^{+}Ki67^{+}Sig^{-}CD95^{+}CD10^{+}CXCR4^{+}$	BAFF-$R^{hi}TACI^{lo}$	(48, 52, 53, 56, 70, 71, 74, 76, 77)
GC B-cells centrocytes	$CD19^{+}CD20^{+}CD95^{+}CD10^{+}CD38^{+}CD83^{+}SIgM/A/G^{+}$	BAFF-$R^{hi}TACI^{+}$	(78, 79)
Resting memory	$CD19^{+}CD20^{+}SIgD^{-}SIgG/A^{+}CD27^{+}CD21^{+}CD95^{+}$	BAFF-$R^{+}TACI^{hi}$ (short > long isoform)	(48, 69, 72, 73, 80)
Activated memory	$CD19^{+}CD20^{hi}SIgD^{-}SIgG/A^{+}CD27^{+}CD21^{lo}CD95^{+}$	BAFF-$R^{int}TACI^{+}BCMA^{+}$	(68, 81–83)
Tissue-like memory	$CD19^{+}CD20^{hi}SIgD^{-}SIgG/A^{+}CD27^{-}CD21^{lo}CD95^{+}$	BAFF-$R^{int}TACI^{+}BCMA^{+}$	
Plasmablasts	$CD19^{+}CD20^{lo}CD21^{lo}CD27^{hi}CD38^{hi}CD138^{lo}$	BAFF-$R^{lo}TACI^{lo}BCMA^{+}$	(64, 68, 81)
Plasma cells	$CD19^{lo}CD20^{-}CD27^{hi}CD38^{+}CD138^{hi}$	BAFF-$R^{lo}TACI^{hi}BCMA^{hi}$	(55, 57, 58, 64)

bone marrow signals (64), likely osteoclast-derived BAFF/APRIL and IL6 (65). Accordingly, TACI^{hi} myelomas are expected to be more responsive to BAFF-related immunotherapies. Based on these data in malignant cells, normal circulating plasmablasts are thought to be TACI^{lo} in contrast to long-lived plasma cells present in bone marrow that would be TACI^{hi}.

TACI: A MULTIFACETED RECEPTOR FOR BAFF/APRIL IN MICE AND HUMANS

Conventional and Regulatory Functions of TACI in Mice

BAFF-R is expressed by most follicular B-cells whereas TACI is absent (or very low) from naïve B-cells but highly present on marginal zone (MZ) and class-switched memory B-cells (48, 72, 73). TACI-deficient mice fail to respond to type-2 TI antigens (TI-2) but retain normal TD response (**Table 2**). However, they have reduced serum IgM and IgA levels, but normal IgG levels (48). *In vitro*, Castigli et al. have established that the murine TACI–APRIL pair is mandatory for IgA class switching and plays a dominant role over the BAFF-R–BAFF pair in IgG class switching (56). In another mouse model, TACI deficiency induces hyperplasia, enlarged MZ B-cell pool (66) and lupus-like autoimmune manifestations in aged mice (84). In agreement with TACI controlling exacerbated B-cell activation, knock-in mouse carrying a C76R mutation that impairs TACI-induced NF-κB activation develops splenomegaly with increased proportions of MZ and follicular B-cells (74). So, murine TACI that positively controls response to TI-2 antigens and IgA class switching can also deliver inhibitory signals that dampen abnormal B-cell activation and expansion. *In vitro*, Figgett et al. recently demonstrated that BAFF binding to TACI selectively limits TI innate response of TLR4-activated MZ B-cells by promoting FAS/FASL-mediated apoptosis (75). This process is thought to prevent inappropriate TI B-cell responses such as the expansion of self-reactive B-cells, and therefore to safeguard peripheral immune tolerance. Thus, membrane TACI controls excessive expansion/response of various mouse B-cell subsets.

BAFF-R and TACI in Humans: Lessons from Patients with Genetic Immunodeficiency

Spontaneous mutations occurring in individuals or families offer the opportunity to compare the biological importance of key molecules between mice and humans (**Table 2**). Studies in two patients with common variable immunodeficiency (CVID) carrying a homozygous deletion in BAFF-R gene, that precludes its membrane expression, confirm the key role of BAFF-R in human B-cell development. However, the phenotype of these patients is less severely compromised than that of BAFF-R-deficient mice,

TABLE 2 | Consequences of deficiency in BAFF-R and transmembrane activator and CAML interactor (TACI) in genetically modified mice and common variable immunodeficiency (CVID) patients.

Receptor	Phenotype	Reference
BAFF-R KO mice and A/WySnJ mice	Blockade of B-cell development at the T1/T2 transition Small proportions of marginal zone (MZ) and follicular B-cells Normal proportions of B1 cells Rudimentary GC but rapid involution, residual TD response Impaired class switching	(53, 76)
CVID patients with BAFF-R deficiency	No BAFF-R membrane expression Reduced numbers of mature B-cells, in particular MZ B-cells Expansion of T2 B-cells in blood Substantial numbers of naive and memory B-cells Reduced levels of IgM and IgG but normal IgA levels	(77)
TACI KO mice	Normal MZ and B1 cells Impaired response to TI-2 Ags with low IgM/A levels Normal IgG levels Normal TD responses	(48)
	B-cell lymphoproliferation and enlarged MZ B-cell pool Overproduction of Ig in response to TD Ags Lupus-like autoimmune manifestations in aged mice Increased production of self-reactive antibodies	(66, 84)
CVID patients with TACI deficiency	No or reduced TACI membrane expression Impaired NF-κB signaling Impaired IgA and IgG class switching Reduced response to TI-2 Ags B-cell lymphoproliferations, splenomegaly Increased frequency of autoimmune diseases Lack of lupus-like symptoms	(56, 70, 71)
TACI KI C76R mice	Normal membrane expression of TACI Impaired NF-κB activation Increased proportions of MZ and follicular B-cells Splenomegaly	(74)

with significant numbers of circulating memory B-cells and normal IgA levels, despite B-cell lymphopenia and low levels of circulating IgM and IgG (77).

Similarly, the phenotype of CVID individuals with TACI deficiency differs from that of TACI-deficient mice (70, 71). These individuals combine Ab-deficiency syndrome, B-cell lymphoproliferation, and increased frequency of autoimmune manifestations without symptoms of lupus-like disease. Two homozygous mutations at positions C104R (the human equivalent of murine C76R) and S144X impair class switching to IgA but also to IgG, unlike TACI-deficient mice (71). Whereas TACI was expressed on B-cells from all individuals with heterozygous mutations (including C104R), its signaling was impaired leading to abnormal Ig production *in vitro* (70). Consistent with data in TACI-deficient mice, individuals with TACI deficiency have a strongly reduced response to TI-2 antigens with recurrent infections and more frequently develop splenomegaly. Thus, human TACI is mandatory for response to TI-2 antigens and IgA/G class switching. Splenomegaly and autoimmune manifestations in these patients clearly indicate that TACI also acts as negative regulator of B-cell expansion/response in humans.

Moreover, two recent studies evidenced the release of soluble TACI and BAFF-R, acting as soluble decoy receptors. Surface TACI is constitutively cleaved by ADAM17 from human and murine B-cells, producing a homotrimer acting as a soluble decoy receptor for BAFF and, to a lesser extent, for APRIL. Subsequent cleavage of its remaining membrane-bound C-terminal domain by γ − secretase prevents residual NF-κB activation (85). While ADAM17 cleaves BAFF-R from dark zone GC B-cells (centroblasts), BAFF-R cleavage by ADAM10, which depends on BAFF binding and TACI expression, occurs in memory and MZ B-cells as well as in light zone GC B-cells (centrocytes) (26). By amplifying BAFF-R cleavage from centrocytes, BAFF excess might impair B-cell selection and high affinity Ab maturation. Taken together, these results highlight a previously unexpected role for TACI as a key modulator of BAFF-mediated responses.

A supplementary level of complexity was introduced by the identification of two isoforms of human TACI produced by alternative splicing of the unique encoding gene. One isoform with two extracellular ligand-binding domains resembles murine TACI whereas the second isoform, which contains only one binding domain, was referred to as TACI-short by authors (80). *In vitro* studies have established that TACI-short binds APRIL and BAFF with higher affinity than the other isoform and that its triggering by either ligand leads to a more potent activation of canonical NF-κB pathway (86) and plasma cell differentiation (80). Consistent with previous data (87), intense NF-κB activation downstream TACI-short correlates with enhanced recruitment of MyD88. In particular, messengers of both TACI isoforms were found in isolated resting memory (RM, $CD21^+CD27^+$) and MZ B-cells, with TACI-short mRNA being present in higher amounts (80). It is therefore possible that the response to BAFF/APRIL is finely modulated through binding to TACI trimers containing various ratio of each isoform. Mechanisms favoring preferential TACI-short expression *in vivo* remain to be identified but, *in vitro*, TLR9 ligands strongly upregulate it in $CD27^+$ B-cells. To what extent each TACI isoform contributes to the biology of memory B-cells and long-lived plasma cells remains to be studied. Since survival of memory B-cells is less dependent on BAFF *in vivo* than that of transitional and naïve B-cells, TACI-short expression might confer them an exceptional responsiveness to limited BAFF amounts. Whether TACI-short is released and whether it differently modulates BAFF-mediated BAFF-R cleavage on RM B-cells should be examined.

EVIDENCE FOR SOLUBLE AND MEMBRANE BAFF OVEREXPRESSION DURING HIV/SIV INFECTION

Elevated circulating levels of BAFF and/or APRIL are associated with autoimmune diseases, chronic inflammation (14, 88), or occur after CD20 B-cell depleting therapy (89, 90). Because chronic inflammation and hypergammaglobulinemia are hallmarks of chronic HIV-1 infection, serum BAFF levels were first measured in chronically HIV-infected individuals (91). In this pioneer report, authors observed increased BAFF levels in most individuals, correlating with levels of self-Abs only in individuals with more than 200 CD4 T-cells per microliters. In these individuals, classical monocytes ($CD14^{hi}$) overexpressing mBAFF were identified as a major source of soluble BAFF. Extending these first results, Fontaine et al. have evidenced increased levels of serum BAFF in HIV-infected people, with a sustained increase from the acute phase of infection in rapid and normal progressors (16). In these HIV-infected individuals, mBAFF expression was preferentially upregulated in blood myeloid dendritic cells (DC) (defined as $HLA\text{-}DR^+CD11c^+$) and their precursors ($HLA\text{-}DR^+CD14^+CD11c^+$) (16). In a cohort of untreated individuals with primary HIV infection, we found that circulating BAFF levels were consistently increased at diagnosis (20–45 days after infection) but rapidly decreased toward baseline levels by 2–3 months of infection (1 month of follow-up) (19). Whereas mBAFF was mainly present in intermediate monocytes ($CD14^+CD16^+$) of healthy individuals, its expression was preferentially enhanced in $CD1c^+$ DC and non-classical ($CD14^{lo}CD16^{hi}$) monocytes in individuals with primary HIV infection (19). A similar trend was observed in $BDCA\text{-}3^+$ DC and intermediate monocytes but did not reach significance. *In vitro*, the virus itself can directly drive mBAFF expression and its subsequent release in monocytes as well as in monocytes-derived DC and macrophages. *In vivo*, type I and II IFN could also contribute to BAFF increase. This virus-mediated effect is essentially independent on replication since it was observed with AT2-inactivated virus. Extending our results, Gomez et al. recently showed that HIV-1 does not induce BAFF expression in monocyte-derived macrophages displaying a M1 phenotype (92). Unexpectedly, our findings showed that mBAFF was expressed by a majority of pDC in healthy individuals, an expression that strongly decreased in patients with primary HIV infection. However, this loss was not due to BAFF release since pDC are unable to cleave mBAFF (19, 38, 39). Preferential cognate interactions of pDC with MZ and memory B-cells (93, 94) might relay on mBAFF binding to TACI-short, highly expressed by these B-cell subtypes (80).

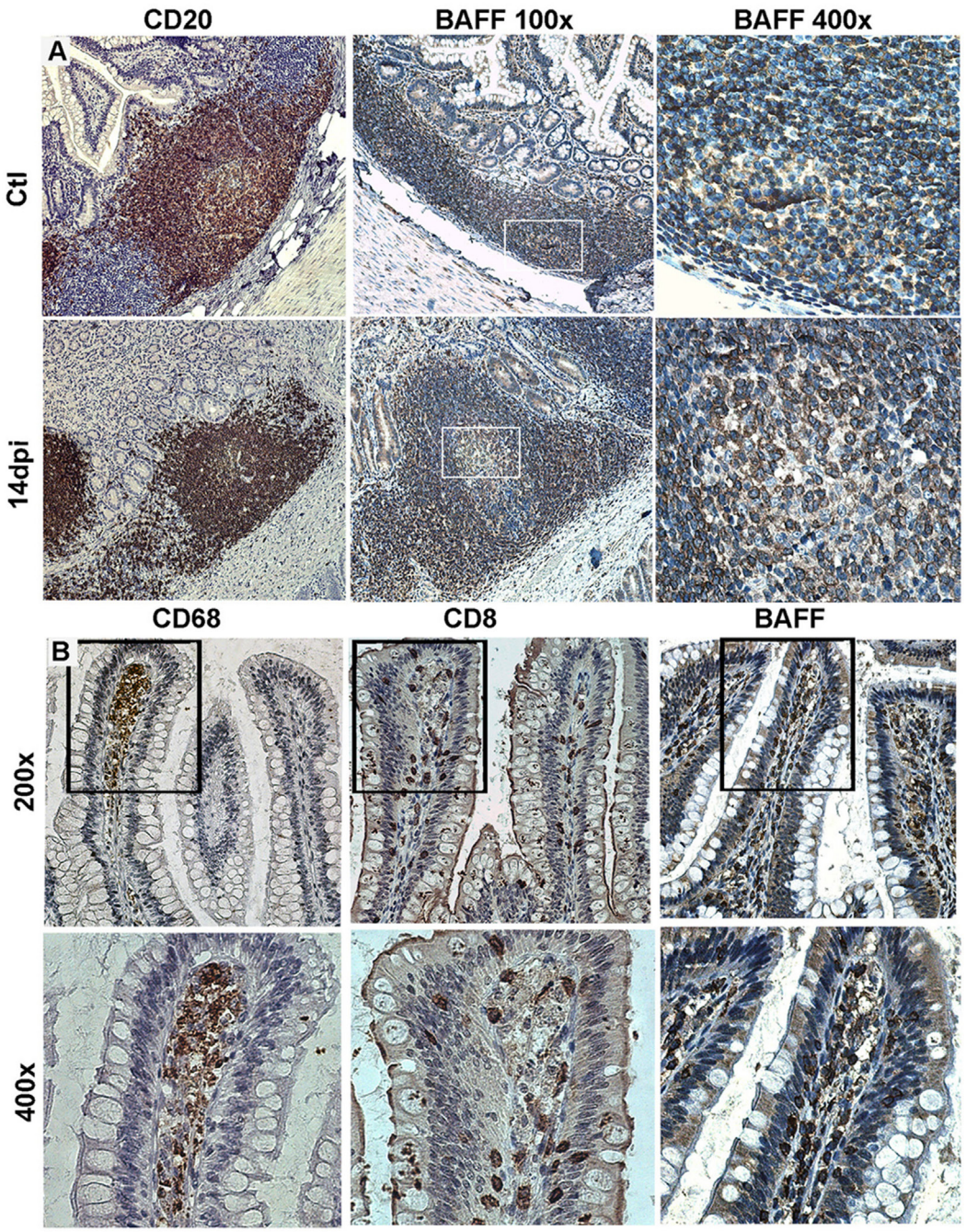

FIGURE 1 | Tissue expression of B-cell-activating factor (BAFF) in SIV-infected macaques. **(A)** Terminal ileum sections from control macaques (upper panel) and macaques infected for 14 days (lower panel) were stained with anti-CD20 (B-cells, left panels) or anti-BAFF (clone Buffy 2, middle and right panels) antibodies (Abs). Original magnification: 200× for CD20, 100× and 400× for Buffy 2. **(B)** Terminal ileum sections with clear villi present were stained with CD68 (macrophages), CD8 ($CD8^+$ and intraepithelial T-cells), and Buffy 2 (BAFF expression) Abs, respectively (original magnification 200×). Inserts from upper panels are shown in the lower panels (original magnification 400×). Reproduction authorized by SpringerNature.

In acutely SIV-infected macaques, we consistently observed a transient increase in BAFF plasma levels by 2 weeks of infection. BAFF levels correlate with total IgG levels, plasma viral loads and inversely with CD4 T-cell counts (21). However, steady BAFF overexpression was observed in spleen and intestinal mucosa (duodenum and terminal ileum) until 1 month post-infection. This BAFF signal was more intense in the spleen MZ, follicular mantle zone and within GC (21) but was also present all along the ileum villi in macrophages and in intraepithelial cells, likely $CD8^+$ (**Figure 1**). According to previous data in humans, these latter cells might correspond to BAFF-expressing type-3 innate-lymphoid cells (ILC3) (95, 96). Retrospective measurement

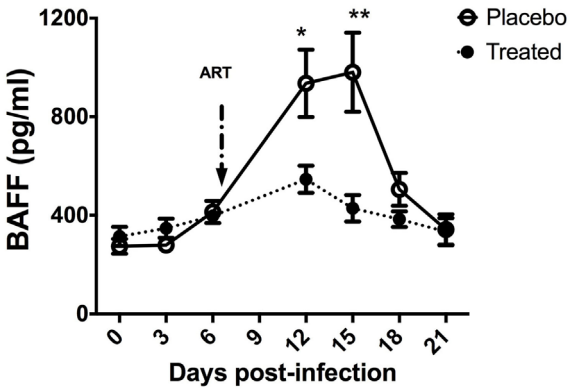

FIGURE 2 | B-cell-activating factor (BAFF) levels in SIV-infected macaques upon antiretroviral therapy. Two groups of five macaques infected for 7 days by SIVmac 251 (50AID50) were treated or not (placebo) with antiretroviral therapy for 2 weeks and euthanized at day 21 post-infection (pi). Plasma BAFF concentration was determined using the BAFF Quantikine ELISA kit (R&D systems) in samples collected before infection and every 3 days pi. At each time point, mean value ± SEM is indicated for each group. At each time point, significant differences between treated and placebo groups were calculated by a Wilcoxon sign-ranked test (two-tailed, unpaired, and non-parametric *t*-test). The *p* values *($p < 0.05$) and **($p < 0.01$) were considered as significant.

of blood BAFF levels in two groups of SIV-infected macaques treated or not by a 2-week antiretroviral therapy initiated at day 7 post-infection (97) showed a significant reduction of BAFF levels in treated animals at days 12 and 15 (42 and 56% reduction, respectively) (**Figure 2**). In these animals, the plasma viral load was concurrently reduced by 10^3-fold and the proportions of memory B-cells increased in blood and spleen. Median value of plasma IgM returned to pre-infection level and SIV-specific Abs were no longer detectable after treatment (97). Thus, early initiation of antiretroviral therapy dampens BAFF increase but inhibits early virus-specific Ab production. In agreement with our data, Poudrier et al. recently showed a transient BAFF increase during the first week of SIV infection and a progressive return to baseline values after 2 months before re-increasing by 3 months post-infection (early chronic infection) in progressor animals only. These authors established that granulocytes massively contribute to BAFF production during acute and chronic phases of infection (22). This observation fits well with increased proportions of activated neutrophils in the blood of chronically HIV-infected people (98, 99). Therefore, elevated BAFF levels might constitute a good predictor of disease progression at the early chronic phase (22). This conclusion is consistent with data of comparative transcriptomic analysis showing that upregulation of *TNFSF13B* (encoding BAFF) messenger is associated with disease progression during pathogenic HIV/SIV infections (20).

In conclusion, non-classical monocytes and $CD11c^+$ DC strongly contribute to elevated levels of soluble BAFF during HIV/SIV infection (16, 19, 91), but macrophages, granulocytes/neutrophils, epithelial cells, and ILC3 can also contribute to its local production in spleen and mucosa (21, 22, 100). Membrane BAFF-expressing pDC, which preferentially migrate into the vaginal mucosa and into the large intestine during pathogenic SIV infection (101, 102), might support TI B-cell response through cognate interaction with infiltrating B-cells. Through its binding to cell-type specific receptors, the virus can directly induce membrane/soluble BAFF overexpression but also the release of type I and II IFN that are keys inducers of BAFF expression. In our studies, IL1β, IL6, and TNFα are unable to modulate membrane and/or soluble BAFF overexpression by myeloid cells or pDC (19).

Preventing progression toward the chronic phase of virus infection generally requires the rapid production of potent neutralizing Abs that is rarely observed during acute HIV/SIV infection. That prompted us to interrogate the pathways of Ab production and the development of plasmablasts/cells as well as the nature of virus responsive B-cells.

SELF-REACTIVE B-CELLS: THE LAST CHANCE FOR NEUTRALIZING HIV Abs?

Whereas GC hyperplasia is one the first sign of ongoing B-cell response described in HIV-infected patients (1), the virus-specific Ab production is delayed and globally inefficient in containing virus replication and in preventing the establishment of viral reservoirs (103). Even when present, most virus-specific Abs have limited and transient capacities to neutralize the virus. Whereas pioneers studies have evidenced that inactivated purified SIV or fixed SIV-infected cells can elicit protective virus-specific Abs during infection with autologous virus (104, 105), most candidate vaccines subsequently fail to clear HIV (8). Potent bNAbs are nevertheless produced by a minority of HIV-infected individuals, generally at low titers and only after years of infection. Analyses of bNAbs that target HIV-1 envelope trimer have considerably extended our knowledge on envelope epitopes susceptible to neutralization and therefore identified new targets for vaccine trials (106). The vulnerability sites include: the membrane-proximal external region (MPER) of gp41, the CD4-binding site of gp120, an exclusively glycan epitope on the outer domain of gp120, an extended region including residues from both gp120 and gp41 between the MPER and gp120 protomers, a gp120 V2-glycan site at the apex of the envelope trimer and a gp120 V3-glycan site centered at Asn332 and the fusion peptide of HIV-1 (106, 107). Whereas passive infusion of bNAbs in humans has limited impact on HIV-1 viral loads and disease progression, two recently identified bNAbs directed against the CD4-binding site (VRC01 and 3BNC117) have significant antiviral effects (108–110). Unexpected results have shown that a subset of bNAbs concurrently recognizes nuclear or cytoplasmic human (self) antigens or proteins of commensal pathogens. These self/poly-reactive Abs preferentially recognize the CD4-binding site and the MPER region (111–114). Rare poly-reactive Abs recognizing the gp120-V3 loop have been also cloned from memory B-cells of HIV-infected patients (115, 116). bNAbs have a high degree of somatic mutation, deletions and insertions and/or elongated highly hydrophobic heavy chain complementary-determining region 3 with development of breadth correlating with acquisition of self/poly-reactivity

(8, 107). Whereas these features predict negative selection, current studies reveal that ancestors of B-cells producing bNAbs are frequently self-reactive (117). A clever study recently demonstrated that breaching tolerance in mice favors the generation of cross-reactive HIV-1 self-Abs (114). Early non-neutralizing Abs directed against HIV-1 gp41 subunit are also poly-reactive (118, 119) and derive from commensal bacteria-specific memory B-cells generated in terminal ileum before infection. These B-cells acquire cross-reactivity with HIV gp41 upon T-cell driven affinity maturation, which involves GC reaction in constitutive follicles (Peyer patches or mesenteric lymph nodes) or in virus-induced isolated follicles (118, 120). Therefore, HIV might preferentially interact with self/poly-reactive B-cells in different tissues.

In physiological settings, self-reactive B-cells are eliminated at the following three major checkpoints: (i) in the bone marrow before the surface IgM-positive immature B-cell stage; (ii) in spleen MZ (or peri-follicular zone in humans) when new emigrants mature into follicular or MZ B-cells, and finally (iii) within GC during Ab affinity maturation (121). In bone marrow, 50–75% of early B-cells are self-reactive, most of which are eliminated by central tolerance mechanisms before they reach the periphery. Despite this elimination based on "tonic" BCR signaling, a substantial proportion of self/poly-reactive B-cells are still present in the blood of healthy individuals and more frequent among immature and MZ B-cells than among naïve B-cells (122, 123). Given its capacity to support the survival of transitional and MZ B-cells through BAFF-R, BAFF overproduction might abnormally rescue self-reactive B-cells as shown in murine models (124, 125) or in patients with systemic lupus erythematosus (126). If BAFF-R can directly deliver survival signal to transitional B-cells, BAFF-R signaling also interferes with BCR signaling in mice and might thus abnormally rescue early B-cells expressing self-reactive BCR (127–129). More recently, self-reactive transitional B-cells (T1 and T2) abnormally expressing TACI have been identified in BAFF transgenic mice as a consequence of BAFF excess. These $TACI^{hi}$ transitional B-cells co-express AID (activation-induced cytidine deaminase), an enzyme mandatory for somatic hypermutation and isotype class switching, and T-bet, a transcriptional factor associated with IFNγ production and IgG class switching. Accordingly, binding of these $TACI^{+}$ transitional B-cells by self-antigens promotes AID-mediated hyper-somatic mutations that spontaneously produce self-reactive IgG, *ex vivo* (25). Although less numerous, $TACI^{+}$ transitional B-cells are present in wild-type mice with physiological BAFF settings. Increased proportions of T1-like ($CD10^{+}CD21^{lo}$) B-cells related to disrupted homeostasis have been reported in lymphopenic HIV-infected people with more advanced disease (67–69). Unfortunately, neither circulating BAFF level nor proportions of self-reactive B-cells, potentially HIV cross-reactive, have been estimated at the time of these studies. Whereas $TACI^{+}$ transitional B-cells might also contribute to hypermutated Ab production during HIV infection, only rare transitional B-cells were found to express T-bet in healthy and chronically HIV-infected individuals (130). However, this might occur in highly lymphopenic HIV-infected individuals with more advanced disease.

Marginal zone B-cells that express diverse IgV_H genes more frequently used by self/poly-reactive Abs including by bNAbs directed against CD4bs (131), might be a "natural reservoir" for HIV cross-reactive B-cells. As mentioned earlier, human MZ B-cells highly express TACI, in particular TACI-short, and are in close contact with different BAFF/APRIL-producing cells such as macrophages, DC, neutrophils, or ILC3 in the splenic peri-follicular zone (132, 133). Thus MZ B-cells likely produce a first pool of virus-specific Abs. Indeed, we showed that the frequency of spleen MZ B-cells decreased soon after the peak of plasma viral load whereas plasmablasts/cells, mainly expressing IgG or IgM, were more numerous in the MZ 1 month post-infection in SIV-infected macaques (97). Similarly, Fontaine et al. identified a circulating population with mixed features of transitional and MZ B-cells, thought to rapidly mature into MZ B-cells upon abnormal BAFF release by myeloid cells in viremic HIV-infected people (16). Together, these data suggest that HIV induces an early differentiation of MZ B-cells into plasmablasts/cells followed by a transient lymphopenia, which tends to be compensated by accelerated repopulation of the MZ B-cell pool in patients with higher levels of replication and/or inflammation (including high BAFF levels). Studying the expansion of early self-reactive B-cells, potentially expressing T-bet, in concert with BAFF levels during pathogenic SIV/HIV infection might be valuable. Whether this repopulation favors expansion of HIV/SIV cross-reactive B-cells or their deletion remains to be studied.

BAFF, B-CELLS, AND T_{FH} IN GCs: FROM PHYSIOLOGICAL SETTINGS TO HIV/SIV INFECTION

Memory B-cells and long-lived plasmablasts/cells are generated within the GC through a complex process including several cycles of somatic mutations/selection as elegantly described elsewhere (134, 135). Through somatic hypermutations of V_H genes, an integrated process mandatory to Ab affinity maturation, the GC reaction constitutes an important stage where self-reactive B-cells are physiologically generated. Such self-reactive B-cells escaping peripheral tolerance and maturing into circulating memory IgG^{+} B-cells have been associated with autoimmunity (136) but might alternatively contribute to production of bNAbs (7). This directly questions the function of GC reaction with the generation of effectors B-cells (memory B-cells and long-lived plasmablasts/cells) in the context of chronic inflammation, where BAFF (and APRIL) can be overproduced.

Residual development of GC and efficient affinity maturation of Abs in response to TD antigen occur in BAFF or BAFF-R-deficient mice (50, 52, 53). However, GC more rapidly involute in these mice with reduced numbers of proliferating GC B-cells (centroblasts), impaired network of follicular dendritic cells (FDC) and reduced trapping of immune complexes (76). By contrast, BAFF overexpression in GC increases autoimmunity by reducing the competition between B-cell clones for T-cell help and survival signals, at least in mice (124, 137). Fibroblastic reticular cells throughout the body and FDC in GC are the main sources of BAFF in homeostatic settings (138) but also of CXCL13,

which attracts B-cells to build B-cell follicles (139). During a TD response, T_{FH} constitute not only the major source of BAFF, mandatory for the B-cell survival and the selection of high affinity B-cell clones (79) but also of CXCL13 as shown in vaccinated mice and primates (140). Consistent with ongoing TD response in HIV-infected patients, high blood CXCL13 levels have been reported with concomitant decrease of CXCR5 expression by circulating B-cells (141). Whether this decrease is due to B-cell activation or to an enhanced BAFF-mediated internalization of CXCR5, it likely perturbs the recruitment of B-cells into follicles. Given that BAFF enhances the CXCL13-mediated chemotactic response of $CD27^+$ human B-cells, *in vitro* (142), it could potentiate the entry of recently antigen-activated B-cells (GC founders) or of memory B-cells into the follicle during a first or second exposure to antigen/pathogen, respectively. Within GC, BAFF overproduction might either increase the CXCL13-mediated response of B-clones in the light zone or accelerate the internalization of CXCR5 in centrocytes (light zone B-cells), favoring their rapid return to the dark zone. In both case, the asymmetric recycling of B-cell clones between dark and light zones and the selection process would be impaired leading to an abnormal pattern of mutation/selection of B-cell clones required for affinity maturation (143, 144). Alternatively, BAFF excess in GC might enhance BAFF-mediated BAFF-R cleavage on $TACI^+$ GC B-cells (centrocytes). Decreased BAFF-R expression might consequently abolish BAFF effect on the CXCL13 chemotactic response of centrocytes or decrease the survival of high-affinity B-cell clones (26). At which step HIV cross-reactive B-cells clones appear and why they are not eliminated as self-reactive B-cells in the context of high amount of viral antigens is far from being clear. Vulnerability sites frequently buried in the envelope structure or masked by glycans are probably weakly accessible. This might favor their ignorance during the GC selection process.

In addition to its action on GC B-cells, two independent studies implied TACI in T_{FH} expansion (**Figure 3**). In the first study, Ou et al. showed that BAFF preferentially binds BAFF-R and upregulates ICOSL expression by GC B-cells in physiological settings. When it is locally overproduced, BAFF also binds to TACI on GC B-cells leading to down modulation of BAFF-R-mediated ICOSL expression and subsequently T_{FH} expansion (78). According to recent data on BAFF-R processing (26), one hypothesis could be that BAFF excess leads to TACI-mediated BAFF-R cleavage, which reduces BAFF-R signaling and thus down-modulates ICOSL expression. In a second study, IL21

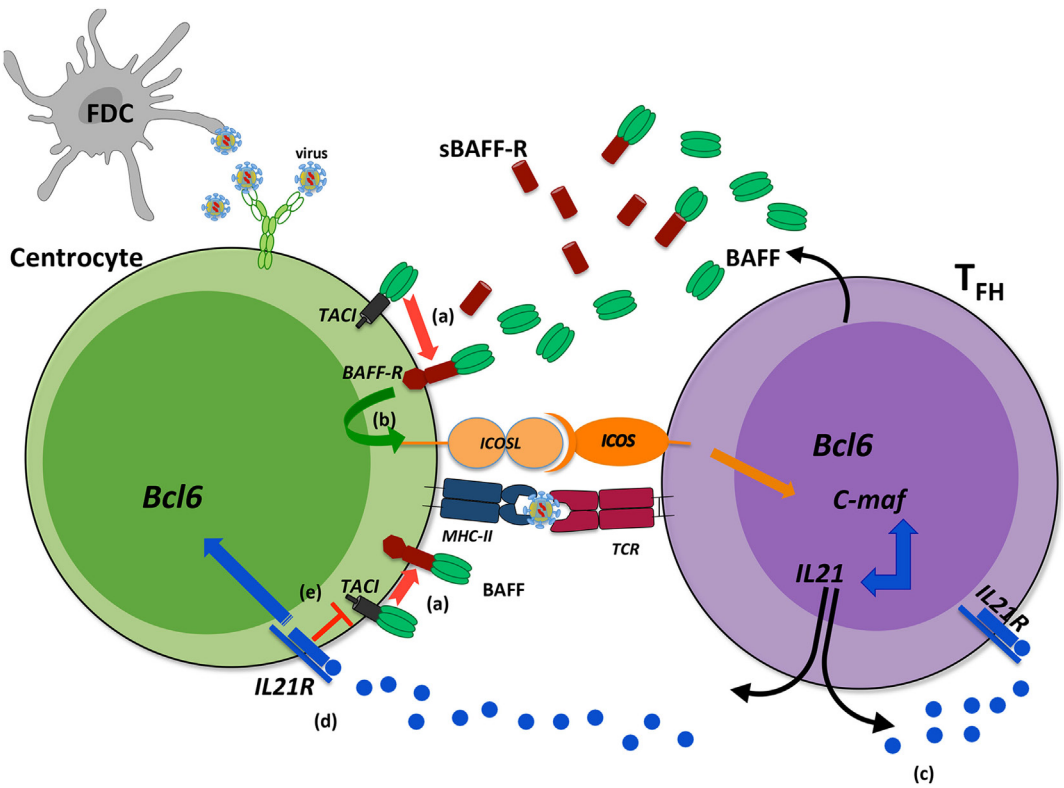

FIGURE 3 | Transmembrane activator and CAML interactor (TACI) as a key regulator of B-cell-activating factor (BAFF)-dependent BAFF-R cleavage in germinal center (GC). During a T-dependent response, follicular helper T-cells (T_{FH}) produce BAFF that can bind either to BAFF-R or to TACI. When BAFF is locally released in excess, its binding to BAFF-R can induce the cleavage of BAFF-R in a TACI-dependent manner from the surface of centrocytes (a). Reduced BAFF-R signaling leads to decreased ICOSL expression on B-cells (b) and therefore dampens ICOS signal, mandatory for T_{FH} maintaining and IL21 production. This might constitute a physiological regulatory mechanism, exacerbated when high amounts of antigens are maintained within GC. IL21 is a key cytokine for T_{FH} that ensures their survival (c) and that of light zone GC B-cells (d). In addition, IL21 decreases TACI expression that might prevent early TACI-dependent BAFF-R cleavage (e). Such regulatory roles would imply that IL21 and BAFF are produced sequentially during the GC reaction with possible consequences on recycling and differentiation of GC B-cells.

produced by T_{FH}, besides supporting the survival of both T_{FH} and GC B-cells through IL21R, also diminishes TACI expression thus preventing premature loss of T_{FH} (79). As T_{FH} concurrently produce BAFF and IL21, a delicate balance is thought to control efficient GC reaction. The existence of two human TACI isoforms could further complicate our understanding of the role of BAFF and its receptors, BAFF-R and TACI, in GC reaction.

Impaired helper functions of T_{FH} at the chronic phase of HIV/SIV infection (2, 6, 145) likely contribute to inefficient B-cell response to HIV/SIV. However, early functional T_{FH} are present at elevated frequencies in nodal GC from the acute phase of HIV infection and their presence correlates with the breadth of bNAbs at the chronic phase (146). Thus, generation of bNAbs is dependent on the preservation of T_{FH} functions, likely impaired in CXCR3$^+$ T_{FH} (147). As recently shown, human T_{FH} express BAFF-R and release more IFNγ after culture with BAFF (148), thus BAFF excess might contribute to T_{FH1}-expansion during HIV/SIV infection. Being produced by FDC and T_{FH} in GC, BAFF likely exerts a physiological role on T_{FH}, during response to TD natural or vaccine antigens. In conclusion, the overexpression of BAFF might impair GC reaction and even modulate T_{FH} functions.

MEMORY B-CELLS: THE WEAK LINK IN HIV/SIV INFECTION

It is now well established that chronically HIV-infected patients have an impaired memory B-cell compartment with lower frequency of HIV-specific and vaccine-specific memory B-cells as well as reduced anti-vaccine Abs (149–151). In addition to lower proportions of memory B-cells, viremic HIV-infected individuals also exhibited increased proportions of CD21^{lo} mature B-cells (68, 81). This subset highly expressed BCMA and TACI but had decreased BAFF-R expression and BAFF binding. Based on this phenotype profile and on the concomitant increase in CD27, CD38, and CXCR3 expression, these CD21^{lo} B-cells were first considered as circulating plasmablasts, prone to apoptosis and Ab production (67, 81) and expanded as a consequence of HIV-induced hyperactivation. After the identification of a subset of CD20^{hi}CD21^{lo} tissue memory cells in human tonsils exhibiting signs of exhaustion (82), the classification of this CD21^{lo} population in HIV-infected patients has been revised. In addition to plasmablasts, the CD21^{lo} B-cell subset comprised CD27$^+$CD21^{lo} and CD27^{lo}CD21^{lo} cells often referred to as activated memory (ActM) and tissue-like memory (TLM) B-cells, respectively. These subsets differ from conventional RM B-cells by their expression of activation, inhibitory and/or apoptotic markers (69). In healthy donors, RM B-cells constitute the predominant fraction of blood memory B-cells with low percentages of CD21^{lo} memory B-cells (152). By contrast, ActM and TLM are overrepresented in blood of chronically HIV-infected patients (153) and in rapidly progressing SIV-infected macaques (154). In contrast to influenza or tetanus-specific Abs enriched in RM B-cells, HIV-specific Abs are enriched in TLM B-cells in untreated individuals (155, 156). More recently, Muema et al. reported increased proportions of ActM, TLM, and plasmablasts but decreased proportions of naïve B-cells in vertically HIV-infected children in a viral-load-dependent manner (83). In agreement with other studies in children, lower IgG levels and proportions of switched memory B-cells against childhood vaccines were observed (150, 157, 158). In these HIV-infected children, circulating BAFF levels were elevated whereas BAFF-R and TACI expression were respectively decreased and increased in most B-cell subsets. B-cell interaction with viral proteins that can induce BCR- or TLR-mediated B-cell activation (9, 10, 159) might also increase TACI expression, possibly stabilized at the membrane by BAFF binding as shown in mice (79). By contrast, decreased BAFF-R expression might be due to potent receptor internalization in the presence of high BAFF levels as suggested during malaria infection (17, 160) or to enhanced BAFF-mediated BAFF-R processing (26).

It is not clear whether increase in TACI expression has any influence on ActM or TLM functions or survival, *in vivo*. Survival of human and simian RM B-cells (BAFF-R$^+$ TACI^{hi}) is less dependent on BAFF than that of naïve and transitional B-cells in physiological settings (161, 162). Moreover, BAFF levels correlate with proportions of MZ and RM B-cells, but not of CD21^{lo} memory B-cells in HIV-infected children (83). By contrast, BAFF levels and proportions of CD21^{lo} memory B-cells were concomitantly increased in individuals infected by *Plasmodium* (17, 160), an infection setting where the frequencies of TLM-like B-cells are increased (18, 163).

Similar to TLR9 ligands that elicit responses in TLM-like B-cells of malaria-exposed people (18, 164), BAFF/APRIL as TACI ligands might deliver differentiation signals to these B-cells through TACI and its downstream TLR-like signaling pathways (87) Whereas overrepresentation of CD21^{lo} memory B-cells is consistently associated with chronic inflammation, the mechanisms leading to this phenotype are largely unknown. Studies in mice and more recently in HIV-infected people showing T-bet expression by CD21^{lo} B-cells suggest simultaneous actions of pathogen-derived nucleic acids, through TLR9/7, and Th1-cytokines (IFNγ) (130, 165–167). According to high TACI expression in HIV-infected children, BAFF overexpression might directly or indirectly (for example, by upregulating IFNγ production by NK or Th1-cells) contribute to the generation or survival of these CD21^{lo} memory B-cells and thus Ab-mediated protection against HIV-1.

CONCLUSION

B-cell-activating factor and its receptors (BAFF-R, TACI, and BCMA) are key actors for the B-cell survival and the immune responses of mature B-cells. Whereas BAFF-R is more widely expressed throughout the B-cell differentiation, TACI now appears as a key regulator of various BAFF-mediated responses. Indeed TACI is spontaneously released upon activation and orchestrates the cleavage of BAFF-R-BAFF complexes. This might have a major impact on memory and MZ B-cells that are TACI^{hi}. Perturbations of these regulatory mechanisms likely impair the GC reaction: GC B-cell selection/survival or recycling between dark and light zones as well as the generation of appropriate effector B-cells during TD responses. Soluble but also membrane

BAFF overexpression by key DC subsets during HIV/SIV infection might subsequently initiate the unexpected expansion of HIV cross-reactive B-cell clones and atypical memory B-cells. In this review, we pointed out previous data arguing for the involvement of BAFF in HIV-mediated B-cell dysfunctions and discussed more recent data on BAFF and TACI in physiological settings. Accordingly, we suggest BAFF-mediated mechanisms that could modulate B-cell response during pathogenic HIV/SIV infection. Our research around BAFF is part of a more global approach that aims to identify B-cell subset(s), which could constitute a reservoir of HIV cross-reactive B-cells, and to understand how to promote their expansion and/or prevent their elimination. This information is likely a prerequisite for the development of next-generation HIV vaccines.

AUTHOR CONTRIBUTIONS

All authors contribute to the writing of this review.

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REVIEW
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Mucosal IgA Responses: Damaged in Established HIV Infection—Yet, Effective Weapon against HIV Transmission

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HIV infection not only destroys CD4+ T cells but also inflicts serious damage to the B-cell compartment, such as lymphadenopathy, destruction of normal B-cell follicle architecture, polyclonal hypergammaglobulinemia, increased apoptosis of B cells, and irreversible loss of memory B-cell responses with advanced HIV disease. Subepithelial B cells and plasma cells are also affected, which results in loss of mucosal IgG and IgA antibodies. This leaves the mucosal barrier vulnerable to bacterial translocation. The ensuing immune activation in mucosal tissues adds fuel to the fire of local HIV replication. We postulate that compromised mucosal antibody defenses also facilitate superinfection of HIV-positive individuals with new HIV strains. This in turn sets the stage for the generation of circulating recombinant forms of HIV. What can the mucosal B-cell compartment contribute to protect a healthy, uninfected host against mucosal HIV transmission? Here, we discuss proof-of-principle studies we have performed using passive mucosal immunization, i.e., topical administration of preformed anti-HIV monoclonal antibodies (mAbs) as IgG1, dimeric IgA1 (dIgA1), and dIgA2 isotypes, alone or in combination. Our data indicate that mucosally applied anti-HIV envelope mAbs can provide potent protection against mucosal transmission of simian-human immunodeficiency virus. Our review also discusses the induction of mucosal antibody defenses by active vaccination and potential strategies to interrupt the vicious cycle of bacterial translocation, immune activation, and stimulation of HIV replication in individuals with damaged mucosal barriers.

Keywords: human immunodeficiency virus-induced IgA, vaccine-induced anti-HIV IgA, systemic IgA responses, mucosal IgA responses, secretory IgA, immune exclusion of HIV, simian-human immunodeficiency virus, passive immunization with dimeric IgA

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OVERVIEW: THE AIDS EPIDEMIC AND HIV-INDUCED DAMAGE OF MUCOSAL B CELLS

Since the beginning of the HIV/AIDS epidemic, more than 35 million people have died (http://www.who.int/gho/hiv/en/); were it not for the introduction of combination antiretroviral therapy, the number of deaths would surpass those caused by the Black Plague in the fourteenth century and the Spanish Flu in 1918, making HIV the worst newly emerged pandemic in human history. An estimated 90% of all new HIV acquisitions occur through mucosal contact, including sexual and

perinatal transmission, in which mucosal fluids and tissues are the first points of contact for HIV. Despite this, inducing protective mucosal immune responses by candidate HIV/AIDS vaccines has not been a major focus for most experimental vaccine approaches. Almost all acute HIV acquisitions involve R5-tropic strains, even when the infected source person harbors predominately dual or X4-tropic HIV strains. As such, prevention of virus acquisition by active and/or passive immunization should focus on blocking mucosal transmission of R5 HIV.

B-cell dysregulation was noted at the very beginning of the HIV/AIDS epidemic, even before the viral etiology of this new syndrome was identified [reviewed in Ref. (1)]. Damage to the B-cell compartment was subsequently described as including lymphadenopathy, loss of normal B-cell follicle architecture in lymph nodes, polyclonal hypergammaglobulinemia, altered expression of homing receptors on the surface of B cells and, therefore, increased turnover of such cells, increased apoptosis of B cells due to activation-induced cell death, and eventually irreversible loss of memory B-cell responses with advancing HIV disease. The latter becomes evident by significant decreases in antiviral antibody titers (1–6).

IgA-producing B cells and plasma cells are not spared from the HIV or SIV-induced damage. Mestecky and colleagues (7, 8) described unusually low anti-HIV IgA responses when compared to IgG responses in mucosal fluids. In this review, we discuss the implications of such B-cell damage in infected individuals. We will contrast these findings with the potential role mucosal IgA can play in protecting uninfected hosts from invading HIV or related primate immunodeficiency viruses. Such protection could be provided by passively administering recombinant anti-HIV antibodies directly into mucosal compartments. Alternatively, vaccine strategies can be designed to induce protective anti-HIV mucosal antibody responses. Our review will summarize relevant data generated in non-human primate (NHP) models.

MUCOSAL ANTIBODY PRODUCTION IN NORMAL HOSTS

In order to understand the dysfunction of the B-cell compartment in HIV infection, it is important to understand the processes involved in generating mucosal antibodies of different classes in healthy, uninfected hosts. Mucosal fluids contain IgM, IgG, and IgA in different forms, especially polymeric versions. These antibodies are produced by local plasma cells in the lamina propria. IgM-producing cells secrete multimeric IgM that contains the joining (J) chain and is generally pentameric. This IgM binds to the polymeric immunoglobulin receptor (pIgR) expressed on the basolateral surface of the epithelial cell barrier. The pIgR–IgM complexes are transported across the epithelial monolayer in transcytotic vesicles and released at the luminal side through a process involving proteolytic cleavage of pIgR. This results in release of the secretory component (SC) that remains associated with IgM, thus generating secretory IgM (**Figure 1A**, top).

IgG is produced in the bone marrow from where it enters the circulation and is distributed throughout the body tissues. IgG can also be generated locally by subepithelial plasma cells; its trans-epithelial transport occurs through the neonatal Fc receptor (FcRn) (**Figure 1A**, middle). In contrast to pIgR, FcRn is not degraded upon release of its IgG cargo at the luminal side; this receptor can shuttle back and forth between the luminal and the basolateral aspects of the epithelial cells and carrying IgG-antigen immune complexes from the luminal side across the epithelium into the subepithelial space (**Figure 1A**, middle) (9).

Like the other immunoglobulin (Ig) classes, IgA destined for mucosal secretions is also produced locally by plasma cells in the lamina propria. B cells release dimeric IgA (dIgA), which consists of two IgA monomers linked at their Fc alpha ends by the J chain. Like IgM, dIgA molecules bind to pIgR at the basolateral aspect of epithelial cells and get transported in transcytotic vesicles to the luminal side, where pIgR undergoes cleavage into a remnant stump and SC, giving rise to secretory IgA (SIgA) (**Figure 1A**, bottom) (10) found in mucosal fluids. The relative ratios of mucosal IgM:IgG and IgM:IgA vary and depend on the active participation of $CD4^+$ T-helper cells, which provide critical stimulatory signals to B cells to undergo Ig class switching.

In humans, IgA exists as closely related subclasses, IgA1 and IgA2, which differ predominantly in the hinge region. In IgA1, the latter contains 19 amino acids (aa) as well as several O-linked oligosaccharides. In contrast, the IgA2 hinge region is only 6 aa long and lacks glycosylation. Due to their open hinge region, IgA1 molecules have a T-like shape with a distance between Fab fragments of approximately 16 nm. Conversely, IgA2 is *Y*-shaped, and the distance between Fab regions measures only 10 nm due to the shorter, stiffer hinge region. The structural differences between IgA1 and IgA2 molecules likely result in different biological activities. Of note, only humans and great apes have the IgA1 subclass with the remarkably open hinge. Rhesus macaques only encode the IgA2-like subclass [reviewed in Ref. (10)].

Among the three classes of mucosal antibodies, IgA in its various forms clearly stands out. The human body generates more IgA per day than all other classes of Igs combined (11), and since IgA ranks only second in the plasma concentration after IgG, it is obvious that the majority of IgA is destined to enter mucosal fluids that need to be replaced continuously. These facts imply a critical role for IgA function in the mucosal compartment.

MUCOSAL ANTIBODY PRODUCTION IN HIV/SIV-INFECTED HOSTS

Skewing of Mucosal Antibody Production in HIV/SIV Infection

Severe depletion of $CD4^+$ T cells in the lamina propria and in epithelial tissues during acute SIV infection was first described by Smit-McBride and colleagues (12). This observation was followed by the recognition that the significant loss of the gut-associated lymphoid tissue (GALT) had serious consequences: loss of barrier integrity as demonstrated by increases in plasma concentration of lipopolysaccharide (13, 14). Mattapallil et al. (15) confirmed the loss of GALT $CD4^+$ T cells and characterized the affected cell population as memory $CD4^+$ T cells.

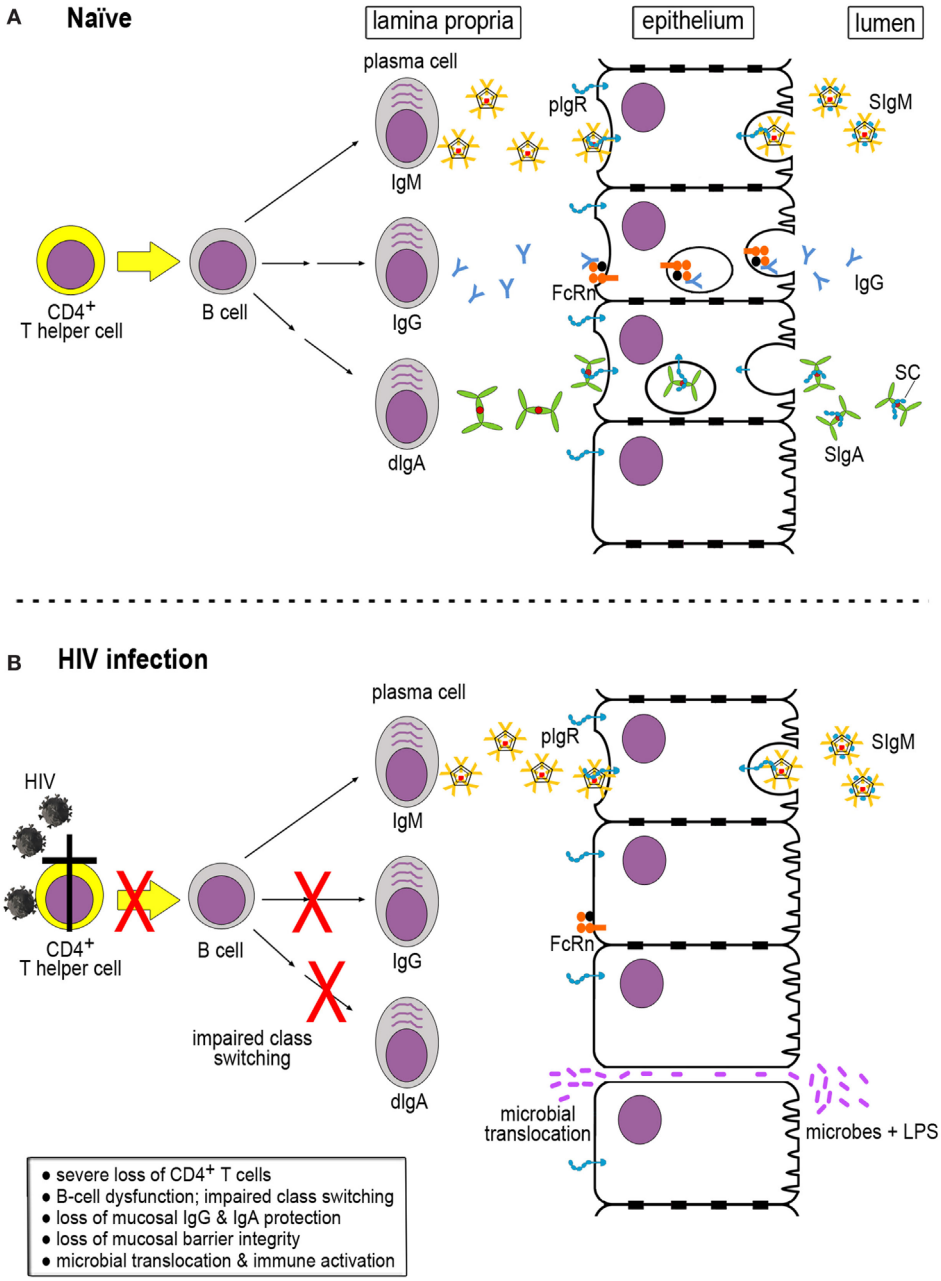

FIGURE 1 | Generation of immunoglobulins (Igs) in naïve and HIV-infected hosts. **(A)** In a naïve host, multimeric IgM, IgG, and dimeric IgA (dIgA) are produced in the lamina propria by mature plasma cells. The latter are derived from B cells that have received help from CD4+ T cells for Ig class switching. IgM and dIgA interact with the polymeric immunoglobulin receptor (pIgR), which exports the Igs across the epithelial cells. pIgR is cleaved by proteolysis at the luminal side, resulting in the generation of secretory component (SC) that is retained by IgM and dIgA, giving rise to secretory IgM (SIgM) and IgA (SIgM and SIgA, respectively). IgG binds to the neonatal Fc receptor (FcRn) expressed by epithelial cells that transports IgG across to the luminal side. Unlike pIgR, FcRn does not undergo proteolytic cleavage at the luminal side and can shuttle back and forth. **(B)** During HIV infection, severe loss of CD4+ T cells occurs, resulting in impaired Ig class switching and B-cell dysfunction. As a consequence, IgG and SIgA are markedly decreased in the mucosal compartment. Lack of sufficient mucosal barrier defenses leads to loss of barrier integrity, microbial translocation, and immune activation.

Among CD4+ T cells affected early and severely during HIV/SIV infection is the T helper 17 (Th17) population, a favorite target of primate immunodeficiency viruses. Th17 cells tend to localize preferentially to the gastrointestinal tract where they express a number of genes found to be involved in the maintenance of epithelial cells, including interleukin-22 (IL-22) (16, 17). Favre et al. (18) made an important contribution toward understanding the consequences of severe losses in Th17 cells. These authors compared the acute stage of experimental infection with SIVagm in the setting of a pathogenic host–virus interaction in Asian

pig-tailed macaques with that in African green monkeys (AGMs), the natural SIVagm host where the infection remains non-pathogenic. Only pig-tailed macaques but not AGMs suffered immune activation and severe, selective depletion of Th17 cells systemically and in mucosal tissues.

Loss of CD4$^+$ T-helper cell function greatly reduces Ig class switching in subepithelial B cells, which results in a significant loss of IgG and SIgA in mucosal fluids (**Figure 1B**). The serious loss of IgG and dIgA production in the lamina propria leads to a strong skewing of the IgG:IgM and dIgA:IgM ratios, with the IgA content of mucosal fluids in HIV/SIV infection being most severely affected [reviewed in Ref. (19)]. This relative lack of mucosal IgA and IgG results in impaired immune exclusion of bacterial pathogens and makes the epithelial barrier vulnerable to breaches (**Figure 1B**, bottom). Indeed, during acute SIV as well as HIV infection, bacterial translocation occurs, which results in immune activation and further upregulation of virus replication, starting off a vicious cycle.

Bacterial Translocation: Adding Fuel to the Fire

Bacterial translocation has serious, deleterious consequences to the host. The most important one is triggering inflammatory responses, resulting in general immune activation. Macrophages, instead of phagocytosing bacteria or bacterial products that may have crossed the epithelial barrier in normal epithelial homeostasis, now send out inflammatory signals that in turn create a more fertile ground for HIV/SIV to spread locally in mucosal tissues [reviewed in Ref. (17)]. Factors involved in this immune activation include tumor necrosis factor-α, which is released from macrophages, and interferon-α (IFN-α), which is produced by plasmacytoid dendritic cells and macrophages. Activated monocytes/macrophages produce soluble CD14 (sCD14) and soluble CD163 (sCD163). High plasma levels of sCD14 were found to be an independent poor prognostic sign for survival of HIV-infected individuals (20).

Mucosal dendritic cells (DCs) play an important role in the local immune activation following bacterial translocation. While DCs are key players in the adaptive immune defenses that benefit the host, these cells also contribute to local immune activation. They release inflammatory cytokines as well as type 1 interferons that damage the Th17 cell population while favoring T regulatory cells in intestinal tissues (18). In addition, DCs can trans-infect CD4$^+$ T cells. Such responses greatly intensify local mucosal virus replication [reviewed in Ref. (21)]. Ultimately, bacterial translocation and the ensuing immune activation lead to further damage of mucosal integrity through a vicious cycle of increased virus replication followed by increased loss of CD4 T-helper cell function, which ultimately leaves the mucosal barrier devoid of the protective IgA and IgG antibodies (**Figure 1B**).

Compromised Mucosal Antibody Production and HIV Genetic Diversity Worldwide

We hypothesize that loss of epithelial integrity, which leads to bacterial translocation, immune activation, and ultimately to increased numbers of activated HIV target cells, will have another serious consequence: superinfection with new strains of HIV. The compromised local mucosal environment will facilitate transmission of new HIV strains and support high levels of replication of the incoming strain. If the latter infects a cell already harboring the preexisting virus, the two HIV genomes will recombine to generate circulating recombinant forms (CRFs). This is a frequent event in the ongoing HIV pandemic as reflected by the ever increasing complexity of viral genomes with an increasing fraction of CRFs. Inter and intra-clade recombinations are known to occur (https://www.hiv.lanl.gov/content/sequence/HIV/CRFs/CRFs.html) (22–24). In the case of an individual with HIV infection, broad, anti-HIV cell-mediated immune responses encompassing multiple epitopes, which controlled the primary virus, have not prevented superinfection during structured treatment interruption (25).

We propose that the loss of mucosal barrier function is one of the main drivers of the rapidly evolving genetic complexity of HIV during the ongoing pandemic (26). At the entire human population level, superinfection with unrelated HIV strains is problematic. The increasing multitude of genetically evermore divergent strains increases the level of difficulty to find protective HIV vaccines. Superinfection is also deleterious at the level of the superinfected individual, who will experience a second phase of acute viremia. Neutralizing antibodies against the new HIV strains will most likely not exist. If so, high viral loads will ensue and increase immune activation throughout the body. This in turn will increase the damage to the CD4$^+$ T-helper cell population and accelerate disease progression. Given the increasing prevalence of CRFs, we hope that our hypothesis will stimulate research on a possible link between loss of mucosal barrier integrity and the prevalence of CRFs. We feel that very early onset of antiretroviral therapy may limit mucosal damage and thus lower the risks of superinfection, which would result in slowing the rate of CRF prevalence. As long as access to antiretroviral drugs remains limited in developing countries, the rates of superinfection may not decline. Finding ways to protect mucosal barriers in already infected individuals and thereby lowering the chances of HIV superinfection could be additional protective mechanisms for individuals with chronic HIV infection.

HARNESSING MUCOSAL IgA TO PROTECT THE HOST

The potential role of IgA in providing protection to the host has been controversial. The RV144 phase III efficacy trial performed in Thailand showed a modest but significant lowering of the risks of HIV acquisition among the vaccinees of 31.2%, a result that gave impetus to analyze the immune responses responsible for this effect (27). Surprisingly, neither neutralizing antibodies nor cell-mediated immune responses were linked to the lowering of virus acquisition risks. Rather, serum IgG with antibody-dependent cellular cytotoxicity (ADCC) activity targeting the V1V2 region was associated with beneficial outcome. The latter seemed to be counteracted by serum IgA responses targeting

HIV envelope. Mucosal samples were not available for analysis in this trial (28).

Passive Immunization: A Tool to Show Cause and Effect between Antibodies and Protection

To settle the issue whether anti-HIV Env IgA responses harm or help the host, passive immunization is the tool of choice. This avoids influences of any other immunological principles and restricts the experimental parameters to only the passively administered antibody. This approach is required to dissect the potentially protective roles of anti-HIV humoral responses in the systemic as well as in the mucosal compartments. To our knowledge, passive immunization with systemically administered anti-HIV IgA has not been performed to assess prevention of simian-human immunodeficiency virus (SHIV) acquisition in NHP models. In contrast, we have used passive immunization with topically administered, monoclonal dIgAs to ask whether such monoclonal antibodies (mAbs) could prevent SHIV acquisition after mucosal challenge (29, 30). Of note, passive immunization is the best tool to give cause/effect information between a well-characterized monoclonal antibody and the degree of protection, as no other immunological mechanisms are provided to account for the outcome of the study.

Passive immunization with mucosally administered, monoclonal dIgAs is depicted in **Figure 2A**. Any significant prevention of SHIV acquisition would have to occur in the mucosal lumen by trapping infectious virion in large complexes to prevent mucosal transcytosis; this process is called immune exclusion (10, 31). This information needs to be generated in order to assess the role of mucosal B cells and anti-HIV mucosal antibodies in preventing virus acquisition—key data for future vaccine design against a pathogen that is predominantly transmitted *via* mucosal routes.

Passive Mucosal Immunization with Monoclonal dIgAs

Our group has performed passive mucosal immunization studies with monoclonal dIgAs to test their protective potential against intrarectal SHIV challenge. We generated dIgA1, dIgA2, and IgG1 versions of a neutralizing anti-HIV mAb, HGN194, which targets the conserved V3 loop crown of HIV gp120 (32). This mAb neutralized all tier 1 strains tested and selected tier 2 strains in which the V3 loop crown was accessible. When given intravenously (i.v.) at full dose (50 mg/kg) 24 h prior to high-dose intrarectal challenge with a clade C SHIV (SHIV-C), this mAb provided 100% cross-clade protection to the rhesus macaques (33). Next, we sought to test whether administering the dIgA1, dIgA2, and IgG1 isoforms of HGN194 intrarectally would protect RMs against subsequent intrarectal SHIV-C challenge. All three isoforms neutralized the challenge virus equally well in tissue culture systems. However, the *in vivo* study yielded a surprising result: the dIgA1 isoform was significantly more potent in preventing intrarectal SHIV transmission compared to the dIgA2 form ($p < 0.05$) (29). In this first proof-of-concept study of passive mucosal immunization with recombinant dIgAs, better *in vivo* protection by dIgA1 compared to dIgA2 was linked to better virion capture *in vitro* and inhibition of transcytosis of cell-free virus in a transwell assay (29).

Protective Mechanisms of IgA in the Mucosal Lumen and Barrier

IgA in mucosal fluids can mediate protection by direct neutralization, immune exclusion, or inhibition of transcytosis. In our passive immunization studies, we have shown that administering of neutralizing dIgA intrarectally prevented SHIV acquisition after intrarectal virus challenge (**Figure 2A**). The incoming SHIV could either be directly neutralized by dIgAs in the mucosal lumen. Alternatively, large immune complexes could be formed that trap the incoming virus and prevent it from traversing the epithelial barrier *via* transcytosis. Such a mechanism is termed immune exclusion.

IgA responses induced by vaccination can also block HIV/SHIV infection (**Figure 2B**). The HIV-specific dIgAs produced by subepithelial plasma cells and transported across the epithelial layer into the lumen could mediate protection through immune exclusion or by inhibiting transcytosis. Another interesting mechanism of IgA-mediated protection is intracellular neutralization (**Figure 2C**). This occurs when a virion is taken up by an epithelial cell and enters the same transcytotic vesicle in which dIgA-pIgR cargo is being carried toward the lumen. Virus is bound by dIgA, forced to make a U-turn, and excreted back into the lumen. This phenomenon was described by Burns et al. (34) for the rotavirus murine model.

The Interplay between Mucosal dIgAs and IgGs

Mucosal fluids are known to contain not only IgA in the form of SIgA but also IgG. We sought to test whether the dIgA version would interfere with the protection provided by the IgG1 forms—as had been implied by the systemic IgG and IgA antibodies in the RV144 trial. Since the hypothesis was that the IgA form would decrease the protective effect of the IgG, we deliberately selected the dIgA2 version of HGN194, which had given suboptimal protection of only 17% when used as a single agent. In contrast, the dIgA1 version had provided 83% protection. Instead of delivering the IgG mucosally, we decided to administer a suboptimal dose i.v. 24 h before intrarectal SHIV-C challenge to allow the antibody to distribute in tissues and enter mucosal fluids.

We enrolled three groups of RMs; one group was given only the i.v. IgG1form of HGN194 24 h before virus challenge, the second group was given the same IgG treatment and an additional intrarectal passive immunization 30 min before virus challenge. The controls were left untreated. By itself, the low-dose i.v. IgG form gave no protection. The dIgA2 version by itself had given 17% protection in the previous study (29). Surprisingly, the low-dose i.v. IgG1 combined with the intrarectally administered dIgA2 yielded 100% protection (30). This *in vivo* synergy cannot be explained by synergistic neutralization *in vitro*. Rather, we postulate that local interactions with mucins and epithelial barrier structures may underlie this remarkably potent protection. This unexpected result has since been replicated and again yielded 100% protection (unpublished data).

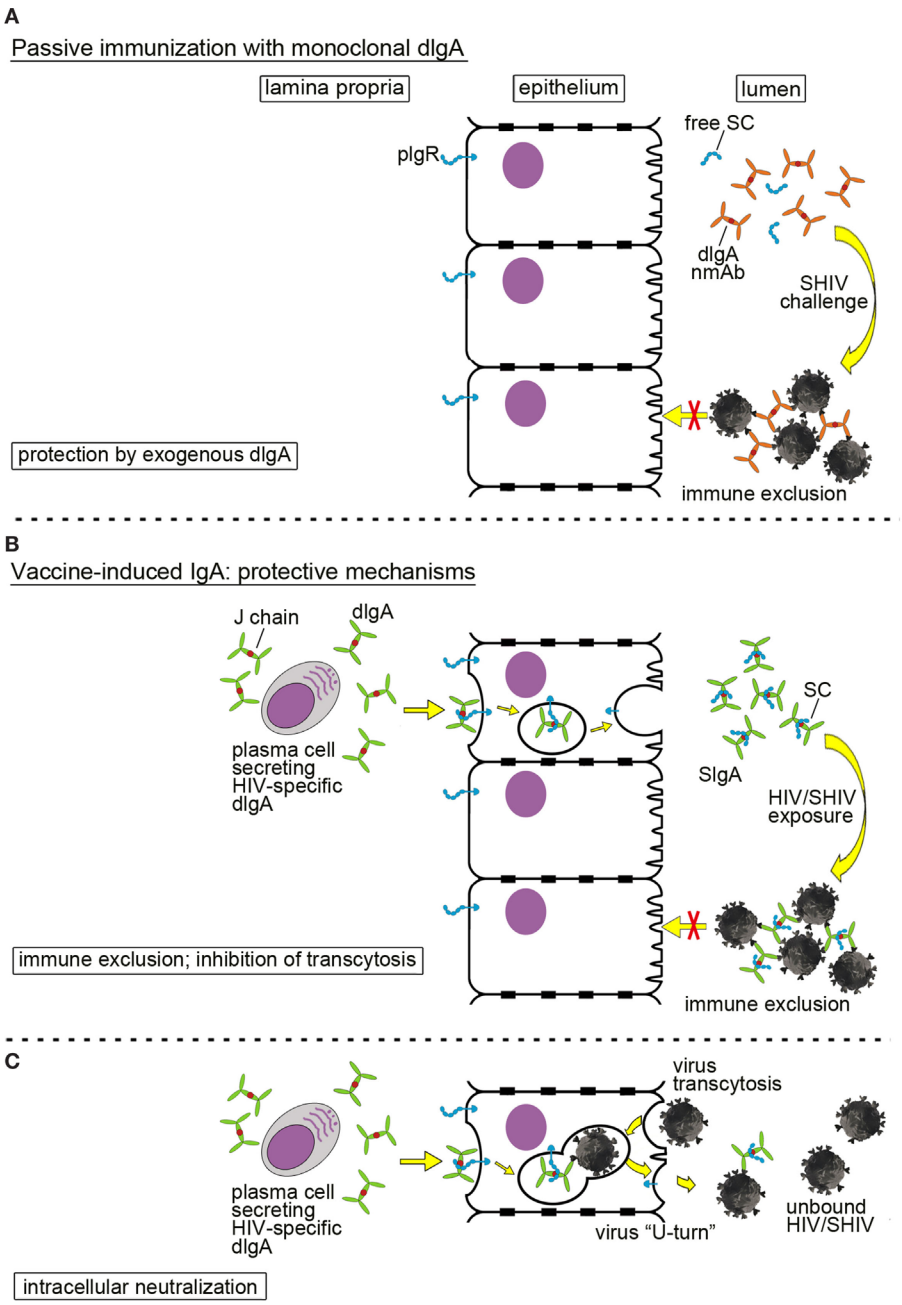

FIGURE 2 | Mucosal defenses by dimeric IgA (dIgA) or secretory IgA (SIgA) against HIV/simian-human immunodeficiency virus (SHIV). **(A)** Passive immunization with a neutralizing monoclonal anti-HIV Env dIgA. Topically administered neutralizing dIgA monoclonal antibodies prevent SHIV from crossing the epithelial barrier by formation of large immune complexes, leading to immune exclusion. To indicate the exogenous source and the recombinant nature of the monoclonal dIgA, the latter are colored in ochre in contrast to dIgAs produced locally by the host (green dIgAs; Figures 1A and 3B,C). **(B,C)** Mucosal SIgA induced by active immunization and different mechanisms of protection; **(B)** immune exclusion and inhibition of transcytosis. Plasma cells in the lamina propria produce virus-specific dIgA that interacts with the polymeric immunoglobulin receptor (pIgR; blue) on the basolateral surface of epithelial cells; pIgR transports dIgA across epithelial cells in transcytotic vesicles. Proteolytic cleavage of pIgR at the luminal side generates secretory component (SC) that is retained by dIgA molecules. The latter complexes are released as SIgA into the lumen. Virion invasion of epithelial cells is blocked by formation of large immune complexes between SIgA and SHIV leading to immune exclusion. **(C)** Intracellular neutralization. This phenomenon occurs when virions are taken up by epithelial cells and enter transcytotic vesicles, in which dIgA-pIgR cargo is being exported toward the lumen. Virions are bound by specific dIgA, and the entire virion-dIgA-pIgR complex is excreted. Essentially, the virion in the complex is forced into a U-turn. This figure has been adapted from the original review article "Are anti-HIV IgAs good guys or bad guys?" by Zhou and Ruprecht (10) (https://retrovirology.biomedcentral.com/articles/10.1186/s12977-014-0109-5). The original article is an open access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

To summarize, passive immunization has revealed a potent protective mechanism at the level of the mucosal barrier that can yield 100% protection by combining mucosal IgG with dIgAs. It will be important to elucidate the mechanisms of this interaction in future studies. It needs to be emphasized also that passive immunization involves only the mAbs administered, in the absence of any other potentially confounding protective mechanisms by the host. These encouraging data provide strong impetus to focus on inducing protective IgG and IgA mucosal antibody responses by targeted active vaccination.

Induction of Protective Mucosal Antibody Responses by Active Immunization

To date, one vaccine strategy specifically was designed to focus on the induction of mucosal antibody responses: virosomes displaying different fragments of HIV gp41. Virosomes are empty particles derived from influenza virus but devoid of any nucleic acid; as such, this vaccine carrier is noninfectious and has a very good safety profile in clinical studies targeting conditions other than HIV (35, 36). Two populations of virosomes were tested in NHP studies, namely virosome-P1, which displayed the extended P1 peptide mimicking the membrane proximal external region (MPER) of HIV gp41, in a second population of virosomes displaying a truncated form of gp41 lacking the immunodominant mini loop. This second form of virosomes was termed virosome-rgp41. When tested in Chinese-origin rhesus monkeys, 100% of the vaccinated animals were protected from persistent systemic infection when given the combination of the two virosomes by two intramuscular vaccinations followed by two intranasal boosts. This group of vaccinees showed no seroconversion to SIV Gag after multiple low-dose intravaginal challenges with an upfront heterologous R5 tier 2 SHIV, although some of the animals had low level blips of viremia initially (37). These authors performed an extensive analysis to determine the correlates of protection. None of the systemic antibody responses showed any link, including neutralizing antibody responses and systemic ADCC. In contrast, vaginal fluid IgA was linked to protection through inhibition of virus transcytosis in a transwell system and vaginal IgG showed neutralizing and ADCC activity. In other words, only mucosal IgA and IgG but not systemic IgA and IgG responses correlated with protection.

We have independently confirmed these data during the first half of the multiple low-dose vaginal challenges, where we noticed between 78 and 87% protection against the initial challenge virus dose. These repeat studies were conducted in Indian-origin rhesus monkeys. When comparing the virus challenge dose with the viral RNA copy numbers of the average HIV inoculum likely transferred from a HIV positive man to a female partner, the SHIV inoculum used in our study was 70,000 times higher. When we had to increase the SHIV challenge dose in the Indian-origin monkeys in a second part of the virus challenge phase as had been done in the earlier study (37), protection was lost. This virus challenge dose was greater than 100,000 times the average HIV inoculum passed from an infected man to a female partner. We interpret these findings as promising data that warrant optimizing vaccine strategies based upon this platform (unpublished data).

Are Highly Exposed Persistently Seronegative (HEPS) Individuals Protected by Anti-HIV Mucosal IgA?

A few groups have reported an intriguing link between individuals who despite frequent sexual HIV exposures have remained uninfected—and IgA responses [reviewed in Ref. (10)]. HIV-specific IgA responses have been correlated with resistance to HIV acquisition in sex workers and in persistently uninfected sexual partners of HIV-positive individuals; the methods to isolate mucosal IgA were based upon jacalin resins that preferentially bind to the O-linked oligosaccharides in the wide hinge region of human IgA1 [jacalin specifically binds to IgA1 hinge O-linked oligosaccharides (38–40) reviewed in Ref. (10)]. Epitope mapping revealed that mucosal IgAs targeted relatively conserved MPER epitopes HIV gp41 (41, 42). Mucosal IgAs isolated from HEPS subjects exhibited cross-clade neutralization (43). Other investigators noted that HIV-specific mucosal antibody responses were either not detectable or found in only a low fraction of HEPS in some cohorts (44–47). The disparate findings regarding mucosal IgA isolated from HEPS individual may stem from assay conditions, including the choice of protease inhibitors and the timing of their addition to mucosal fluids, the use of jacalin-based IgA isolation methods that yield predominantly IgA1 isotype antibodies, and assay sensitivity.

More recently, Hirbod et al. (48) described that neutralizing IgA1 in the foreskin of uncircumcised men was associated with lower risks of HIV acquisition. These authors performed blinded analyses on foreskin swabs collected in a randomized Ugandan trial of male circumcision for HIV prevention. The study's goal was to assess correlates of HIV acquisition risks in foreskin using a case-control design. IgA was isolated by Jacalin column chromatography from swabs, a method that predominately yields IgA1 as mentioned above. The presence of IgA neutralizing capacity in foreskin samples was associated with an odds ratio (OR) of 0.31 for HIV acquisition in these uncircumcised men at initial enrollment and 0.21 at the last visit when cases were still seronegative. These data parallel those obtained in high-risk Kenyan sex workers, where the OR of HIV infection among study subjects with neutralizing IgA in cervical/vaginal secretions was 0.31 (30). Together, data from both studies imply a protective effect of mucosal IgA against sexual HIV transmission.

The presence of neutralizing anti-HIV IgA in the cervicovaginal secretions of HEPS women in Kenya and Uganda enrolled in the Partners pre-exposure prophylaxis (PrEP) study was confirmed by Lund et al. HEPS women on oral PrEP had significantly higher levels of neutralizing IgA antibodies as compared to placebo controls (49).

In summary, studies on HEPS subjects imply that mucosal anti-HIV IgA responses may be linked to prevention of persistent systemic HIV infection. Understanding the mechanism of protection among these populations will be important in designing effective vaccines.

CONCLUSION

The goal of this review was to provide a juxtaposition between the potential of mucosal antibodies in normal hosts to protect against

immunodeficiency virus acquisition versus the severely damaged status of mucosal antibody-producing cells in established HIV/SIV/SHIV infections. In uninfected hosts, IgA in mucosal fluids can prevent mucosal virus transmission through a process termed immune exclusion. This was demonstrated in the first proof-of-concept passive mucosal immunization studies involving recombinant monoclonal dIgAs. Thus far, active induction of protective mucosal IgA together with IgG has been achieved only in the vaccine study by Bomsel et al. (37) and by our group (unpublished data).

During the course of natural HIV/SIV/SHIV infection, the production of mucosal antigen-specific IgG and IgA is severely compromised, which leads to a skewing of the IgG:IgM and IgA:IgM ratios in mucosal fluids. It is likely that the low production of mucosal IgA and IgG compromises mucosal barrier integrity. This can lead to microbial translocation that is associated with severe immune activation, an additional mechanism that upregulates virus replication in mucosal tissues. Together, such damages inflicted on mucosal cells, tissues, and barrier function also weaken anti-HIV mucosal antibody responses. This may be a key risk factor in the frequently observed superinfection of HIV-positive individuals, resulting in inter- or intra-clade recombination events and the generation of CRFs. Their ever increasing genetic diversity may be an indirect indicator of loss of mucosal barrier protection due to the damage inflicted upon the mucosal B-cell compartment. Strategies aimed at improving humoral mucosal defenses and prevention of microbial translocation in HIV-infected individuals—perhaps by therapeutic vaccination—may improve the overall health status of individuals with chronic HIV infection.

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Plasma CXCL13 but Not B Cell Frequencies in Acute HIV Infection Predicts Emergence of Cross-Neutralizing Antibodies

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Immunological events in acute HIV-1 infection before peak viremia (hyperacute phase) may contribute to the development of broadly cross-neutralizing antibodies. Here, we used pre-infection and acute-infection peripheral blood mononuclear cells and plasma samples from 22 women, including 10 who initiated antiretroviral treatment in Fiebig stages I–V of acute infection to study B cell subsets and B-cell associated cytokines (BAFF and CXCL13) kinetics for up to ~90 days post detection of plasma viremia. Frequencies of B cell subsets were defined by flow cytometry while plasma cytokine levels were measured by ELISA. We observed a rapid but transient increase in exhausted tissue-like memory, activated memory, and plasmablast B cells accompanied by decline in resting memory cells in untreated, but not treated women. B cell subset frequencies in untreated women positively correlated with viral loads but did not predict emergence of cross-neutralizing antibodies measured 12 months post detection of plasma viremia. Plasma BAFF and CXCL13 levels increased only in untreated women, but their levels did not correlate with viral loads. Importantly, early CXCL13 but not BAFF levels predicted the later emergence of detectable cross-neutralizing antibodies at 12 months post detection of plasma viremia. Thus, hyperacute HIV-1 infection is associated with B cell subset changes, which do not predict emergence of cross-neutralizing antibodies. However, plasma CXCL13 levels during hyperacute infection predicted the subsequent emergence of cross-neutralizing antibodies, providing a potential biomarker for the evaluation of vaccines designed to elicit cross-neutralizing activity or for natural infection studies to explore mechanisms underlying development of neutralizing antibodies.

Keywords: B-cell subsets, acute HIV, CXCL13, cross-neutralizing antibodies, BAFF

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INTRODUCTION

The development of a successful vaccine for HIV-1 will likely require the elicitation of broadly neutralizing antibodies (bNAbs), i.e., antibodies that target fairly conserved epitopes on the HIV envelope spike and, therefore, neutralize the majority of HIV isolates; however, to date, it is not fully understood how such responses can be induced through vaccination. In natural infection, bNAbs only appear after years of infection, developing in a small subset of individuals, although cross-neutralizing antibodies with narrower breadth can be detected earlier and in higher numbers of people (1–8). Thus far, plasma viral load, CD4 count and inflammation have been described as predictors of neutralizing breadth but these would be irrelevant in the context of vaccine trials (1–3, 6, 9). A report investigating bNAb lineages from early infection showed that reverted germline versions bound early autologous envelopes, potentially initiating key B cell selection processes and downstream antibody evolution pathways (10). This observation points to the potential influence of events occurring during hyperacute HIV-1 infection—before peak viremia—on development of cross-neutralizing antibodies, an area that remains unexplored to date.

In primary and chronic untreated HIV-1 infection (PHI and CHI), prior studies, largely cross-sectional in nature, have shown that B cell subset frequencies, defined by surface expression levels of CD21 and CD27, are disrupted (11, 12). Specifically, HIV-1 infected individuals have increased frequencies of immature/transitional B cells, increased tissue-like memory (TLM) B cells with signs of premature exhaustion and decreased frequencies of resting memory (RM) B cells (11–13). Although combination antiretroviral therapy (cART) initiated during chronic infection results in normalization of most B cell subsets, memory B cell defects persist and only show significant recovery if patients initiate treatment early in the course of infection (14–20). It remains unknown whether pre-infection B cell subset frequencies and changes occurring during hyperacute HIV-1 infection (or immediately following encounter with antigen following vaccination) might be used to predict the emergence of early cross-neutralizing antibodies and thus help guide vaccine strategies to drive this activity.

HIV-1 bNAbs generally have unusual features including high levels of somatic hypermutation in both complementarity-determining region (CDR) loops and framework regions, long heavy chain CDR 3 (CDRH3), and a propensity toward autoreactivity (21–24). Indeed, accumulating data now show that levels of the chemokine CXCL13, produced by T follicular helper cells (Tfh), play a key role in the quality of the germinal center (GC) reaction and predict development of cross-neutralizing antibodies in HIV-infected patients (25–27). The B cell-associated cytokine B cell activating factor (BAFF) can also potentially influence the survival and class switching of unique autoreactive B cells likely to generate cross-neutralizing antibodies (28–30). Thus far, BAFF has been shown to augment development of cross-neutralizing antibodies in animal models when used as an adjuvant or supplied exogenously (31, 32) although this was not true in a cohort of subtype B infected individuals (25). Whether the levels of these two key B cell associated cytokines during hyperacute HIV-1 infection can predict subsequent development of cross-neutralizing antibodies later remains to be determined.

We sought to understand the dynamics of the B cell response, with respect to subset changes and B cell associated cytokines, prior to infection, and during hyperacute infection and how they might influence development of cross-neutralizing antibodies. Additionally, the impact of cART initiated during the acute phase of infection on these factors was evaluated. We used pre- and post-HIV-1 subtype C infection samples from young women enrolled in a study termed Females Rising through Education, Support and Health (FRESH) in the KwaZulu-Natal province of South Africa (33). We measured the dynamics of B cell subsets, plasma levels of BAFF and CXCL13 before infection and longitudinally during hyperacute HIV-1 infection and determined their influence on the emergence of cross-neutralizing antibodies at approximately 1 year postinfection (PI). Our data demonstrate that B cell defects reported in PHI and CHI emerge during hyperacute HIV-1 infection in women who do not initiate early treatment and are abrogated with immediate treatment, indicative of the influence of viral load on the observed changes. However, these dramatic B cell changes occurring in hyperacute infection did not predict the emergence of cross-neutralizing antibodies. In contrast, changes in BAFF and CXCL13 during hyperacute infection were not directly associated with viral loads. Importantly, we found higher levels of CXCL13 during hyperacute infection in individuals who subsequently developed detectable cross-neutralizing antibodies within 1 year of infection compared to those who did not. Hence our data from subtype C hyperacute infection confirm the utility of CXCL13 levels early in infection as a biomarker for possible superior GC activity associated with emergence of cross-neutralization antibodies.

MATERIALS AND METHODS

Study Population and Blood Samples

Females Rising through Education, Support and Health is a longitudinal cohort study of 18- to 23-year-old HIV-1-negative women at high risk of HIV-1 infection established in the Umlazi Township of Durban, KwaZulu-Natal, South Africa. Cohort recruitment and follow-up details have been comprehensively described elsewhere (33–35). Briefly, blood samples were obtained at study entry and every 3 months thereafter from HIV-1-negative study participants. Study subjects attended twice-weekly sessions in which trained counselors offered a comprehensive life and job skills, empowerment and HIV-1 prevention curriculum. During the twice-weekly visits, finger prick blood samples were taken for monitoring of plasma HIV-1 RNA, with results available within 24 h. Participants with a positive RNA test were contacted immediately, counseling was provided, and blood samples were collected. Subsequently, longitudinal PI venous blood samples were obtained at regular intervals through peak viremia and beyond. Peripheral blood mononuclear cells (PBMCs) were frozen from each venous blood draw for future analysis. Initially, participants identified with onset of

plasma viremia were closely monitored and referred for cART if meeting eligibility according to South African guidelines (36). Beginning July 2014, the study protocol was amended and participants with onset of HIV-1 plasma viremia were initiated on cART immediately using a standard 3-drug regimen of tenofovir disoproxil fumerate 300 mg, emtricitabine 200 mg, and efavirenz 600 mg (TDF/FTC/EFZ). From July 2015, participants with acute viremia received early treatment with TDF/FTC/EFZ plus raltegravir (RAL) 400 mg twice-daily, with RAL withdrawn two months after suppression of plasma viremia to undetectable levels.

B Cell Phenotyping

Frozen PBMCs were thawed and allowed to rest for 2 h before being used for phenotypic analysis using a panel of fluorescently labeled monoclonal antibodies reactive with the following cell surface markers: BV711 conjugated antihuman CD3 (BioLegend, San Diego, CA, USA), BV450 mouse antihuman CD21 (BioLegend, San Diego, CA, USA), Qdot 605 mouse antihuman CD19 (Life Technologies, Carlsbad, CA, USA), PE mouse antihuman CD27 (BD Biosciences, San Jose, CA, USA), Alexa Fluor 700 mouse antihuman CD38 (BD Biosciences, San Jose, CA, USA), and aqua viability dye (Life Technologies, Carlsbad, CA, USA). Rested PBMCs were stained with 200 µl of diluted viability dye and allowed to incubate in the dark for 15 min at RT. Thereafter, cells were washed twice in phosphate-buffered saline (PBS) and then 100 µl of the cocktail of antibodies was added to 2×10^6 cells and incubated for 15 min at room temperature. Thereafter, tubes were washed with 3 ml PBS and centrifuged at $600 \times g$ for 5 min. Supernatant was discarded and 100 µl of 2% paraformaldehyde was added to each tube. Samples were then acquired on the LSRFortessa (Becton Dickinson, Franklin Lakes, NJ, USA) and data analyzed on FlowJo version 9.8.3 (FlowJo LLC, Ashland, OR, USA).

Determination of Plasma BAFF and CXCL13 Levels

BAFF and CXCL13 levels were determined by ELISA (R&D systems, Minneapolis, MN, USA) using the manufacturer's protocol. Plasma samples were thawed slowly on ice, spun down and the clear supernatant used immediately for the assays.

Neutralization Assays

Neutralization activity was determined using a previously described standard TZM-bl cells based assay (NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) (37). This assay measures Tat-induced luciferase reporter gene expression after infection by HIV-1 Env-pseudotyped viruses with neutralization quantified by reduction in relative light units in TZM-bl cells in the presence of HIV-1-positive plasma. Samples were used at 1:50 dilution, and the ID50 was calculated as the reciprocal dilution at which 50% of the virus was inhibited.

Data Analysis

Non-parametric Spearman's rank tests were used to test for correlations and a 2-tailed Mann–Whitney test was used to evaluate unpaired groups. Wilcoxon matched signed-rank test was used to evaluate paired samples. To assess the relationship between each B cell subset and time, varying viral load, CD4 count, BAFF, and CXCL13 adjusted for days PI, linear mixed effects models with random (subject specific) intercepts were fitted to the B cell data. Due to the complex non-linear evolution of B cell subsets over time, an unstructured mean was considered. The variables of interest (CD4 counts, viral load, CXCL13, and BAFF levels) were treated as time dependent covariates in the model, separately. B cell subsets (the outcome) were log transformed. By comparison of Akaike information criterion and Bayesian information criterion, the most suitable model was that with a random intercept and residuals which follow an autoregressive (1) structure. p-Values less than 0.05 were considered significant. Data analysis was performed in Graphpad Prism version 6 (Graphpad Software, San Diego, CA, USA) and Stata version 13.0 (Statacorp, College Station, TX, USA).

Ethics Statement

Study subjects provided written informed consent for participation in the study. Ethical approval was provided by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal and the Institutional Review Board of Massachusetts General Hospital.

RESULTS

Rapid but Transient Changes in Frequencies of B Cells and B-Cell Subsets in Acute HIV-1 Subtype C Infection

Pre-infection samples were obtained from all participants in this study. Among the 12 untreated participants, the initial PI samples were obtained in Fiebig stage I for 11 participants and Fiebig stage III for one participant, providing us the opportunity to study very early changes in B-cell subsets and associated cytokines, and to determine how early events might influence the emergence of cross-neutralizing antibodies. Multiple samples were also obtained from participants prior to peak viremia, and during resolution of peak viremia to a viral load set-point. Ten early treated women were also studied, representing a subset of persons within our cohort who initiated standard first line treatment (TDF/FTC/EFZ) within less than 3 days of HIV-1 RNA detection. Among them, the initial PI samples were obtained in Fiebig stage I for 8 participants and Fiebig stage V for two participants. If a participant did not have a sample at 3 months after HIV-1 RNA detection, an alternative sample at 2 months was used (**Figures 1** and **2**).

It has previously been reported that HIV-1 uninfected people have geography- and gender-dependent differences in lymphocyte counts (38–40). We, therefore, first established the baseline (pre-infection) frequency of B cells defined as the percentage of $CD3^-CD19^+$ cells of the total live peripheral blood lymphocyte population in the 12 untreated women. We found that on average these cells accounted for 7% of the peripheral blood lymphocytes at baseline (range 3.9–12.1%), which was lower than what has been observed in geographically different cohorts from Uganda

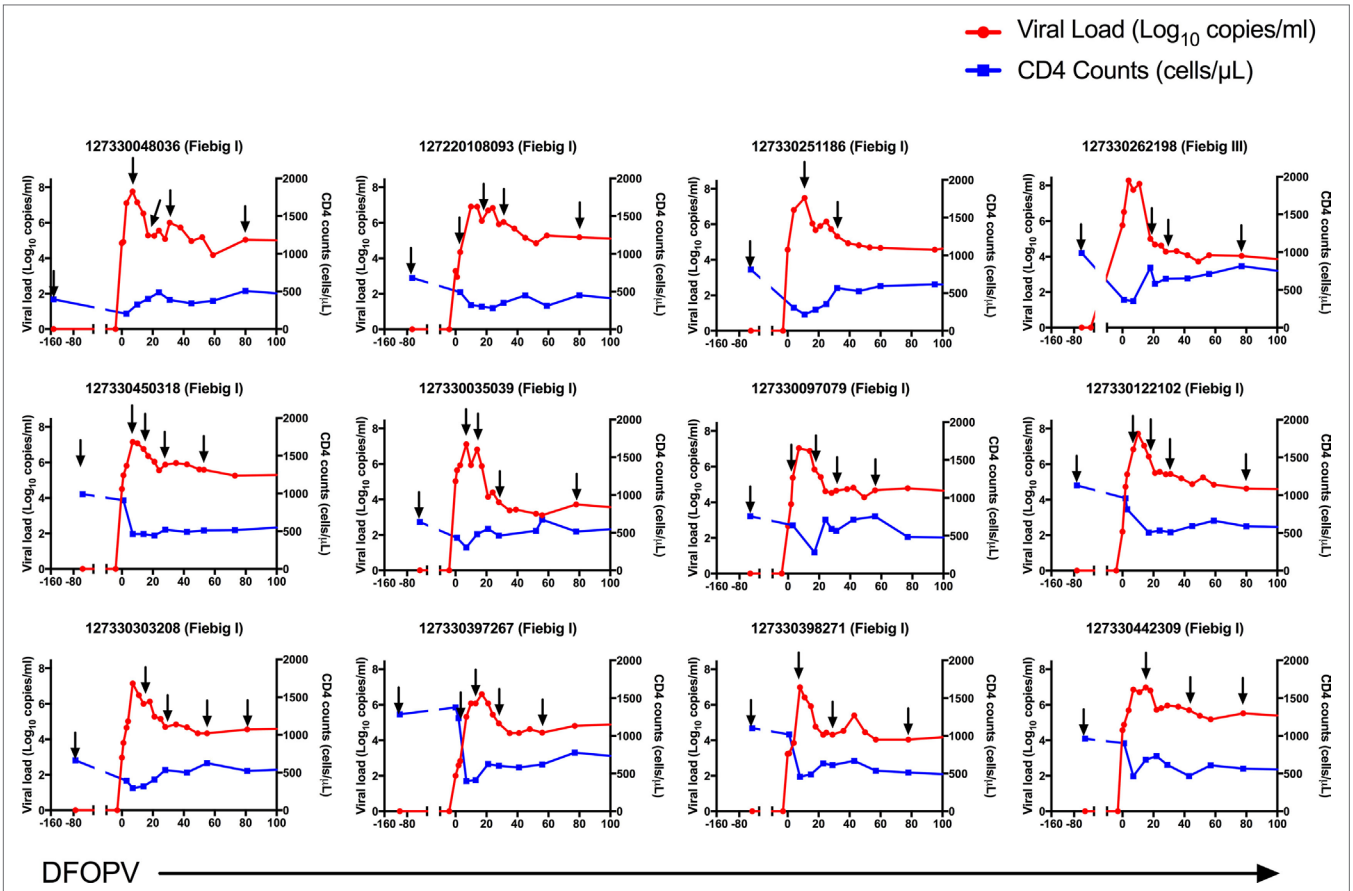

FIGURE 1 | Dynamics of plasma viral loads and CD4 counts during hyperacute HIV-1 subtype C infection in absence of early antiretroviral treatment. Plasma HIV-1 RNA levels (red) and absolute CD4 counts (blue) before HIV infection and following onset of detectable plasma viremia in 12 subjects with hyperacute HIV infection that were not initiated on early antiretroviral treatment. The arrows indicate time-points used for B cell analysis. DFOPV, days following onset of plasma viremia.

(40). Following infection, three untreated individuals showed a transient increase in frequency of total B cells at days 7 and 14, although these populations decreased thereafter (**Figure 3A**). Overall, the median frequency of total circulating B cells was significantly lower compared to baseline pre-infection levels at 30 days ($p = 0.024$) and 90 days (0.048) following onset of plasma viremia (DFOPV) (**Figure 3A**). These data suggest that HIV-1 subtype C infection in an African population alters B cell frequencies presumably through indirect killing or redistribution of B cells, or through expansion of other lymphocyte populations, resulting in decreased proportions of B cells in the periphery over time.

The availability of pre-infection and hyperacute infection samples allowed us to determine baseline frequencies and subsequent kinetics of alterations in B cell subsets with the goal of defining early signatures associated with emergence of cross-neutralizing antibodies. Different clades of HIV-1 differ in pathogenicity and rates of disease progression. Thus, we hypothesized that the B cell kinetics in this clade C cohort might be unique if clade specific features, such as replicative capacity, are a determinant of B cell subset alterations (41–43).

We first determined the kinetics of the four previously described B cell subsets [activated memory (AM), RM, TLM, and naïve cells (11, 12)] defined by the expression of CD21 and CD27 on $CD19^+$ mature B cells as shown in representative data (**Figure 3B**). There was a rapid decrease in the frequencies of RM cells ($CD21^+CD27^+$) noted at 7 DFOPV (medians; 26.55 and 16.5%, range 7–43.9 and 1–21.5% for baseline and 7 DFOPV, respectively), concurrent with an increase in TLM cells ($CD21^-CD27^-$) (medians; 12.7 and 27.85%, range 7.94–38.3 and 7.49–67.7% for baseline and 7 DFOPV, respectively). The frequencies of RM cells remained significantly lower than baseline throughout the time-points tested thereafter in the first 3 months PI ($p = 0.008$, 0.001, 0.005, and 0.019 for 7, 14, 30, and 90 DFOPV, respectively) (**Figure 3C**). Compared to baseline, frequencies of TLM cells were significantly higher at 7 and 14 DFOPV ($p = 0.039$ and 0.0001, respectively). Thereafter, frequencies of TLM cells remained elevated in most individuals though not statistically significant through to 90 DFOPV (**Figure 3D**). Importantly, neither RM nor TLM frequencies were restored to baseline values by ~90 DFOPV. We observed a significant expansion of AM cells ($CD21^-CD27^+$) by 14 DFOPV ($p = 0.005$) that persisted at 30 DFOPV ($p = 0.010$) when a peak was reached followed by contraction to near baseline values in some of the individuals by 90 DFOPV ($p = 0.083$) (**Figure 3E**). No changes were observed in the frequency of naïve B cells ($CD21^+CD27^-$) following HIV-1 infection (**Figure 3F**).

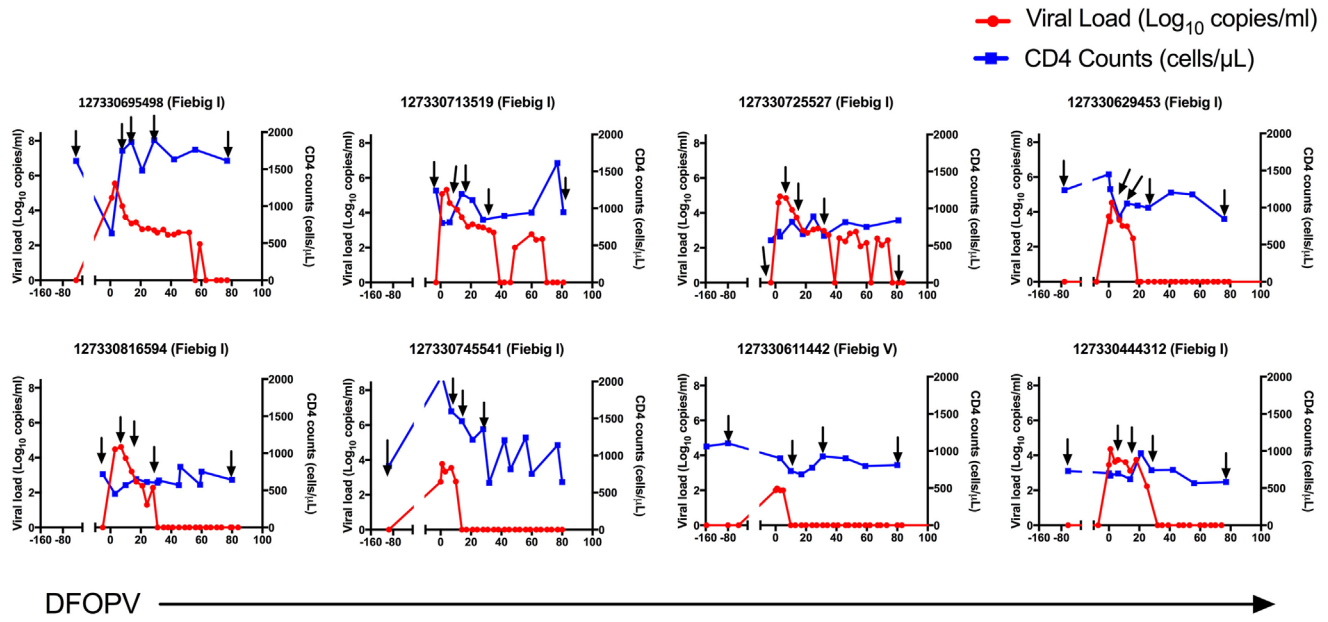

FIGURE 2 | Dynamics of plasma viral loads and CD4 counts during hyperacute HIV-1 subtype C infection with early initiation of antiretroviral treatment. Plasma HIV-1 RNA levels (red) and absolute CD4 counts (blue) before HIV infection and following onset of detectable plasma viremia in eight subjects with hyperacute HIV infection that were initiated on early antiretroviral treatment. The arrows indicate time-points used for B cell analysis. DFOPV, days following onset of plasma viremia.

Plasmablasts (PBs) represent immunoglobulin secreting terminally differentiated B cells, which are transiently enriched in blood during infection or vaccination (44–46). To define PB kinetics in HIV-1 infection, we assessed the frequencies of $CD3^-CD19^+CD27^+CD38^{+++}$ cells before and upon HIV-1 infection. At pre-infection baseline, the median frequency of PBs was 1.26% (range 0.321–11.4%) of the total B cell population. Upon infection, there was a transient expansion of the PB population as shown in the representative example (**Figure 3G**) that peaked by ~14 days (medians 1.26 and 6.58%, range 0.321–11.4% and 0.532–28.6% for baseline and 14 DFOPV, respectively). Following HIV-1 infection, frequencies of the PB population remained significantly elevated at all time-points tested ($p = 0.016$, 0.002, 0.002, and 0.019 for 7, 14, 30, and 90 DFOPV, respectively) (**Figure 3H**). Thus, these data illustrate that untreated subtype C acute HIV-1 infection is associated with rapid changes in frequencies of circulating B cell subsets characterized by an increased frequency of AM, TLM, and PBs but a decrease in RM cells.

Increase in Plasma BAFF and CXCL13 Levels in Acute HIV-1 Subtype C Infection

Given the early increase in PBs and alterations in B cell subsets, and considering that acute HIV infection has previously been associated with a cytokine storm that may have profound long-term immunological consequences (47), we next sought to determine whether there were changes following HIV infection in soluble factors associated with B cell activation, survival, and maturation. Specifically, we investigated the levels and kinetics of BAFF, a cytokine important for B cell survival, and CXCL13, a chemokine responsible for B cell trafficking to GCs and potentially responsible for the expansion of PBs (26, 48). The median plasma level of BAFF at baseline was 795 pg/ml (range 536–1,121 pg/ml). These levels increased rapidly and significantly upon infection peaking by 7 DFOPV at a median of 1,817 pg/ml (range 1,457–4,119 pg/ml, $p = 0.0005$) and remained significantly higher throughout the first 90 DFOPV ($p = 0.005$ for 14 DFOPV and $p = 0.0005$ for both 30 and 90 DFOPV) (**Figure 4A**). The median plasma CXCL13 level at baseline was 76 pg/ml (range 40–282 pg/ml). Similar to BAFF, CXCL13 levels were elevated upon infection although the increase was progressive with the highest median of 275 pg/ml (range 125–511 pg/ml) being registered 90 DFOPV (the last visit analyzed). Compared to baseline, the measurements remained significantly higher throughout the time-points analyzed in the first 90 DFOPV ($p = 0.003$, 0.0005, 0.0005, and 0.0039 for 3, 14, 30, and 90 DFOPV, respectively) (**Figure 4B**). Thus, acute HIV-1 infection is associated with rapid and gradual increase in plasma levels of B cell-associated cytokines BAFF and CXCL13, respectively.

Viral Loads Directly Drive Changes in B Cell Subset Frequencies but Not Levels of Plasma BAFF and CXCL13

Viral loads and associated immune activation in chronic infection have been linked to changes in B cell subsets and development of bNAbs during chronic infection (9, 49, 50). To determine whether viral replication was associated with the observed changes, we first assessed the relationship between contemporaneous viral loads, $CD4^+$ T cell counts and B cell frequencies over time. We found a negative trend and significant relationship between PBs and CD4 counts at baseline (rho = −0.52, $p = 0.080$) and 7 DFOPV (rho = −0.82, $p = 0.023$), respectively (data not shown). Next, we used linear mixed effect models to investigate

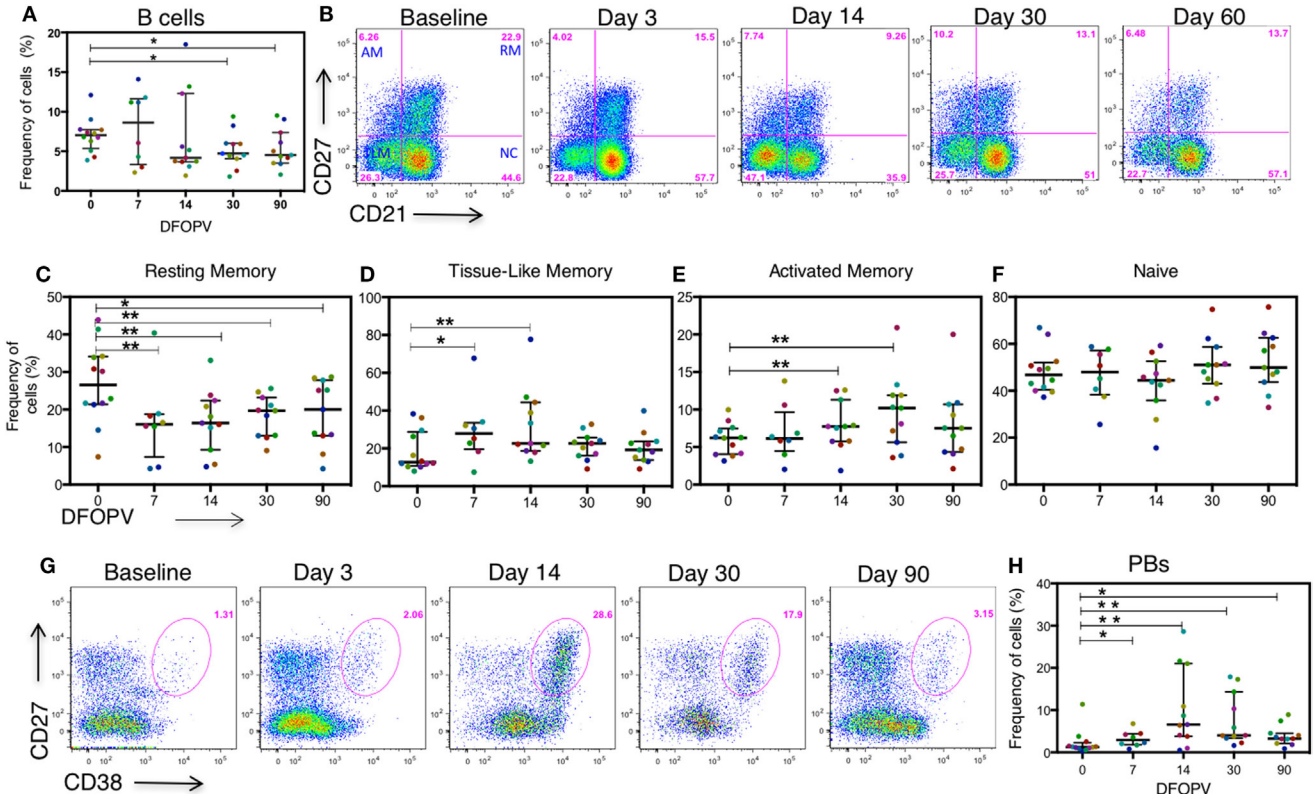

FIGURE 3 | Frequency of B cells before and during acute HIV-1 subtype C infection in absence of early antiretroviral treatment. B cells were defined by the expression of CD19 on $CD3^-$ peripheral blood lymphocytes. B cell subsets were defined by the expression of CD27 and CD21 on $CD3^-CD19^+$ lymphocytes. Plasmablasts (PBs) were defined as $CD27^+CD38^{+++}$ cells on $CD3^-CD19^+$ peripheral lymphocytes. Subsets were analyzed on longitudinal AHI samples obtained in the first ~90 DFOPV and compared to matched baseline values. Panel **(A)** shows a summary of the frequency of B cells as a percentage of lymphocytes overtime. Panel **(B)** is representative data showing B cell subsets from baseline (before infection) to ~60 DFOPV, example from participant 127-033-0097-079. Panels **(C–F)** represent frequencies of B cell subsets; **(C)** resting memory, **(D)** tissue-like memory, **(E)** activated memory, and **(F)** naïve cells. **(G)** Representative data from participant 127-033-0108-093 shows kinetics of PBs from baseline to ~90 DFOPV. **(H)** A comparison between frequencies of PBs at baseline and longitudinal time-points up to ~90 DFOPV. Horizontal lines represent median values and each color represents one patient. DFOPV, days following onset of plasma viremia, and time-point "0" represents baseline (visit prior to infection). *p*-Values were calculated by Wilcoxon matched signed-rank test (*$p < 0.05$, **$p < 0.005$).

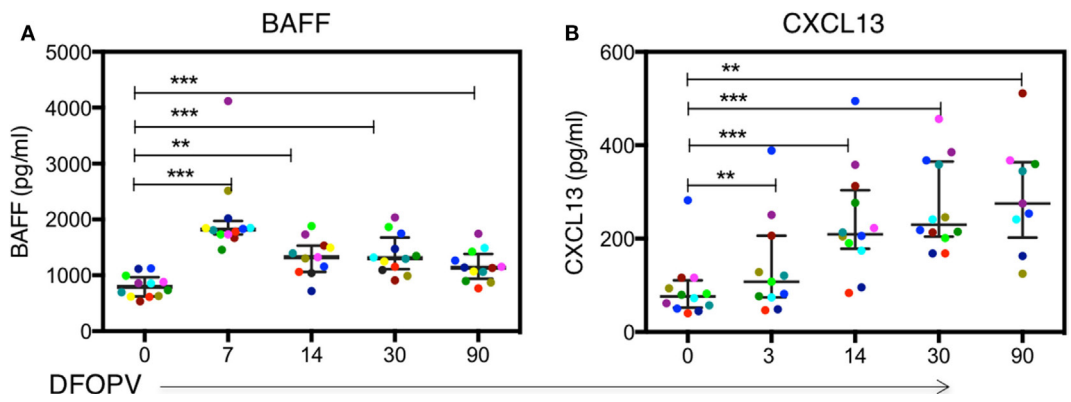

FIGURE 4 | Dynamics of plasma BAFF and CXCL13 levels following acute HIV-1 subtype C in absence of early antiretroviral treatment. Panels **(A,B)** show kinetics of BAFF and CXCL13 levels determined by ELISA using plasma samples obtained longitudinally from untreated patients within the first ~90 DFOPV and compared to matched baseline values. Horizontal lines represent median values and each color represents one patient. DFOPV, days following onset of plasma viremia. *p*-Values were calculated by Wilcoxon matched signed-rank test (**$p < 0.005$, ***$p < 0.0001$).

the overall relationship between the rapid changes in viral loads, $CD4^+$ T cells and observed changes in B cell subset frequencies over time. Viral load was negatively associated with RM cell frequencies ($p < 0.0001$), positively associated with TLM cells ($p = 0.005$) but no significant associations with AM and PBs were observed (**Table 1**). In contrast, $CD4^+$ T cell counts were positively associated with RM cells ($p = 0.001$) and negatively associated with TLM cells ($p = 0.039$) and AM cells ($p = 0.009$) (**Table 1**). Further, we used a model of a similar form to determine the relationship between changing levels of BAFF, CXCL13, and markers of disease progression. Interestingly, there was no significant relationship between viral loads and BAFF ($p = 0.511$) or CXCL13 ($p = 0.940$). Furthermore, no association was found between CD4 cell counts and BAFF plasma levels; however, we observed a negative association between $CD4^+$ T cell counts and CXCL13 plasma levels ($p < 0.0001$) (data not shown). We also found that BAFF levels were significantly associated with high frequencies of AM ($p = 0.006$) and PBs ($p = 0.026$) cells (**Table 1**). In contrast there was no significant relationship between plasma levels of CXCL13 and any B cell subset frequencies (**Table 1**). Taken together, these data confirm the direct relationship between viral loads and B cell subset frequencies but not BAFF and CXCL13. We, therefore, show for the first time that accumulation of TLM cells, which has mostly been associated with chronic infection, manifests within days of infection and associates with viral loads. Furthermore, the observation of a positive correlation between BAFF levels and specific B cell subsets (AM and PBs) during hyperacute HIV-1 infection may suggest a direct stimulation and/or maintenance of these subsets by this cytokine.

Early cART Blocked Changes in B Cell Subset Frequencies and Plasma Levels of BAFF while Diminishing Changes in Levels of Plasma CXCL13

Following our observation that changes in B cell subset frequencies are influenced by viral load, we next determined whether in the absence of persistent antigenemia the levels of the different B cell subsets, as well as B cell associated cytokines BAFF and CXCL13, would remain normal. Remarkably, there were no significant B cell subset changes observed (representative data **Figures 5A,B** and summary **Figures 5C–E**) except for an increase in PBs at 7 DFOPV ($p = 0.039$) (**Figure 5F**) but at lower frequencies than what was observed in untreated women (**Figure 3**). Indeed, frequencies of AM cells at 30 and 90 DFOPV trended toward being lower than baseline ($p = 0.109$ and 0.078, respectively, data not shown).

Furthermore, we did not observe significant changes in median plasma BAFF levels up to 90 DFOPV (**Figure 6A**). However, CXCL13 levels trended toward being higher upon infection and were significantly higher at 90 DFOPV compared to baseline despite complete suppression of viral loads in most of the individuals (**Figure 6B**). The levels of BAFF and CXCL13 were significantly different between the untreated and early treated individuals at all time-points tested except at baseline and 7 DFOPV for CXCL13 (**Figures 6C,D**). Our data confirm that viremia drives the changes in B cell subset frequencies, an effect that is blocked by early treatment. Furthermore, although early cART largely abrogated the cytokine surge, there was no direct relationship between viral loads and the cytokines in untreated persons, suggesting that the early cytokine responses may be induced by infection-associated changes other than viremia.

Emergence of Cross-Neutralizing Antibodies within 1 Year of HIV-1 Subtype C Infection

Given the rapid changes in frequencies of B cell subsets and increased levels of BAFF and CXCL13 observed during acute HIV-1 infection, we next determined whether the enrichment of a particular B cell subset or cytokine was associated with the emergence of cross-neutralizing antibodies, as an early predictor of cross-neutralization activity. We first probed for presence of cross-neutralizing antibodies for the 12 antiretroviral-naïve individuals using plasma collected at ~1 year PI. Antibody cross-neutralization activity was determined by standard TZM-bl assay against 12 viruses of different subtypes (C, B, and A) and tiers (1 and 2) (51) (**Figure 7**). As expected, we found that all individuals had detectable cross-neutralizing antibodies at 1 year PI against the tier 1 subtype C strain MW965 (100%) and most had activity against the tier 1 subtype B viruses MN.3 (92%) and SF162.LS (83%). One patient 127-33-0108-093 neutralized all three tier 1 viruses with the greatest potency at the time-point prior to initiation of cART (**Figure 7**). Among all subjects tested,

TABLE 1 | Linear mixed effect models for the relationship between viral loads, CD4 counts, CXCL13, and BAFF over time and B cell subsets in absence of early antiretroviral treatment.

		HIV-1 infected untreated				
	B cell subset	**Activated memory**	**Resting memory**	**Tissue-like memory**	***Naive***	**Plasmablasts**
Viral Loads	Coef (SE)	0.0604 (0.037)	−2.5253 (0.5238)	0.1083 (0.0388)	−0.3031 (1.2403)	0.1384 (0.0973)
	p-Value	0.103	**<0.0001**	**0.005**	0.807	0.155
CD4 counts	Coef (SE)	−0.0006 (0.0002)	0.0119 (0.0035)	−0.0005 (0.0002)	0.0028 (0.0074)	−0.0010 (0.0005)
	p-Value	**0.009**	**0.001**	**0.039**	0.705	0.078
CXCL13	Coef (SE)	0.0008 (0.0006)	−0.0160 (0.0106)	0.0007 (0.0007)	0.0026 (0.0188)	0.0010 (0.0013)
	p-Value	0.207	0.13	0.309	0.889	0.424
BAFF	Coef (SE)	0.0003 (0.0001)	−0.0015 (0.0025)	0.00002 (0.0001)	0.0038 (0.0040)	0.0007 (0.0003)
	p-Value	**0.006**	0.553	0.909	0.373	**0.026**

Significant p values are shown in bold.

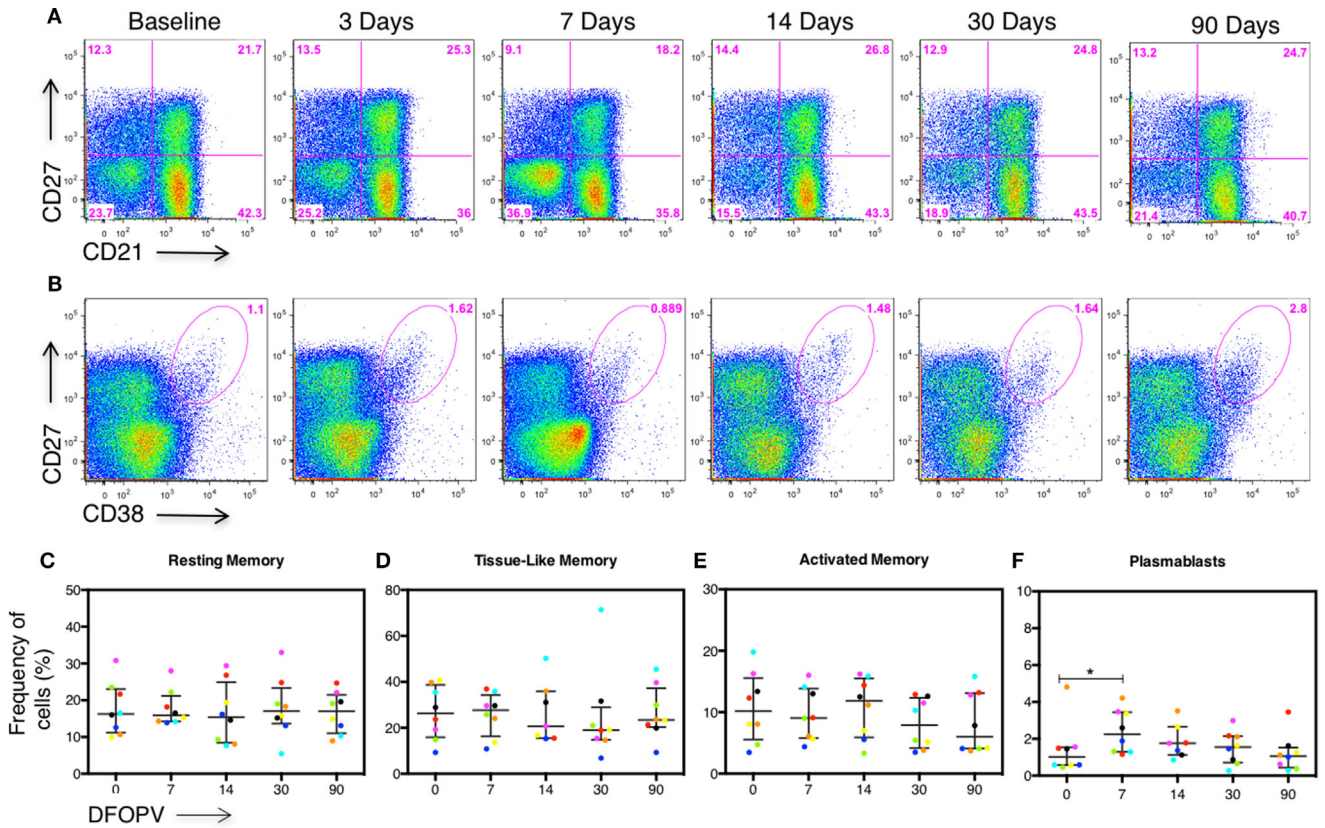

FIGURE 5 | Early combination antiretroviral therapy (cART) blocks B cell subset changes. Peripheral blood mononuclear cells obtained longitudinally from eight individuals who initiated cART during Fiebig stage I–V were used to define B cell subsets and plasmablasts (PBs) by the expression of CD27, CD21, and $CD27^{+}CD38^{+++}$ cells on $CD3^{-}CD19^{+}$ peripheral lymphocytes, respectively. B cell frequencies from the first ~90 DFOPV were compared to matched baseline values. Panels **(A,B)** are representative data from participant 127-033-0629-453 showing kinetics of B cell subsets and PBs, respectively, from baseline (before infection) to 90 DFOPV. Panels **(C–F)** represent frequencies of B cell subsets; **(C)** resting memory, **(D)** tissue-like memory, **(E)** activated memory, and **(F)** PB cells. Horizontal lines represent median values and each color represents one patient. DFOPV, days following onset of plasma viremia, and day "0" represents baseline (visit prior to infection).

there was weak cross-neutralization activity detected against 4/9 (44%) tier 2 viruses tested. Three patients (127-33-0048-036, 127-33-0108-093, and 127-33-0450-318) had detectable but weak cross-neutralization activity against the tier 2 subtype B viruses tested. No activity was detected against tier 2 subtype A viruses at 1 year PI (**Figure 7**).

To enable us to perform further analyses, individuals were categorized into those that did or did not have detectable cross-neutralization activity (regardless of the potency) against any of the tier 2 viruses (6). Using this stratification, five individuals were classified as having detectable cross-neutralization activity and seven as having no detectable cross-neutralization activity (**Figure 7**), and these strata were used in subsequent analysis.

Plasma Levels of CXCL13 Early in Infection Predict Emergence of Cross-Neutralizing Antibodies 1 Year PI

We next investigated whether events occurring early upon infection could predict the emergence of cross-neutralizing antibodies 1 year PI. We found no differences between individuals with and without detectable cross-neutralization activity when comparing viral load set-point ($p = 0.268$) and contemporaneous viral loads ($p = 0.404$). Contemporaneous CD4 counts also did not distinguish between the two groups ($p = 0.458$). Notably, among individuals with detectable cross-neutralizing antibodies at 1 year, 3/5 (60%) qualified for and initiated cART due to low CD4 count within 2 years of infection compared to 2/7 (28%) of those who did not, though that relationship between emergence of cross-neutralizing antibodies and deterioration in CD4 counts was also not statistically significant ($p = 0.558$, Fisher's exact test). One participant in the group with no detectable cross-neutralization was initiated on treatment outside of normal criteria due to pregnancy. To determine whether the expansion of a specific B cell subset following hyperacute infection was predictive of the emergence of cross-neutralizing antibodies, we compared the peak frequency of AM, TLM, and PBs and nadir levels of RM cells in the individuals with and without cross-neutralization activity and found no apparent differences in this small group of 12 individuals (data not shown).

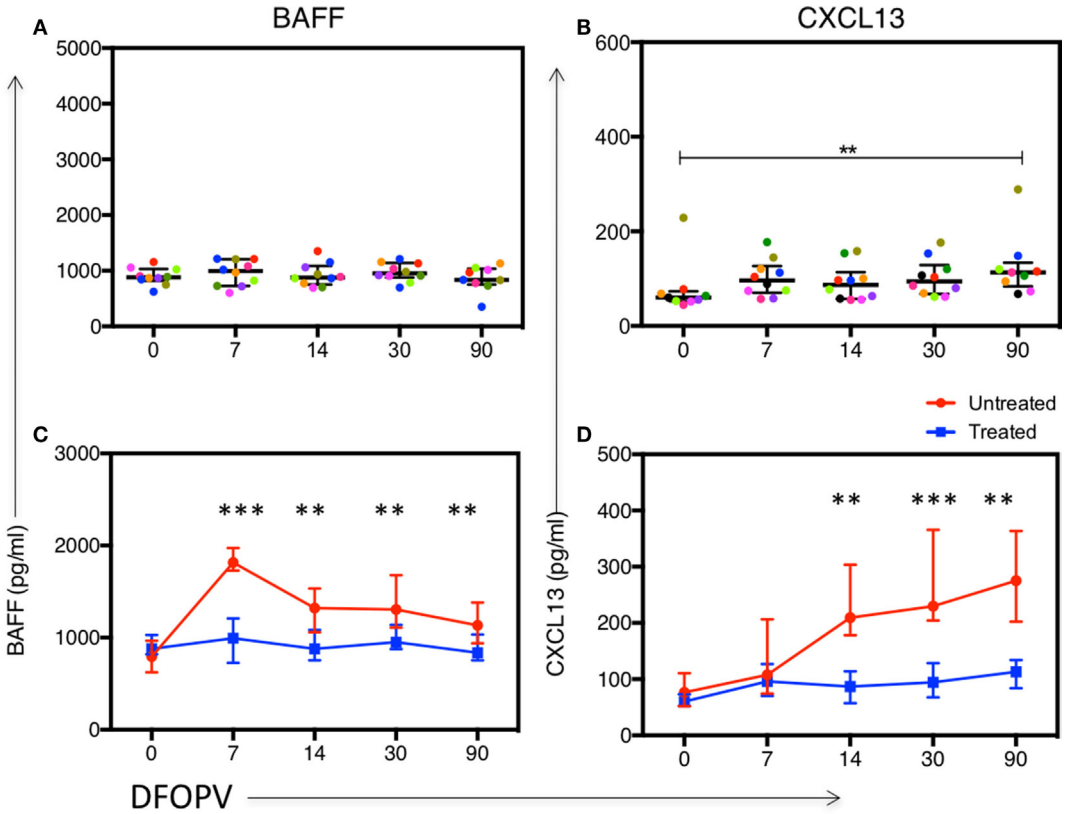

FIGURE 6 | Early combination antiretroviral therapy (cART) blocks or reduces changes in plasma levels of BAFF and CXCL13. Panels **(A,B)** show kinetics of BAFF and CXCL13 levels, respectively, determined by ELISA using plasma samples obtained longitudinally from 10 women who initiated cART during Fiebig stages I–V. Panels **(C,D)** show a comparison between kinetics of BAFF and CXCL13, respectively, between untreated and early treated women. Lines represent median values. DFOPV, days following onset of plasma viremia. *p*-Values for **(A,B)** were calculated by Wilcoxon matched signed-rank test and for **(C,D)** by Mann–Whitney test (**$p < 0.005$, ***$p < 0.0001$).

Similarly, we sought to investigate whether plasma levels of CXCL13 and BAFF were associated with the emergence of cross-neutralizing antibodies. There was no significant difference between BAFF levels in the two groups at all time-points tested (data not shown). In contrast, plasma CXCL13 levels were significantly higher in those with detectable cross-neutralization activity at all early time-points tested ($p = 0.012$, 0.010, 0.030, and 0.018 for 7, 14, 30, and 90 DFOPV, respectively) (**Figures 8A–D**). Hence, high levels of CXCL13 early in infection were associated with emergence of cross-neutralizing antibodies within 1 year PI.

DISCUSSION

Development of an effective vaccine able to induce bNAbs remains a high priority for the HIV field but how these responses evolve in natural infection remains unclear. It has previously been reported that interactions between B cells and transmitted founder virus soon after infection likely shape the evolution of such antibodies (10). Thus understanding factors that influence the humoral response to HIV-1 early in natural infection could open new insights into designing an effective vaccine. We took advantage of a unique cohort in which specimens were available prior to HIV-1 infection and longitudinally during the earliest phases of infection, and determined the relationship between frequencies of B cell subsets and key B cell activating cytokines (BAFF and CXCL13) on the emergence of cross-neutralizing antibodies 1 year following infection. We show that in the absence of cART, the impact of HIV-1 infection is rapid and greatly impacts the frequencies of circulating RM, TLM, and PBs subsets, within 7 DFOPV. These frequencies rebounded although never to the baseline values by ~90 DFOPV which coincides with early stages of viral load set-point. These subset changes were associated with viral load in the regression analyses, confirming that viremia drives them. Plasma levels of BAFF and CXCL13 were also elevated in untreated people but did not show association with viral loads within that group. While BAFF levels showed a steady decrease after a peak on day 7, the levels of CXCL13 continued to rise and remained high 90 DFOPV possibly due to effects of immune activation or ongoing viral replication within lymphoid tissues. Frequencies of B cell subsets and plasma levels of BAFF did not influence emergence of cross-neutralizing antibodies. However, individuals with high plasma levels of CXCL13 early in infection were more likely to have detectable but weak cross-neutralizing antibodies at 1 year PI.

	Subtype C Tier 1	Subtype B Tier 1		Subtype C Tier 2						Subtype B Tier 2	Subtype A Tier 2		Control
PID	MW965	MN.3	SF162	ConC	ZM109	Du156	Du151	CAP255.16	CAP256.SU	6535	Q23.17	BG505	MuLV
127-33-0048-036	4967	2438	2260	106	<50	<50	81	<50	<50	75	<50	<50	<50
127-33-0108-093*	15807	8879	2369	152	<50	<50	<50	<50	<50	66	<50	<50	<50
127-33-0251-186	8507	792	862	<50	<50	70	<50	<50	<50	<50	<50	<50	<50
127-33-0450-318	12965	1329	1233	1809	77	<50	<50	<50	<50	121	<50	<50	<50
127-33-0262-198	1991	258	80	<50	<50	50	<50	<50	<50	<50	<50	<50	<50
127-33-0303-208	2368	166	407	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
127-33-0396-267	1533	443	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
127-33-0398-271	15855	1780	133	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
127-33-0097-079	6013	1341	846	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
127-33-0122-102	13690	135	400	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
127-33-0035-039	321	79	61	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
127-33-0442-309*	1050	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50

FIGURE 7 | Emergence of cross-neutralization activity in plasma obtained within 1 year of HIV-1 subtype C infection. The emergence of cross-neutralization activity in plasmas from 12 patients ~1 year post detection of plasma viremia was evaluated against viruses from different clades (C, B, and A) and tiers (1 and 2) as indicated at the top. The values shown are the reciprocal dilution of plasma at which 50% of the virus was neutralized (ID50). Cases where no cross-neutralization was detected were assigned an ID50 of <1:50. ID50s are color coded for clarity; ID50 < 1:50 (gray), 1:50 to 1:100 (blue), 1:101 to 1:200 (yellow), 1:201 to 1:1,000 (orange), and >1:1,000 (red). Individuals with detectable cross-neutralization of tier 2 viruses (5/12) are grouped together. * indicates that plasma samples tested were obtained prior to 1 year of infection. MuLV was used as the negative control. Experiments were performed at least two independent times and the mean values are reported.

CXCL13 has previously been documented to be a biomarker of the GC activity in mice, non-human primate models, vaccine recipients and HIV-1 infected people (25–27). In those studies, participants were infected with a range of subtypes but the samples tested were mainly from primary or chronic HIV-1 infection. We report a similar observation in our cohort of 12 young African women infected with HIV-1 subtype C and in hyperacute HIV-1 infection. Importantly, pre-infection samples allowed longitudinal tracking of changes following infection, clearly demonstrating that CXCL13 is induced following infection. Similar to previous reports, viral load did not have a direct influence on the CXCL13 levels in the first 60 DFOPV. However, there was a trend toward a positive correlation by 90 DFOPV, which might be an indication of a shift toward chronic infection, a period during which CXCL13 levels and viral loads correlate positively in the absence of treatment (52–55). CXCL13 plays a crucial role in the organization of B cell follicles of secondary lymphoid organs by recruiting B cells and specific T cell subsets through its receptor CXCR5 (56, 57), thus its ability to predict emergence of cross-neutralizing antibodies is not surprising.

We report a dramatic decline in frequencies of circulating RM cells that might reflect the impact of GC destruction immediately upon establishment of HIV-1 infection (5). The mechanism by which HIV-1 results in depletion of RM cells is unclear but has significant implications for maintenance of humoral immunity. Future studies need to understand whether it is active virus replication that is responsible for RM changes or a particular viral protein, and if the latter, this would suggest potential caution in the inclusion of that protein in potential immunogens to avoid unintended detrimental immunological consequences. Of note, all the observed B cell subset changes were successfully blocked by cART initiated during Fiebig stage I-V except for an initial spike of PBs, a possible reflection of GC events where infected CD4 Tfh cells may continue to stimulate B cells within the follicles before death (58).

Despite viral loads being a good predictor of development of cross-neutralizing antibodies, which are precursors for bNAbs (6), only about 25% of individuals displaying high viral loads develop bNAbs suggesting a role for other factors. The rate of depletion of CD4 T cells has also been reported to predict the development of bNAbs (2). In our study, neither viral loads nor CD4 counts predicted the emergence of cross-neutralizing antibodies at 1 year PI. However, the independent prediction by levels of CXCL13 suggests a complex multifactorial determination of the development of cross-neutralizing antibodies. Indeed, other factors, in addition to viral loads and CD4 counts, have been reported to predict the development of cross-neutralizing activity and could have influenced the associations that we observed here. For instance, early follicular helper T cell responses, measured by the frequencies of $CXCR5^+$ CD4 T cells and which we did not assess in this study, has been show to predict of the development of neutralization breadth (25, 59). In addition, the development of bNAbs has been associated with reduced control of autoreactivity (60). Importantly, the observations reported here could be limited due to the small numbers of patients available which might preclude our ability to detect associations. Furthermore, we probed for cross-neutralizing antibodies within 1 year of infection, which is very early in the development of cross-neutralizing antibodies, and certainly before the development of any bNAbs in any of the study participants. These limitations could have also

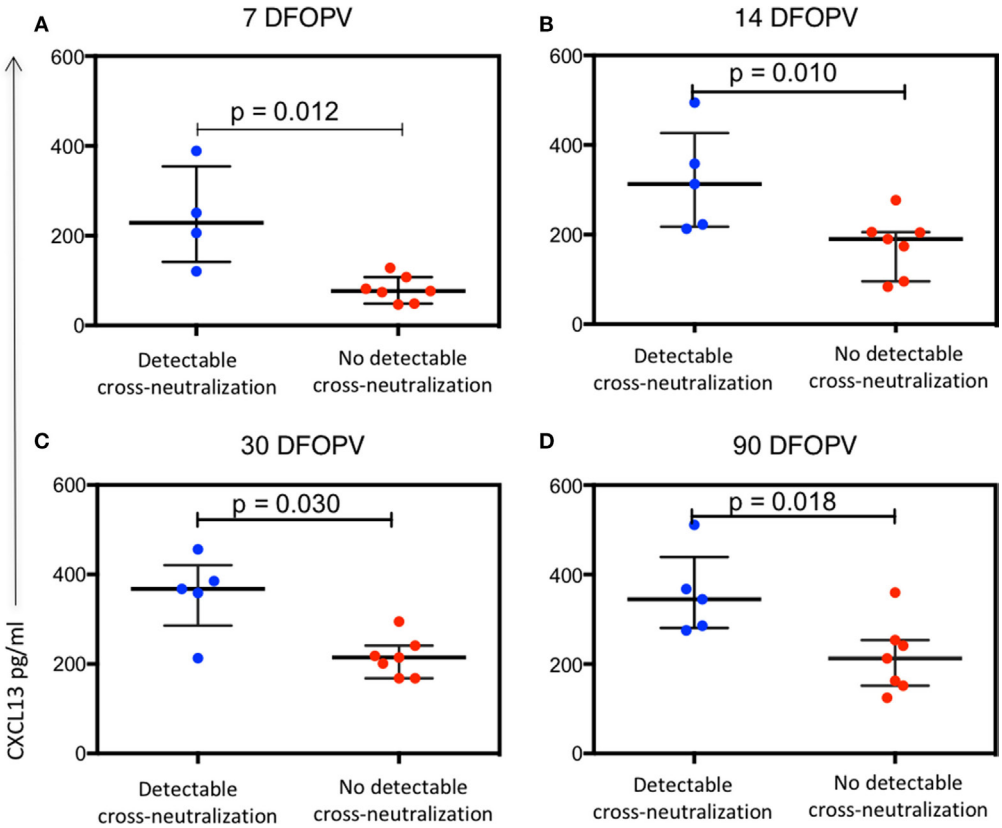

FIGURE 8 | Plasma levels of CXCL13 are associated with emergence of antibody cross-neutralization activity. Panels **(A–D)** show the difference over time in plasma CXCL13 levels between individuals with detectable cross-neutralization activity (detectable cross-neutralization) and those without (no detectable cross-neutralization). *p*-Values were calculated by Mann–Whitney test. DFOPV, days following onset of plasma viremia.

reduced our ability to detect associations between B cell subsets and the emergence of cross-neutralizing antibodies.

In conclusion, acute HIV-1 subtype C infection is associated with rapid changes in B cell subsets that do not predict the emergence of cross-neutralizing antibodies within the first year of infection. Instead, our data showing an association between CXCL13 levels in acute infection and emergence of cross-neutralizing antibodies adds to growing evidence suggesting that plasma CXCL13 might be a surrogate for a functional GC compartment and serve as a biomarker to evaluate candidate vaccines for their ability to stimulate a rapid and robust GC reaction.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Biomedical Research Ethics Committee of the University of KwaZulu-Natal and the Institutional Review Board of Massachusetts General Hospital with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal and the Institutional Review Board of Massachusetts General Hospital.

AUTHOR CONTRIBUTIONS

JM, A-SD, ZE, LM, BW, GA, and TN conceived the study. JM, YR, NI, AM, and KD participated in the acquisition of the data. JM, DM, and TR performed data analyses. JM drafted the manuscript with assistance from all authors. All authors gave the final approval for publication.

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The Role of Maternal HIV Envelope-Specific Antibodies and Mother-to-Child Transmission Risk

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Despite the wide availability of antiretroviral therapy (ART) prophylaxis during pregnancy, >150,000 infants become infected through mother-to-child transmission (MTCT) of HIV worldwide. It is likely that additional intervention strategies, such as a maternal HIV vaccine, will be required to eliminate pediatric HIV infections. A deeper understanding of the fine specificity and function of maternal HIV envelope (Env)-specific responses that provide partial protection against MTCT will be critical to inform the design of immunologic strategies to curb the pediatric HIV epidemic. Recent studies have underlined a role of maternal HIV Env-specific neutralizing and non-neutralizing responses in reducing risk of MTCT of HIV and in prolonging survival rates in HIV-infected infants. However, critical gaps in our knowledge include (A) the specific role of maternal autologous-virus IgG-neutralizing responses in driving the selection of infant transmitted founder (T/F) viruses and (B) Env mechanisms of escape from maternal autologous virus-neutralizing antibodies (NAbs). A more refined understanding of the fine specificities of maternal autologous virus NAbs and ways that maternal circulating viruses escape from these antibodies will be crucial to inform maternal vaccination strategies that can block MTCT to help achieve an HIV-free generation.

Keywords: mother-to-child transmission, HIV, vaccines, vertical HIV transmission, neutralizing antibodies, non-neutralizing antibodies, maternal vaccines

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INTRODUCTION

According to the 2016 UNAIDS global report, >150,000 infants became infected with HIV-1 *via* mother-to-child transmission (MTCT) in 2015 (1). This is despite the great success in expanding the availability of antiretroviral therapy (ART) worldwide. Ongoing challenges for the elimination of pediatric HIV infection include the following: lack of universal HIV testing and treatment during pregnancy, late maternal presentation for clinical care, maternal HIV acquisition in late pregnancy, and lack of maternal adherence to ART therapy during breastfeeding (1, 2). Thus, it is likely that alternative strategies, such as a maternal or infant HIV vaccine, will be required to eliminate pediatric HIV infections.

Mother-to-child transmission of HIV can occur *via* three distinct routes: during pregnancy (antepartum), during labor and delivery (peripartum), and during breastfeeding (postpartum). Maternal ART has been highly successful in reducing MTCT of HIV to as low as 2% transmission risk; however, poor maternal adherence to ART therapy, ART-associated toxicity in infants, and limited ART availability in resource-limited areas remain outstanding challenges in preventing

MTCT of HIV (2). Interestingly, in the absence of maternal ART prophylaxis during pregnancy or at delivery, only 30–40% of HIV-infected mothers vertically transmit HIV to the infant, suggesting that maternal factors may provide partial protection against vertical transmission of HIV infection (2, 3). These factors could include maternal immune responses capable of mediating partial protection against MTCT of HIV. Efforts to develop immune-based strategies that can synergize with current ART prophylaxis to further reduce MTCT risk have focused on understanding the role of maternal HIV envelope (Env)-specific antibodies in mediating protection against HIV transmission. Interestingly, maternal IgG is transferred to fetus across the placenta throughout gestation and mediates protection against neonatal infections during the first few months of life (4–6). Given that maternal HIV Env-specific IgG responses are present at the time of infant infection, MTCT provides a unique setting to elucidate the role of maternal passively acquired Env-specific IgG in mediating protection against virus acquisition in infants. Understanding the determinants of how infant T/F viruses initiate infection in the presence of maternal neutralizing and non-neutralizing antibodies (NAbs) in the fetus could also shed light on mechanisms of virus superinfection in adults. Furthermore, defining viral escape mechanisms from autologous virus NAbs is not only relevant in the setting of MTCT but may also have important applications in our understanding of more general virus escape mechanisms from host immune responses.

THE ROLE OF MATERNAL NAbs IN MTCT

The role of maternal autologous virus NAbs in protecting the neonate against HIV transmission remains unclear. Although some studies reported higher levels of NAbs in serum among non-transmitting mothers (7–10), other studies have not confirmed this association (11–13). These contradictory results may be due to the small mother infant pair sample sizes, unclear timing of infant HIV-1 infection, disparate maternal and infant sample collection times, unknown route of vertical transmission, ART prophylaxis or treatment during pregnancy, delivery or postpartum, and lack of control for factors that impact MTCT, such as maternal plasma viral load and peripheral CD4+ T cell count (14, 15). Given the extensive genetic and antigenic diversity of HIV within a host, elucidating the fine specificity of maternal NAb responses against conserved vulnerable regions of the HIV Env may provide a better understanding of maternal immune correlates of protection against MTCT. The conserved vulnerable regions on the HIV Env include the following: the CD4 binding site, the variable loop regions 1 and 2 (V1V2), variable loop region 3 (V3), and the gp41 membrane-proximal external region (MPER) (16).

Our group recently identified maternal humoral correlates of protection against peripartum HIV transmission in a large cohort of $n = 248$ HIV-infected women from the Women and Infant Transmission Study (WITS), a historic North American observational cohort of HIV clade B virus-infected, ART naïve pregnant women (9). Importantly, this maternal humoral correlates of protection analysis accounted for known maternal MTCT risk factors such as maternal plasma viral load, peripheral CD4+ T cell count, infant gestational age, and delivery mode, thereby isolating the role of maternal HIV Env-specific IgG responses in mediating partial protection against MTCT of HIV. In the WITS cohort, maternal V3-specific IgG binding responses, tier 1 virus-neutralizing responses, and CD4 binding site-blocking responses all correlated and were independently predictive of reduced MTCT risk (9). Further analyses of the fine specificity and function of the potentially protective maternal V3-specific IgG responses revealed that both binding and neutralizing responses targeting the C-terminal region were associated with reduced MTCT risk (8). Thus, these findings provide proof of principle that maternal HIV Env-specific IgG responses targeting vulnerable epitopes on the HIV Env can afford partial protection against peripartum MTCT of clade B HIV. Furthermore, a separate study observed that Env-specific responses targeting MPER region in gp41 were associated with reduced MTCT risk (17), suggesting that multiple regions in HIV Env may be targets of potentially protective maternal humoral responses. However, the association of maternal humoral responses to defined vulnerable Env epitopes with reduced vertical virus transmission has not been consistently confirmed in other cohorts of HIV-infected women. For example, the association of maternal V3-specific IgG binding responses, tier 1 virus-neutralizing responses, and CD4 binding site-blocking responses were not predictive of reduced MTCT risk in HIV, clade C virus-infected women from the Breastfeeding and Nutrition (BAN) cohort ($n = 88$) (18). However, it should be noted that the majority of transmitting women included in the BAN humoral correlates of protection analysis transmitted *in utero* and in the setting of maternal ART treatment. Nevertheless, these findings suggest that maternal humoral correlates of protection against MTCT of HIV in ART naïve, clade B virus-infected women may not be applicable to other transmission modes (i.e., *in utero* transmission), other viral clades, and/or in the setting of maternal ART treatment. Further defining the fine specificity and function of potentially protective maternal humoral responses will provide immunologic benchmarks used to evaluate future maternal HIV vaccine modalities that may temporarily enhance virus blocking antibody responses during pregnancy. For example, in the moderately protective RV144 vaccine efficacy trial, vaccine-elicited V1V2-specific IgG responses were associated with reduced HIV transmission risk, and thus the elicitation of V1V2-specific IgG responses is currently being used as an immune benchmark in ongoing vaccine efficacy studies (19, 20). Furthermore, given that the fetus is passively immunized with maternal IgG throughout pregnancy, a deeper understanding of the role of NAbs that are present in a host at the time of infection could help inform vaccine strategies.

TRANSMITTED FOUNDER (T/F) VIRUSES THAT INFECT INFANTS AND THEIR SENSITIVITY TO MATERNAL ANTIBODIES

Similar to HIV infection in adults, HIV-infected infants become infected with one to a few HIV viruses, suggesting that a selective virus genetic bottleneck is involved in MTCT (**Figure 1A**)

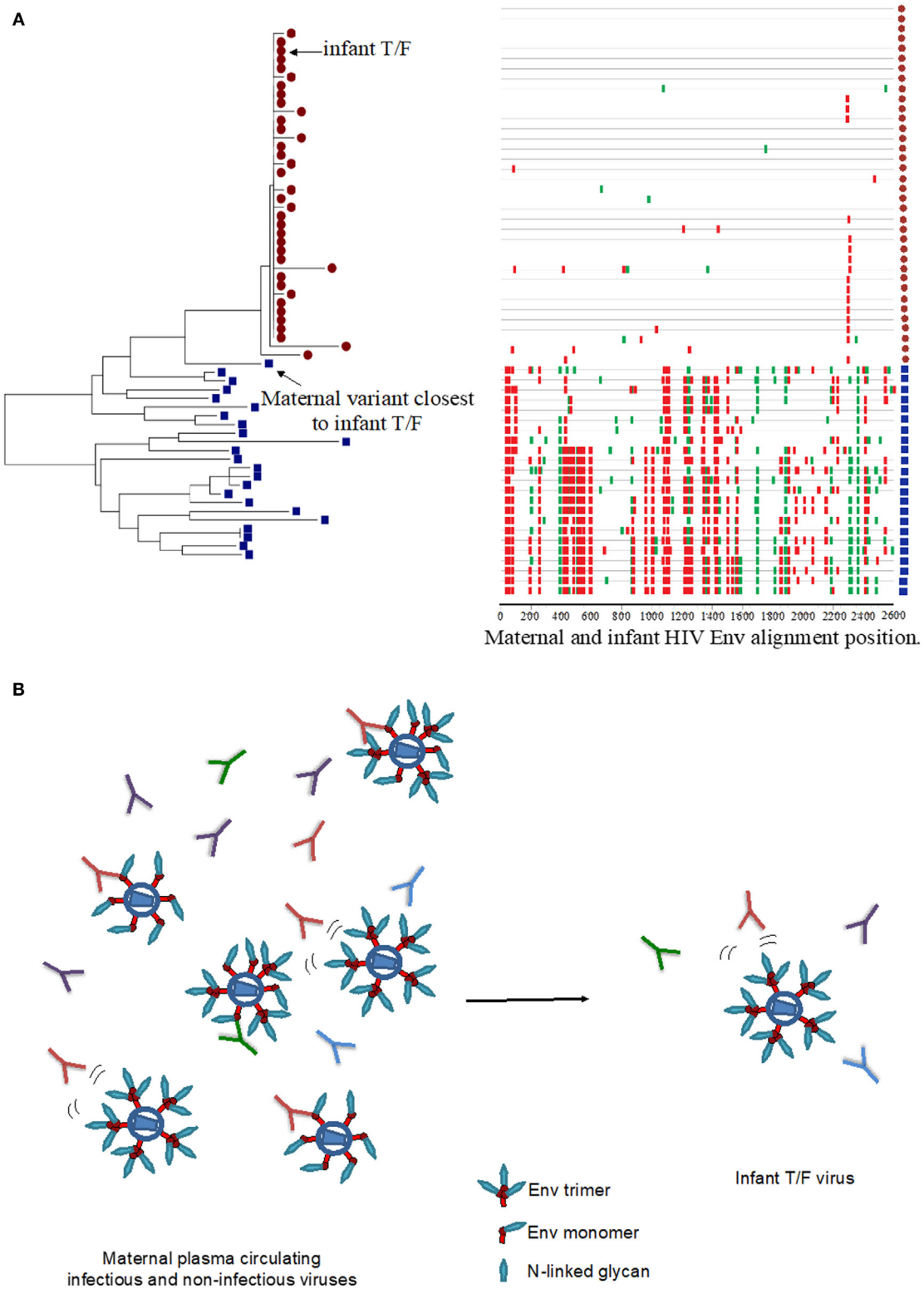

FIGURE 1 | HIV virus escape from maternal neutralizing antibodies (NAbs) in the setting of mother-to-child transmission. **(A)** Neighbor joining phylogenetic tree and highlighter plot of the full HIV envelope (Env) gene (*env*) for one mother–infant pair, showing the transmission of one T/F virus from mother to infant. The red circles represent infant *env* amplicons, and the blue squares represent maternal *env* amplicons in the highlighter plot. Red ticks represent non-silent amino acid mutations, and green ticks represent silent amino acid mutations in the HIV Env region. Neighbor-joining tree was generated using MEGA7, and the highlighter plot was generated using the Los Alamos National Laboratory HIV tools: highlighter plot. **(B)** Maternal infectious and non-infectious virus quasispecies in the presence of a wide pool maternal autologous-virus NAbs may select for infectious neutralization-resistant viruses that infect the infant.

(7, 11, 12, 14, 15). However, factors that drive this selective virus genetic bottleneck are not clear. Env-specific IgG responses can mediate immune pressure on autologous circulating viruses and therefore could contribute to the selection of infant T/F viruses (**Figure 1B**). While some studies have suggested that viruses transmitted from mother to infant may be resistant to neutralization by maternal antibodies (10, 21, 22), other studies have not confirmed these observations (13, 23). The reported increased resistance of infant T/F viruses to maternal NAbs may be explained by genetic differences compared to maternal non-transmitted viruses at key sites including Env glycan motifs. Furthermore, mutation of distal amino acid residues relative to Env neutralizing epitopes could also confer neutralization resistance to maternal autologous virus NAbs (24) (**Figure 1B**). A recent study in HIV, clade A virus-infected women examined the neutralization sensitivity of maternal autologous circulating viruses to paired plasma in 10 transmitting and 10 non-transmitting women and found no association in autologous virus-neutralizing activity and transmission risk (23). This study also reported that transmitting and non-transmitting women had a similar proportion of neutralization-resistant viruses to paired maternal plasma, suggesting that maternal autologous NAbs may not be associated with infant protection. However, to date, no study has evaluated whether neutralization resistance to paired maternal plasma NAbs is a defining feature of infant T/F viruses compared to maternal non-transmitted variants. Given that maternal autologous virus NAbs will only need to block the viruses that initiate infection in the infant (i.e., infant T/F viruses), future studies should focus on defining the susceptibility of infant T/F viruses to paired maternal plasma NAbs and monoclonal NAbs with defined epitope specificities compared to non-transmitted maternal variants in a cohort with standardized sample collection and known transmission risk factors.

In contrast to adult HIV transmission in which an HIV vaccine will need to elicit broadly NAbs against difficult-to-neutralize viruses from several clades, MTCT is a unique setting in which vaccine-elicited antibody responses need to only block the maternal virus pool to which the infant is exposed to (**Figure 1B**). Therefore, vaccination strategies aimed at eliciting broadly NAbs against multiple viral clades may be distinct from immunization strategies aimed at the inducing autologous virus NAbs against a defined and limited pool of maternal viruses. As a maternal and/or infant HIV vaccine will most likely be necessary for eliminating pediatric HIV infections, identifying the maternal NAbs that target specific vulnerable Env epitopes in selecting for neutralization-resistant viruses will be important to inform maternal vaccination strategies. Moody et al. recently demonstrated that in an HIV-infected individual, autologous-virus NAbs targeting the V3 loop and CD4 binding site neutralized a large proportion of autologous viruses isolated from plasma (25). Importantly, the autologous virus NAbs in this individual mediated the neutralization of heterologous easy-to-neutralize tier 1 virus isolates but failed to neutralize difficult-to-neutralize heterologous tier 2 virus isolates, suggesting that these seemingly inconsequential weakly NAbs can drive the selection of predominant strains that repopulate the autologous virus pool in HIV-infected individuals. This observation underlines the role of maternal Env-specific NAbs in selecting for neutralization-resistant viruses circulating in the blood. In the setting of MTCT, these maternal plasma tier 1 virus NAbs could select for neutralizing resistant viruses in the maternal blood compartment, and these viruses may be transmitted to the infant. Thus, maternal V3 and CD4bs-specific NAbs may select for maternal autologous circulating viruses that are neutralization resistant and may drive the selection of infant T/F viruses. Therefore, it will be important to define both the fine specificity and neutralizing function of maternal autologous virus NAbs.

THE ROLE OF MATERNAL NON-NEUTRALIZING HUMORAL RESPONSES AND MTCT RISK

The role of maternal non-neutralizing humoral responses in mediating partial protection in the setting of MTCT of HIV also remains unclear. A study reported that in a small cohort of ($n = 19$) HIV clade A virus-infected Kenyan women, breast milk Env-specific IgG responses with antibody-dependent cellular cytotoxicity (ADCC) activity were associated with reduced MTCT risk (26). Interestingly, these ADCC-mediating IgG responses in breast milk were found to have limited neutralizing activity, suggesting that maternal ADCC responses may be important in limiting postpartum transmission of HIV. However, it should be noted that these findings were from a small cohort of 9 transmitting and 10 non-transmitting HIV-infected women and have not been validated in a larger cohort of clade A HIV-infected women. Pollara et al. examined the role of maternal Env-specific IgG responses in breast milk and found no association of ADCC-mediating responses and decreased MTCT risk in a cohort of ($n = 87$) of HIV clade C infected breastfeeding Malawian women (27). The inability to validate the association of maternal ADCC-mediating breast milk Env-specific IgG responses and reduced MTCT risk may be due to distinct cohort sizes, potential virologic differences in clade A and clade C viruses, and differences in fine specificity and function in these distinct cohorts of HIV-infected women. Despite the seemingly contradictory findings of the role of breast milk Env-specific ADCC-mediating IgG responses and postpartum MTCT risk, maternal passively acquired ADCC-mediating IgG responses have been associated with reduced infant mortality in HIV clade A, peripartum-infected infants, suggesting that maternal passively acquired ADCC responses may prolong infant survival in pediatric HIV-infected patients (28). Together, these studies highlight the potentially protective role of maternal ADCC-mediating Env-specific IgG responses and their importance in increasing infant survival rates in HIV-infected pediatric patients.

THE TRANSPLACENTAL TRANSFER OF MATERNAL HIV ENV-SPECIFIC IGG RESPONSES AND MTCT RISK

In the setting of pregnancy, maternal IgG is passively transferred to the fetus throughout gestation, with the majority of the

transplacental transfer taking place in the third trimester (29). However, in the setting of maternal HIV infection, the transplacental transfer of maternal IgG to the fetus is poorly efficient (29–34). Despite the observed poor transplacental transfer of maternal IgG responses to the fetus in the setting of maternal HIV infection, maternal Env-specific IgG neutralizing responses may be efficiently transferred to the infant (35). However, the efficient transplacental transfer of maternal HIV Env-specific IgG neutralizing responses has not been found to be associated with decreased MTCT risk (35). As the role of maternal HIV Env-specific IgG in mediating infant protection against HIV infection remains unclear, it is not known if the poor transplacental transfer of potentially protective maternal Env-specific IgG responses leads to increased infant HIV transmission risk. However, some studies suggest that the transplacental transfer of maternal Env-specific IgG responses with antiviral functions may be important for infant protection (17). Passively acquired maternal IgG responses in HIV-exposed uninfected infants have been shown to mediate virus transcytosis inhibition *in vitro* in clade C HIV-infected mothers and their infants (17). Furthermore, the fine specificity of passively acquired maternal Env-specific IgG responses was mapped to gp41 epitopes that encompass the MPER, a key site that is commonly targeted by broadly NAbs. Thus, the transplacental transfer of maternal HIV Env-specific IgG responses with antiviral functions may be important for infant protection, as well as survival outcome upon infection (17, 28).

CONCLUSION

It is likely that additional immune-based strategies such as a safe and effective maternal and/or infant HIV-1 vaccine that can synergize with current prophylactic ART treatments will be required to eliminate pediatric HIV infections. Given the growing body of evidence on the role of maternal HIV Env-specific IgG responses and their association with reduced MTCT risk, more studies are needed to further refine the molecular details by which HIV viruses escape maternal NAbs. Larger and better controlled studies that investigate maternal NAbs with defined fine-epitope specificity and their role in preventing or reducing MTCT risk in the setting of ART may provide crucial information for the design of an effective maternal and/or infant HIV-1 vaccine to help achieve an HIV-free generation.

AUTHOR CONTRIBUTIONS

DM and SP conceived the topic. AD, DM, and SP wrote the manuscript and AD prepared the figures.

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REVIEW
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The Role of Natural Antibodies to CC Chemokine Receptor 5 in HIV Infection

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The CC chemokine receptor 5 (CCR5) is responsible for immune and inflammatory responses by mediation of chemotactic activity in leukocytes, although it is expressed on different cell types. It has been shown to act as co-receptor for the human and simian immunodeficiency viruses (HIV-1, HIV-2, and SIV). Natural reactive antibodies (Abs) recognizing first loop (ECL1) of CCR5 have been detected in several pools of immunoglobulins from healthy donors and from several cohorts of either HIV-exposed but uninfected subjects (ESN) or HIV-infected individuals who control disease progression (LTNP) as well. The reason of development of anti-CCR5 Abs in the absence of autoimmune disease is still unknown; however, the presence of these Abs specific for CCR5 or for other immune receptors and mediators probably is related to homeostasis maintenance. The majority of anti-CCR5 Abs is directed to HIV binding site (N-terminus and ECL2) of the receptor. Conversely, it is well known that ECL1 of CCR5 does not bind HIV; thus, the anti-CCR5 Abs directed to ECL1 elicit a long-lasting internalization of CCR5 but not interfere with HIV binding directly; these Abs block HIV infection in either epithelial cells or CD4+ T lymphocytes and the mechanism differs from those ones described for all other CCR5-specific ligands. The Ab-mediated CCR5 internalization allows the formation of a stable signalosome by interaction of CCR5, β-arrestin2 and ERK1 proteins. The signalosome degradation and the subsequent *de novo* proteins synthesis determine the CCR5 reappearance on the cell membrane with a very long-lasting kinetics (8 days). The use of monoclonal Abs to CCR5 with particular characteristics and mode of action may represent a novel mode to fight viral infection in either vaccinal or therapeutic strategies.

Keywords: CC chemokine receptor 5, anti-CC chemokine receptor 5 antibodies, CC chemokine receptor 5 signalosome, HIV infection, HIV protection, CC chemokine receptor 5-based vaccine, CC chemokine receptor 5-based therapy

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INTRODUCTION

The CC chemokine receptor 5 (CCR5) belongs to G protein-coupled receptors (GPCRs), which represent the largest known superfamily of signal transducers and play functional roles in the response to exposure to light and odor as well as in cellular response to different types of signaling molecules (1). They consist approximately 4% of coded human genome (2) and represent one of the most important and largest groups of targets for therapeutics (3). Among them, the chemokine receptors

are responsible for immune and inflammatory responses by mediation of chemotactic activity in leukocytes, even though they are expressed on a wide range of cell types, such as T and B cells, monocytes–macrophages, granulocytes, NK, DC, astrocytes, and neurons, and also on epithelium, endothelium, vascular smooth muscle, and fibroblasts (4–8).

CCR5 has also been implicated in hematopoiesis and it has been demonstrated that it act as co-receptor for the human and simian immunodeficiency viruses (HIV-1, HIV-2, and SIV) either independently of, or together with, the receptor CD4 (9–12). In particular, binding of viral gp120 of HIV-1 to CD4 triggers a conformational change in gp120 itself, which permits its binding to CCR5 and finally the viral entry into the cells (13, 14).

CCR5 is undoubtedly the main HIV-1 and HIV-2 co-receptor, involved in virus entry and cell-to-cell spread (15); interestingly, these R5-tropic viruses (CCR5 dependent strains) are associated with the initial infection (16), while HIV strains using the CXCR4 co-receptor are detected rarely in the early infection (11, 15, 17).

It is well known that chemokine receptor agonists, such as the β-chemokines RANTES (CCL5), MIP-1α (CCL3), and MIP-1β (CCL4), inhibit HIV infection of susceptible cells *in vitro* (18–21).

Interestingly, the number of CCR5 molecules expressed on cell surface is correlated with the levels of viral infection (13) and it has been described a variation of the level of CCR5 molecules among individuals (15), which is due to both environmental and genetic aspects. Indeed, it has been shown that high levels of CCR5, in some developing countries such as Africa, is environmentally driven and it has been hypothesized that it is due to parasitic infections (22). Whereas a CCR5-negative phenotype has been described in either some subjects, which are resistant to HIV infection (exposed to HIV but seronegative subjects, so called ESN) or in Caucasians and in other ethnic groups worldwide; the reduced or absent expression of CCR5 in these populations has been attributed to a genetic mutation, named Δ32, a deletion of 32 base-pair in CCR5 gene that produces a truncated form of the receptor, which is not expressed on the cell membrane (23). Several clinical studies underlined that homozygous mutation affecting the expression of CCR5 confers a total resistance against HIV infection (24–28); whereas heterozygotes for CCR5Δ32 are not associated with complete HIV protection (15) but progress slowly in the infection, most likely due to the reduction of CCR5 levels on the cell surface (29). CCR5Δ32 is spontaneous in 4–18% of Askenazi Jews and European people but it has not been found in Pacific and Asian indigenes (21, 24, 25, 28); this mutation occurs mostly like a heterozygous defect on CCR5 gene (10–20%), with the highest frequencies in Nordic European countries, and only less than 1% is a homozygous mutation, as reported in several study population (24, 25, 30–33). In addition, it has been shown that the frequency of CCR5Δ32 genotype is higher also among ESN and HIV-infected individuals who control disease progression without treatment (so called LTNP) compared to HIV-1 treated seropositive subjects and people from the general population (34, 35). Of note, different levels of CCR5 expression among different individuals do not affect immune functions (36), in fact its absence is not associated with medical dysfunction (37). Nevertheless the prevalence of homozygosity for CCR5Δ32 mutation, which results in the absence of CCR5 expression, has been found increased in either West Nile infected subjects or in tick-borne encephalitis (38, 39) and reviewed in Venuti et al. (21).

More interestingly, anti-CCR5 natural Abs have been discovered and they also showed HIV-blocking properties (40–43).

Overall, several numbers of strategies aimed to the prevention of CCR5 function in the HIV entry has been developed and tested.

MECHANISM OF GENERATION OF ANTI-SELF ANTIBODIES (Abs)

Natural Abs represent the first line of defense against pathogens; they are usually present in human serum as IgG3, IgM, and IgA and are generated in the absence of previous immune activation (44, 45).

The identification of B-1 cells (a subset of B cells), able to produce different self-reactive Abs, has demonstrated the capability of the immune system to interact with self-repertoire (45–47). It has been established that, in human, B cells are able to proliferate and to secrete Abs after exposure to lipopolysaccharide (LPS) from the Gram-negative bacteria membrane independent to the specific B-cell receptor (BCR) (48). Nevertheless, natural human Abs can also cross-react with microbial antigens, thus allowing host protection to pathogen independent of the previous microbial invasion (44).

B-1 cells are detected in the pleural and peritoneal cavity and represent the first line of defense, but they are present in the spleen and bone marrow as well, in which they secrete a higher proportion of circulating natural Abs (40, 45). The activation status of B-1 cells is BCR independent (49) and after their fast redistribution from the body cavities, B-1 cells are able to differentiate and to secrete abundant amounts of IgM and/or IgA (50).

The partial differentiation of B-1 cells and their ability to respond rapidly are fundamental for the Abs production to elucidate host protection to pathogens infection *via* mucosal surfaces and blood. In fact, the production of natural IgM at a steady state by B-1 cells represents a relevant protection against pathogen replication before the development of the antigen-specific response (40, 44, 45, 51, 52).

Many functions have been proposed for natural Abs such as a first line role in host defense and also a regulative part in homeostasis maintenance (40, 45, 53, 54). In addition, B-1 cells produce IgM that stimulate B-2 cells to elicit IgG (45, 55, 56), but they can also lead to induce the IgA production in response to antigen stimulation especially in the serum or in the intestinal lamina propria (40, 57).

Since the Eighties, when the AIDS was first described, several signals of autoimmune dysfunction were reported in subjects infected with HIV, such as B cell altered pathway, with production of high quantity of Abs and also of anti-cell Abs (58–60). These abnormalities, at the beginning, were related to HIV-vs-host activity but other pieces of evidence suggested that some anti-cell Abs may be considered like a host-vs-HIV reactions. Actually, it was shown that some broadly neutralizing human Abs produced during the HIV infection were autoreactive (61).

The finding led to suppose that immunotolerance mechanisms represent a disadvantage for these types of Abs (62, 63). Notably, the studies regarding the follow-up of HIV patients treated with three broadly neutralizing Abs, established that only one of them exhibited a low level of *in vivo* autoreactivity, while autoimmune-related adverse events were not detected in the study (64).

Many healthy donors displayed the presence of natural reactive Abs specific for CCR5 in several pools of immunoglobulins (41). Interestingly, different types of HIV-blocking Abs have been isolated from several cohorts of either ESN or HIV-infected individuals (40). The reason of development of anti-CCR5 Abs in the absence of autoimmune disease is still unknown; however, the presence of these Abs specific for CCR5 or for other immune receptors and mediators probably is related to homeostasis maintenance (40). Virus-induced alterations of self antigens can provide an increase of either auto-immunogenic proteins and the corresponding auto-Abs. Host factors itself, or other concomitant or latent viral infections, could activate these perturbations in the host cells, leading to conformational changes in host receptors and to remodeling from a self protein to a non-self antigenic epitope, as reviewed by Lopalco (40).

CCR5 AND ITS RELATED Abs

CCR5 shows a classic structure composed of seven transmembrane domains with N-terminus and three extracellular loops (ECL1, 2, and 3), which have immunogenic properties. The two longer domains (N-terminus and ECL2) are recruited for HIV binding (65–67). Its preferential ligands are MIP-1α, MIP-1β, and RANTES and the binding of these molecules could interfere sterically with the viral envelope protein (Env) gp120 of HIV binding resulting in an inhibition of viral infection (15). An alternative model of protection is that ligand-induced chemokine receptor internalization eliminates the co-receptor from the cell surface (68); obviously, these two mechanisms are not mutually exclusive.

Anti-CCR5 natural Abs were found also in individuals with Δ32 mutation, sexual partners of subjects who were wild type for CCR5 gene, thus suggesting that CCR5 can be considered as an alloantigen (40, 42, 69, 70). Moreover, hemophilic patients subjected to continuous blood transfusions, ESN and LTNP show Abs to CCR5 directed specifically to the first external loop (ECL1) (21, 29, 41–43, 69, 71–75); these natural Abs have been identified in serum and also in other biological fluids, such as semen, cervicovaginal secretion and saliva in subject with different genetic background (75).

The majority of anti-CCR5 Abs is directed to HIV binding site (N-terminus and ECL2) of the receptor. Conversely, Abs to ECL1–CCR5, which induce a long-lasting internalization of the receptor (29), are capable to block HIV infection in either CD4+ T lymphocytes or epithelial cells, this latter one through transcytosis, which mimics mucosal transmission (76) and this mechanism differs from that induced by all the other ligands directed to CCR5 (40). First of all, the natural Abs recognize ECL1 whereas CCR5 agonists specifically bind to the ECL2 of CCR5. Second and more important, the long-lasting internalization of CCR5 with natural anti-CCR5 Abs seems to be a unique mechanism not demonstrated for other CCR5 modulating molecules so far. Indeed, by using monoclonal antibodies (mAbs) that recognize the N-terminus and the second loop of CCR5, it has been shown a differentially modulation of receptor activity; thus suggesting that each CCR5 extramembrane region can display different properties (65, 77, 78).

A clinical study, related to the presence and the activity of Abs to ECL1 in the sera of some LTNP, clearly demonstrated that the loss of these Abs observed during the follow-up of these subjects was significantly associated with the clinical progression of the disease (29). Moreover, in another studies, a total of 206 Asian and Caucasian ESN subjects have been tested for the presence of anti-CCR5 Abs directed to ECL1 and 9% resulted positive (43, 75, 79), similar percentage (9.8%) have been found in different cohorts of HIV seropositive subjects (total subjects 336) (29, 80), although only in LTNP anti-CCR5 Abs have been associated with resistance and showed anti HIV property *in vitro* (29, 81). Strikingly, anti-CCR5–ECL1 Abs resulted HIV protective only when they were directed to a conformational epitope within ECL1 loop (43, 75). A total of 325 healthy controls have even analyzed as well but none resulted positive for anti-CCR5 Abs, thus suggesting that these Abs could be elicited by low levels of viral antigenic stimulation; that could explain why these Abs have been found in ESN and LTNP people but not in subjects who were not exposed to HIV or progressed and developed AIDS. Another hypothesis could be that anti-CCR5 Abs are elicited during other antigenic stimulations (different from HIV), which induce alterations of self-repertoire, thus eliciting anti-self responses. Finally, the priming due to endogenous retroviral proteins, which share homology with HIV env protein, could elicit in some HIV-exposed subjects a specific immune response.

Of note, these ECL1 specific Abs do not induce alteration in immune functions, as demonstrated by healthy subjects with anti-CCR5 Abs (45) or by elicited anti-CCR5 Abs in animal models such as mice and macaques (82–84) as further specified in the section of CCR5 immunization as vaccination strategy.

The ECL2 domain represents the binding site for both HIV and chemokines, so the Abs that recognize this site can prevent chemokine binding and/or signaling (66), although N-terminus is specific for viral binding only. For example, 2D7 is one of the most potent mAb directed to ECL2 that blocks HIV-1 entry into CD4 T cells, but not the transcytosis carried out with epithelial cells (66, 76, 85). An anti-CCR5 mAb named PRO140 is a humanized mAb that targets a conformational epitope between N-terminus and ECL2 and it deeply blocks viral entry (86). Another fully human IgG4 mAb with a strong activity against various HIV-1 isolates is CCR5mAb004 (87).

A recent study has demonstrated for the first time that the region designated as the membrane-proximal region (MPR), between the N-terminus and the ECL1, is important for HIV-1 infections (16). In fact, the Abs directed to this epitope block the infection of R5-tropic HIV-1 without affecting X4-tropic strain; furthermore, the substitution of MPR with the equivalent region of CCR2b, CXCR4, or CCR3 significantly abrogates viral infection (16). Both these findings provide an argument against the possible use of a target therapy with CCR5-specific Abs.

ENDOCYTOSIS AND *DE NOVO* SYNTHESIS OF CCR5 WITH NATURAL ANTI-CCR5 Abs

Ligands binding to CCR5 leads to conformational changes, which include desensitization and internalization (88). Two major mechanisms of rapid receptor regulation have been distinguished, specifically homologous (agonist-specific) and heterologous (agonist-nonspecific) desensitization, and both mechanisms are really important in fine tuning leukocyte responses (89, 90). Homologous desensitization requires phosphorylation of the receptor binding mediated by members of the GPCR kinases (GRK) family (91). This in turn leads to the association of β-arrestin1/2 with the receptor and to desensitization *via* uncoupling of the receptor and G protein (77, 92); in particular, β-arrestins bound physically with the receptors and initiate endocytosis through clathrin-coated vescicles and also act as scaffold proteins in crosstalk with other signaling pathways (93). Conversely, heterologous desensitization is traditionally defined as a state of cellular refractoriness to different agonists after receptor phosphorylation sites different from GRK mediated by second messenger-activated protein kinases, such as PKC (90).

CCR5 internalization can also induce a different second pathway, which recruits caveolae and it is independent of clathrin-coated pits. Caveolae are microdomains able to be internalized under precise conditions or in a controlled manner (13, 94).

It is well known that, after endocytosis, the GPCR proteins are also classified in receptors that are recycled, slowly or rapidly, to the cell membrane after their resensitization and those that should be degraded (77, 95–97). CCR5 is usually recycled after desensitization (4): after stimulation with natural ligands, CCR5 is internalized into the trans-Golgi network (TGN) *via* the endosome recycling compartment (ERC) (98) and, when the resensitization process is complete, it can return to the cell surface (4, 98). However, rare examples of post-endocytic sorting for GPCRs mediated by ligands have been reported (77, 99–101).

Bönsch and colleagues have recently shown that different ligands of the same GPGR are able to induce different phosphorylation pathways, which may be a relevant factor for the interaction with β-arrestins (77, 102). In addition, ligands trigger a characteristic short-term kinetics of CCR5 internalization, which transiently involves β-arrestins with consequent rapid recycling or degradation on the cell membrane; conversely, natural anti ECL1-CCR5 Abs induce a specific long-lasting kinetics of CCR5 internalization (29) with the recruitment of an ERK1-mediated pathway (70, 77). Of note, a hitherto unrecognized mechanism of CCR5 modulation mediated by G-protein-dependent ERK1 was comprehensively reported; in particular, natural anti-CCR5 Abs led to activation of ERK1 which is localized predominantly in the cytosol and it interacts directly with the CCR5 protein, thus inducing the degradation of CCR5 with a consequent *de novo* synthesis (70); the re-expression of CCR5 on the cell surface needs several days (70). This finding is actually important for the design of suitable microbicide or therapeutic tool that could inhibit HIV infection for several days after application by using a specific molecule able to induce long-lasting internalization and degradation of CCR5.

Furthermore, it is largely reported that GPCRs, considering the stability of interaction with β-arrestins after agonist stimulation, can be functionally divided into two general classes: (i) "Class A" receptors, such as β2 adrenergic receptor (β2AR), develop transient complexes with β-arrestins transiently ubiquinated and with weak activation of ERK1/2; by contrast, (ii) "Class B" receptors, such as vasopressin V2 receptor (V_2R), develop tight receptor–β-arrestins complexes, regulated by its constant ubiquitination and a durable activation of ERK1/2 which is located mainly into the endosomes. Endosomes complexes containing activated GPCRs, activated and ubiquitinated β-arrestins and phosphorylated ERK are called "signalosome" (77, 102, 103). In fact, it is well understood that the ubiquitination status of β-arrestin has a relevant role for its interaction with proteins responsible for endocytosis (e.g., clathrin) and for signaling (e.g., ERK1/2), and influences temporal and spatial dissociation of the complex (104–108). Overall, CCR5 is classified as a "Class A" receptor, but stimulation with anti-CCR5 Abs lead to the translation into a very long-lasting Class B type (77, 102, 106).

Very recently, it has been published the different ability of two RANTES analogous (5P14 and PSC) to induce the development of stable complexes between CCR5 and β-Arrestin1. Briefly, PSC-RANTES is able to induce a long-duration of recruitment of β-Arrestin1 to CCR5 compared to 5P14-RANTES, which elicits a temporary recruitment. Notably, the experiments have been carried out and the results assessed at short time only (50 min) (1). Therefore, it is possible to determine the fate of the internalized receptor by the aid of specific CCR5-ligands, suggesting that the stability of ligand-induced receptor–arrestin complexes has a crucial role in the sorting mechanism (1, 77).

In a very relevant way, these published data underline that the binding of natural Abs induces modifications in CCR5 signaling, which leads ligand-induced post-endocytic sorting in a very long-lasting Class B trafficking (77). Furthermore, in T cell, anti-CCR5 Abs that recognize ECL1 are able to induce a CCR5-negative phenotype, ERK1-mediated, by the strong support of β-arrestin2 (as shown in **Figure 1**); otherwise, it is possible that this mechanism could be specific for T cells only (77, 109).

INDUCTION OF ANTI-CCR5 Abs AS VACCINATION STRATEGY

Published data, obtained in mice and macaques, demonstrate the capability of either anti-CCR5 Abs to display HIV-blocking properties or vaccines against CCR5 to prevent the problem of virus variability and viral escape (82, 110–113). Accordingly, the development of Abs as functional inhibitors of CCR5 is the big goal that could be reached, since Abs can provide protection by causing very low toxicity (113). Several groups have investigated the possibility to use *in vivo* Abs specific to CCR5 (82, 83, 111, 112, 114–116). Interestingly, when a long-term intranasal immunization was performed, it has elicited specific IgA and IgG in both mucosal secretions and sera of the immunized mice. Such systemic and mucosal Abs induce a CCR5-negative

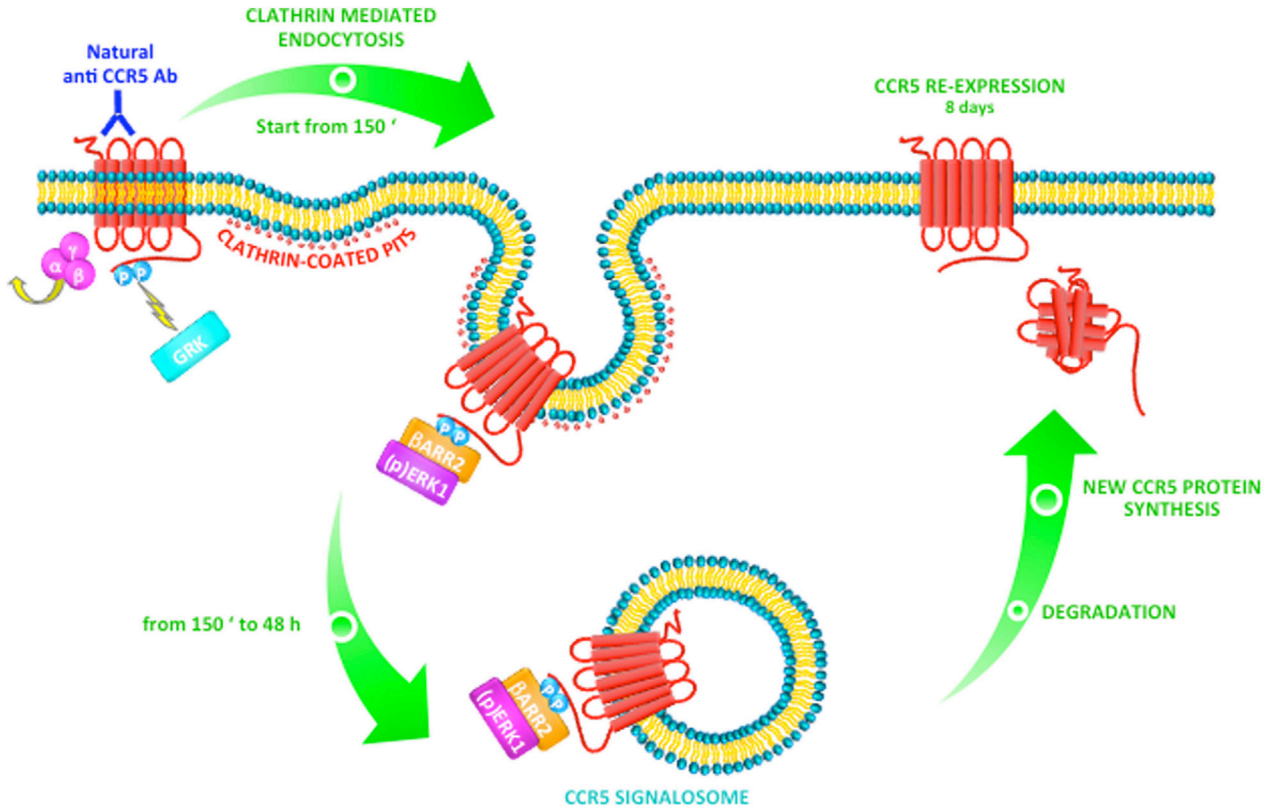

FIGURE 1 | Natural anti-CCR5 antibodies (Abs) to ECL1 triggers a Class B CCR5 trafficking pathway in T cells. After stimulation with anti-CCR5 Abs, the CCR5 receptor associates with G protein and G protein-coupled receptor kinases (GRKs) trigger receptor phosphorylation. β-arrestin1/2 can initiate desensitization (at 150 min) with consequent internalization of CCR5 by clathrin-coated pits. In particular, activated CCR5 together with β-arrestin2 is accumulated into protein complexes and induces the activation and retention in the cytoplasm of MAP kinase ERK1. These events determine the formation of a CCR5 signalosome with β-arrestin2 and ERK1 into the cytosol, which remains stable from 150 min up to 48 h. The signalosome could be targeted for degradation with consequent *de novo* synthesis of the proteins complex (CCR5, β-arrestin2, and ERK1). As a consequence, CCR5 reappears on the cell surface with long-lasting kinetics (8 days).

phenotype on both peripheral and mucosal cells, thus blocking HIV replication *in vitro* (111). In accordance with this result, the use of ECL1–CCR5 peptide, chimeric-generated in the context of the capsid protein of Flock House Virus, elicits Abs able to induce CCR5 internalization and re-expression with a very slow kinetics which needs 4 weeks after immunization to be recovered (82). Furthermore, in a subsequent study, it has been published that the substitution of amino acids within ECL1 in position 95 and 96 elicited Abs, which induced stronger long-lasting internalization of CCR5, whereas amino acid substitutions in position 92, 98 and 99 abrogated biological activity of such Abs (112), thus highlighting the importance of the epitope in driving different trafficking pathway. Moreover, in a recent study performed in mice, several aspects of anti-CCR5 immunization, including the use of all the extramembrane domains of CCR5 have been tested, to better understand the ideal schedule to reach long-lasting and strong immune responses. Interestingly, ECL1 and ECL2 showed stronger responses compared to the N-terminus; they achieved nearly complete CCR5 downregulation, and they blocked HIV infection (82). In addition, in this study was not observed any immune dysfunction in T cell responses or histopathological alterations in organs and tissues in relation to the presence or the induction of Abs specific for CCR5. The possibility of long-term toxicity and any functional impact of anti-CCR5 Abs needs additional studies; however, the findings showed in this latter study are supported by other published studies, where no adverse events were reported in CCR5-immunized macaques after 3 years of follow-up (84). In addition, it has recently published that the prophylactic immunization of macaques with virus-like particle specific for two CCR5 regions is safe and immunogenic and is capable to reduce highly virus replication in a subset of the animals (83). On the other hand, Bogers and colleagues used an immunization approach to target both virus and CCR5 (three extracellular peptides of CCR5, an N-terminal HIV gp120 fragment generated in transgenic plants and recombinant SIV p27) (117); this strategy of vaccination showed a significant block of the virus infection by eliciting good serum and vaginal quantity of Abs (117). More recently, Peabody et al. demonstrated that the immunization with recombinant vectors, which enable the CCR5–ECL2 region to recreate its native conformation, overcomes the issue of tolerance and induces the appropriate immune response (118).

Although several strategies aimed at inducing a CCR5-negative phenotype to prevent HIV-1 entry, the earlier immunization

studies in macaques observed little or no protection against SIV challenge (116, 118), probably due to poor selection of CCR5 antigen or to the correct peptide sequence in the wrong conformation. Indeed, it has previously demonstrated that immunization with ECL1 domain, in a linear conformation, does not elicit serological Abs responses that bind to the native molecule (111) and, moreover, in macaques, the immunization with ECL2 in its native conformation induces immune responses with expected properties (84). Nevertheless, Chain and colleagues have recently defined a new linear epitope of CCR5 within the N-terminus domain recognized by two independently produced mAbs; in particular, they found that RoAb13 Ab is capable to bind to both linear peptide and native form of the epitope and the sulfation of tyrosines at CCR5 N-terminus enhanced its binding to the peptide (119). RoAb13 has been previously reported to block HIV infection (120) but also blocks migration of monocytes after the chemokine binding to CCR5 or in the presence of inflammatory macrophage conditioned medium (119).

A significant challenge in the design of anti-CCR5 Abs is that they must be purely "blocking Abs" that either bind to the epitope in such a way to occlude the viral receptor or Abs binding results in receptor internalization. The most effective anti-pathogen Abs are able to engage host defense mechanisms, such as Complement or ADCC (Antibody-Dependent Cell-mediated Cytotoxicity), thus resulting protective against HIV infection (121) although these functions could result in inhibition of the effectiveness of immune responses. Moreover, as reported by Pastori et al., it is possible to elicit the production of murine serum anti-ECL1–CCR5 Abs at levels 300-fold greater than those found in humans and that the quantity of murine CCR5-specific immunoglobulins reached 50% of total Igs (82). It is noteworthy that such HIV-1 blocking Abs are present in serum and mucosal fluids from subjects with different genetic backgrounds (75), thus suggesting that it is possible to elicit these Abs in subjects coming from both developing as well as developed countries. In addition, an individual who received a stem cell transplant from a CCR5-negative donor, for acute myeloid leukemia treatment, is believed to be the only patient to have been cured of HIV (119, 122).

ANTI-CCR5 Abs IN THE IMMUNE-PROPHYLAXIS AGAINST HIV INFECTION

The Abs can prevent viral infection by several mechanisms of action: (1) can directly block virus attachment to the cell by leading the Abs to bind either virus or receptor and/or co-receptor on host cells; (2) can block fusion at cell surface at the post-binding/pre-fusion state as well (87). For reducing the development of viral escape variant, it has been highly considered to target the conserved cellular receptors, such as CCR5, for treatment of HIV infection. In particular, as HIV needs the presence of one co-receptor in dependence of the strain (CCR5 and/or CXCR4) in association with the receptor CD4, mAbs against cellular proteins have been developed and are being tested in clinical trials. A humanized mAb directed to CD4, named ibalizumab, exert an antiviral property not inhibiting the binding of gp120 but by a post-binding conformational effects, which prevents the interaction between CD4-gp120 and CXCR4 or CCR5 (123, 124). Three clinical trials have been reported, which underlined its efficacy (87). For sure, one emerging therapy is based on the use of CCR5-specific Abs; in particular, CCR5mAb004 appears safe and effective in the reduction of viral load when tested in clinical trials (87). Interestingly, another study involving the mAb PRO140 showed virologic suppression without blocking the response of the receptor to chemokines; however, the highest tolerated dose of this mAb has not been determined, proposing a substantial margin of safety for PRO140 in dependence of the site of administration (87, 125). In all these clinical trials, the use of anti-CCR5 Abs did not induce any alterations in other lymphocyte functions, thus confirming their safety.

Of note, the use of Abs instead of chemokines or classical antiretroviral therapy could reduce the complication related to drugs resistance and also the unwanted interactions with redundant CCR receptors. For example, ST6 is a Fab fragment obtained from a mAb specific for a unique sequence of N-terminus CCR5 and it was engineered in a single-chain antibody (scFv) fused with an ER retention peptide; the usage of such scFv by intracellular immunization was able to downregulate the receptor from cell membrane both in macaques and in human cells, whereas the expression of CXCR4 was not affected. Moreover, the modified cells were not infected with R5-HIV (126). In a subsequent study, it has been demonstrated that transformed primary T cells, with a CCR5 intrabody (an Ab that binds its receptor at intracellular level), were resistant to HIV infection (21). Finally, scFvs directed to CCR5 were utilized, as well, to lead viral pseudotyped lentiviral vectors to cells that express CCR5 (127).

Very recently emerged the evidence that combinations of HIV-blocking Abs will likely be more effective that single one as reviewed by Margolis (128). Alternatively, the bio-engineering, which generates Abs either with different specificities (129) or anchored to target cells (130), has given a proof of concept to generate more potent HIV-blocking Abs.

OTHER STRATEGIES AIMED AT BLOCKING HIV INFECTION THROUGH CCR5

Anti-CCR5 strategies include also the utilization of small molecule drugs, such as Maraviroc, which binds in the transmembrane regions of CCR5 and it is a functional antagonist that prevents CCR5 signaling from cell surface and even if it is currently in clinical trials (131), it has been approved for use in many jurisdictions.[1] Nevertheless, there is low enthusiasm to utilize it as front-line therapy in HIV-infected patients (23), thus it is currently in use in HIV treatment-multiexperienced patients only (132). Moreover, HIV-1 escape mutants to Maraviroc have been described and reviewed by Harada and Yoshimura (133).

Since the discovery that natural ligands of CCR5 (RANTES, MIP-1α, and MIP-1β) show anti-HIV activity (1, 19, 86, 134, 135), a large numbers of modified analogs have been tested due to their short half-lives (<10 min) (134, 136) but no one has been tested in human clinical trial due to low antiviral activity *in vivo*. The most

[1] https://aidsinfo.nih.gov/guidelines/search/1/CELSENTRI/0.

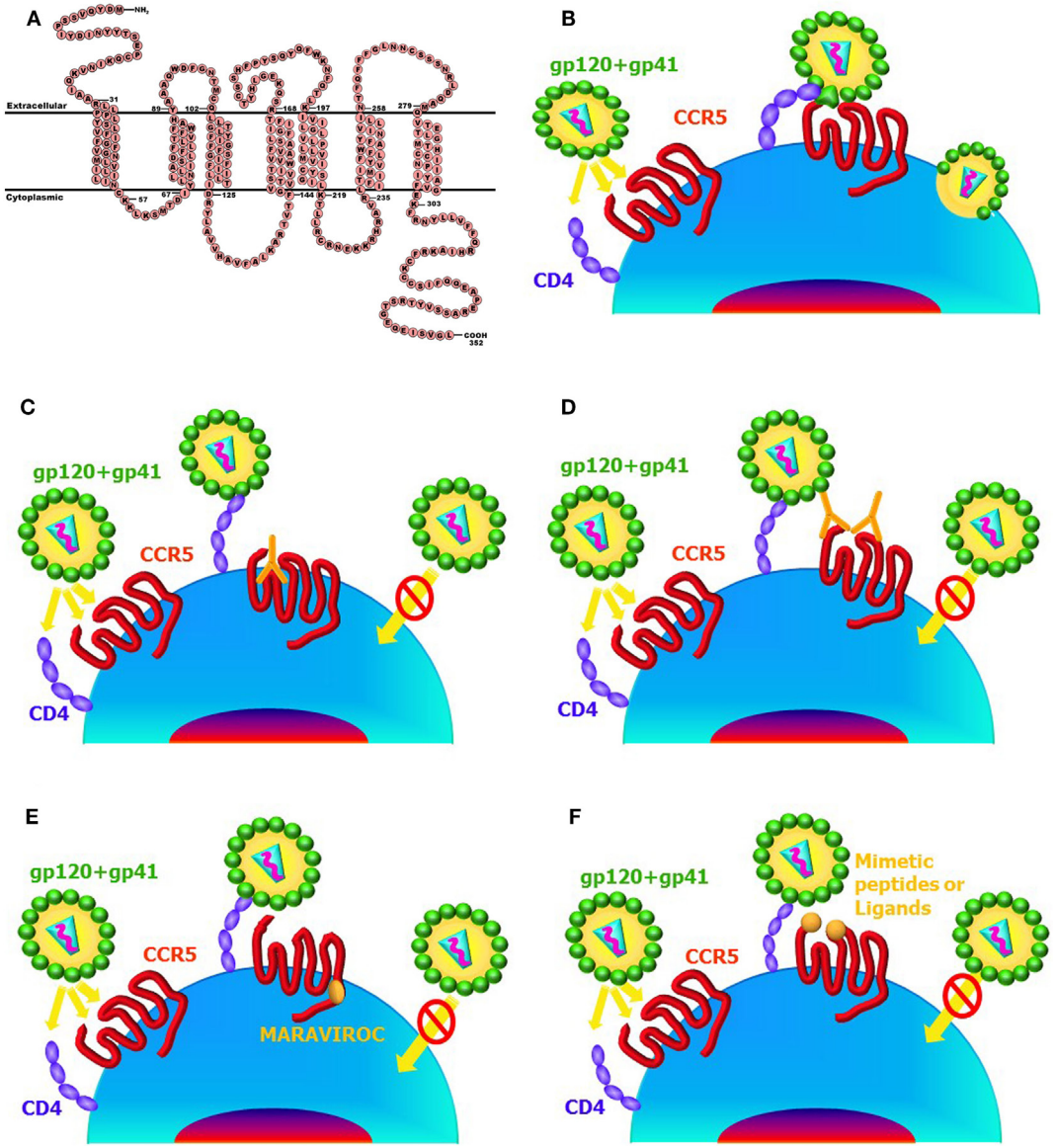

FIGURE 2 | Main anti-CCR5 strategies working at target cell surface. CCR5 protein sequence (https://en.wikipedia.org/wiki/CCR5#/media/File:CCR5_Primary_Protein_Sequence.png) **(A)**. HIV entry process **(B)**. Natural antibodies (Abs) to CCR5 bind to the ECL1 domain, induce long-lasting internalization of the receptor, and block HIV infection **(C)**. Abs to either N-terminus or ECL2 domains of CCR5 compete with HIV-binding site and interfere with HIV infection **(D)**. CCR5 allosteric modulators, such as MARAVIROC, do not allow HIV entry **(E)**. Ligands (such as RANTES or its modified analogous) bind to the ECL2 or mimetic peptides bind to the either N-terminus or ECL2 and interfere with HIV entry process **(F)**.

promising described so far was PSC-RANTES that shows several non-natural, non-coded structures in the N-terminal region (137, 138). It displays an important inhibition of HIV entry, CCR5 dependent, *in vitro* (137) and also a full protection against R5-tropic SHIV infection in a macaque vaginal challenge model (139); although this high potency *in vitro*, it requires high concentration to give protection in macaques (138, 139). Considering that it is capable to induce an intracellular sequestration of CCR5 longer than RANTES, it could be helpful for topical HIV prevention (140). Using a strategy based on phage display, Gaertner and collaborators obtained three different modified PSC-RANTES, which exhibit only natural amino acids: 6P4-RANTES, which prolongs the intracellular sequestration of CCR5; 5P12-RANTES has no detectable G protein signaling and does not bring about receptor sequestration; and 5P14-RANTES, which induces the internalization of CCR5 with no detectable G protein-linked signaling activity (138). Another relevant RANTES derivative is named AOP-RANTES and it was obtained by first generating an aldehyde-like group at the NH2-terminus of RANTES and then reacting with aminooxypentane; it is able to block R5-tropic strain infection on macrophages *in vitro* (141). AOP-RANTES induces >90% downregulation of cell membrane expression of

CCR5 on monocytes/macrophages, lymphocytes and inhibits CCR5 recycling on cell surface whereas RANTES does not (142).

As HIV entry process requires expression of both CCR5 and CD4 on cell membrane, receptor- and co-receptor-mimetic peptides (143, 144) have been proposed as an alternative strategy to block HIV entry but, as for chemokines, no one has been already tested in human clinical trial.

A summary of the immunologic approaches that use CCR5 as target to block HIV transmission/infection is showed in **Figure 2**.

Hematopoietic stem cell transplant using a CCR5Δ32 donor led to the only known cure of HIV-1 infection (122, 145) and T cells treated with engineered nucleases that introduce mutations at the CCR5 locus are resistant to HIV (146–150), accelerating ongoing efforts to develop gene editing- and cell-based therapeutic agents for HIV (15, 151, 152).

Another promising method of gene editing is the use of CRISPR/Cas9 system (Clustered Regularly interspaced palindromic repeats sequences) to target human cells for the disruption of CCR5 gene, otherwise the off-targeting is still a major limit to be overcome (153–155). Furthermore, DNA binding proteins, for example, the transcription activator-like effectors (TALEs), which are vegetal proteins, have been used *in vitro* and showed effects similar to those obtained with engineered nuclease (156).

Zinc finger nucleases (ZFNs) are other common and versatile DNA binding proteins utilized in several cell types. In addition, CCR5–ZFN-modified autologous CD4+ T lymphocytes have been used in a phase I clinical trial and this approach resulted safe (149).

To shutdown CCR5 expression, several RNA-based technologies have been used also with good results, such as RNA silencing (siRNA), antisense RNAs targeting different cellular and viral genes or ribozymes with catalytic activity (157–159); in particular, pseudotyped lentivirus and adenoviruses vectors have been used with good results for transducing siRNA-coding sequence into the cells. In the same way to that described for gene editing, off-targeting activity and over-expression of antisense RNA could cause a toxic effect (160) and could activate innate immune response as well (161).

CONCLUSION

The incidence of natural allo- or auto-responses in healthy people, without symptoms or signals of autoimmune disease, and also the capability of eliciting and maintaining strong and long-lasting HIV-blocking Abs in animal models, suggests that some autoimmune mechanisms could be positively utilized to give a better protection or a higher response to HIV in HIV-exposed individuals and in HIV-positive subjects. Allo- and auto-immune responses could allow a new key to analyze HIV tricks in immune escape and offer unexploited strategies to fight HIV with its own arms. CCR5 is the most important co-receptor in the early stages of infection, and half or more of all infected individuals move to AIDS harboring only CCR5 (R5)-tropic viruses. Epidemiology studies clearly established that CCR5 plays a crucial role in the transmission and pathogenesis of HIV *in vivo*.

As in CCR5-defective individuals were not found inflammatory and immune alterations or disfunctions, CCR5 has been defined as a redundant molecule in humans (12, 141, 162, 163), and as the variability of HIV *env*, CCR5 has become a relevant target to generate drugs and immune modulatory molecules to block HIV transmission and subsequent infection.

Overall, these findings together with the data reported for *in vivo* (clinical trials) and *in vitro* (laboratory findings) studies support the view that CCR5 could represent an excellent target to fight HIV and a good alternative to classical antiviral approaches, although it should be taken into account the concomitant geographical location of *CCR5Δ32* and other pathologies, such as West Nile infection or tick-borne encephalitis.

The development of a sterilizing vaccine capable to prevent HIV infection totally is the highest and the most expected effort, still far from being reached. Over the past 30 years, there has been a huge global effort to develop an effective prophylactic vaccine against HIV/AIDS. This is a significant challenge since no previously licensed vaccine in current use has been designed without the presence of a significant "convalescent population," i.e., patients who have been patently infected and demonstrated subsequent clearance of the pathogen. Such a patient population usually supplies critical information for characterizing adaptive immunological responses associated with "protection." One of the main reasons of failure in developing an effective AIDS vaccine could be the mainstream concept that the most relevant information derive from studying the immune responses in patients who have not cleared the virus. Thus, the design of a CCR5-based vaccine, which takes advantage of data generated in a small but significant clinical cohorts of individuals such as ESN or LTNP could represent an excellent target to generate new vaccination strategy, as these subjects represent a sort of vaccinated/cured subjects and this protective status can be induced and reproduced in all subject. It is relevant underline that natural anti-CCR5 Abs reproduce a protective status similar to that one observed for Δ32 mutation, although an approach based on CCR5 vaccine in individuals who can contract HIV infection may be a more possible and safe goal compared to gene therapy, taking into account the HIV epidemiology and the trouble of implementing CCR5 gene therapy in people living in developing countries.

Nowadays, there are many antiviral drugs used in therapy but the most related problem is the development of drug-resistant strain of virus that invalidates the positive effects obtained with the therapy utilized. Conversely, the possibility of using monoclonal Abs as therapy, with particular characteristics and mode of action, may represent a novel mode to fight viral infection disease. Overall, Abs show low toxicity together with high specificity and versatility.

It is well known that the first effective treatment of infectious disease was the "serum therapy" (administration of hyperimmune sera from immunized animals or human donors) and only after the discovery of antibiotic therapy in association with the development in vaccine design, this treatment was abandoned for mostly of infections (87, 164, 165).

The possibility of usage of Abs in clinical practice was opened from the opportunity of generate and manipulate Abs with different specific epitope recognition, such as the mAbs (87). In fact, in the last years, mAbs have begun a new class

of clinical drug utilized in inflammatory diseases, immunology, and oncology; only their development for infection treatment is going slowly.

Strategies aimed to prevent infection, such as usage of condoms, represent another effective line of defense to fight the HIV epidemic. However, social and ethnic "barriers" impede effective protection of many people. Therapeutic Abs to CCR5 could offer an alternative for primary prevention of HIV and their availability would greatly empower women/men to protect themselves and their partners. Indeed, Abs formulated as a topical product could control the disease without affecting social and procreation aspects. In addition, proceeding directly at the HIV transmission level, the passive immunotherapy approach will help to prevent and reduce both further infection and disease incidence, respectively.

Other strategies involve ART (Anti Retroviral Therapy), which is a strong treatment program utilized to suppress HIV viral replication and the progression of HIV disease. The typical regimen combines three or more different drugs, such as nucleosidic or non-nucleosidic inhibitors of reverse transcriptase, protease, and integrase inhibitors. ART is the only current available treatment for HIV patients and it is being used in many developing countries with the help of WHO.[2] Nevertheless, it has limitations in terms of high cost, intolerance, bad compliance, and insurgence of resistance (166, 167).

For this reason, a new strategy has emerged to identify blocking Abs against the HIV receptors or co-receptors, either as active-immunizations such as a vaccine or passive-immunizations such as the use of CCR5-based immuno-prophylaxis.

Interestingly, natural human Abs that recognize the ECL1 of the receptor induce a long-lasting internalization of CCR5 by triggering the recruitment of β-arrestin2; this event induces the accumulation of the two proteins (CCR5 and β-arrestin2) into the cytoplasm and leads to the activation of ERK1, which is retained into the cytosol as well. This stable CCR5 signalosome persists into the cells at least 48 h; after that, it may be targeted for degradation with consequent *de novo* synthesis of the proteins complex and, consequently, CCR5 reappears on the cell membrane with long-lasting kinetics (8 days) (70, 77). This particular mechanism could be used for designing molecules that work synergistically for stable maintenance of the signalosome into the cells and for driving the complex to degradation; thus permits to reach a long-lasting CCR5 disappearance from cell membrane which could inhibit HIV infection for a long time.

These findings may support the discovery of innovative therapeutic tools where CCR5 is an important player for microbial control and/or elimination (168) and as well as for the regulation T cell function in autoimmune diseases, such as rheumatoid arthritis, type 1 diabetes, multiple sclerosis (169), and in tumorigenesis (170, 171).

AUTHOR CONTRIBUTIONS

AV wrote the review, CP performed the figures and revised the whole manuscript. LL wrote the review and supervised the figures and the whole text.

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Env-Specific Antibodies in Chronic Infection versus in Vaccination

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Antibodies are central in vaccine-mediated protection. For HIV-1, a pathogen that displays extreme antigenic variability, B cell responses against conserved determinants of the envelope glycoproteins (Env) are likely required to achieve broadly protective vaccine-induced responses. To understand antibodies in chronic infection, where broad serum neutralizing activity is observed in a subset of individuals, monoclonal antibodies mediating this activity have been isolated. Studies of their maturation pathways reveal that years of co-evolution between the virus and the adaptive immune response are required for such responses to arise. Furthermore, they do so in subjects who display alterations of their B cell subsets caused by the chronic infection, conditions that are distinctly different from those in healthy hosts. So far, broadly neutralizing antibody responses were not induced by vaccination in primates or small animals with natural B cell repertoires. An increased focus on the development vaccine-induced responses in healthy subjects is therefore needed to delineate how the immune system recognizes different forms of HIV-1 Env and to optimize approaches to stimulate antibody responses against relevant neutralizing antibody epitopes. In this review, we describe aspects of Env-directed antibody responses that differ between chronic HIV-1 infection and subunit vaccination for an increased appreciation of these differences; and we highlight the need for an improved understanding of vaccine-induced B cell responses to complex glycoproteins such as Env, in healthy subjects.

Keywords: B cells, HIV-1, neutralizing antibodies, vaccine, HIV-1 Infection

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B CELL SUBSETS IN NORMAL PHYSIOLOGY

The human adaptive immune system relies on several B-lymphocyte subsets with distinct roles. Circulating B cells can be classified as antigen-inexperienced or antigen-experienced cells. Among the former are the immature, transitional B cells and the mature naive B cells. Human transitional B cells are divided into T1 ($CD10^{+}CD21^{lo}CD27^{-}$) and T2/3 ($CD10^{+}CD21^{hi}CD27^{-}$) B cells, while the mature naive B cells are defined as $CD10^{-}CD20^{hi}CD27^{-}$ cells. Transitional B cells and mature naive B cells express germline-encoded immunoglobulin (Ig) genes of the IgD and/or IgM isotypes. In contrast, memory B cells, plasmablasts, and plasma cells are antigen-experienced cells that in most cases originate from germinal center reactions. Most antigen-experienced B cells have undergone somatic hypermutation (SHM) and class switch recombination to IgG, IgA, or IgE (1), but non-switched memory B cells also exist (2). Resting memory B cells persist by self-renewal, which proliferate and differentiate into plasma cells upon antigen re-exposure. To maintain the lineage following activation, some daughter cells remain as slowly dividing memory B cells, while others become terminally differentiated antibody-secreting cells (ASCs). Whether this is a stochastic process (3) or mediated by directed asymmetric cell division (4)

remains a question of debate. Peripheral ASCs, often referred to as plasmablasts, are short-lived and distinct from the long-lived plasma cells found in bone marrow (BM) or other anatomical niches that support their survival (5, 6).

During late-stage B cell development, immature/transitional B cells exit the BM to enter the circulation where they are subjected to peripheral selection. This is at least in part regulated by B cell-activating factor (BAFF), which is present in limited quantities, thereby setting a competitive threshold for B cell survival (7, 8). The surviving mature naive B cells migrate to secondary lymphoid organs, i.e., the spleen, lymph nodes, and mucosa-associated lymphoid tissue. Upon antigen encounter, extrafollicular plasma cell responses resulting in the production of antibodies that have not undergone SHM may occur. However, most B cell responses against protein antigens are T cell dependent and products of germinal center reactions. Here, antigen-specific B cells undergo hypermutation of the encoded antibody sequences to diversify the antigen-specific repertoire and the resulting B cells interact closely with follicular dendritic cells and follicular helper T (Tfh) cells for selection of high affinity B cell clones. The signals that dictate B cell differentiation into memory B cells or plasma cells in the germinal center reaction are only beginning to be understood (9), including the important roles of Tfh cells (10–12). These processes are of high relevance for vaccine research as both memory B cells and plasma cells are needed for sustained humoral immunity.

B CELL DYSFUNCTION IN HIV-1-INFECTED INDIVIDUALS

During chronic HIV-1 infection, several imbalances in B cell subsets develop (**Figure 1**), affecting the capacity of chronically infected individuals to respond to vaccination and handle co-infections (13–17). Hypergammaglobulinemia and loss of B cell memory are hallmarks of these humoral immunity alterations (18, 19). Dysregulation of B cells is apparent relatively early after HIV-1 infection and worsens during disease progression. Early introduction of antiretroviral therapy to dampen active viremia has positive effects on preserving B cell subsets (20). Dysregulated B cell subsets and functions are also observed in individuals repeatedly exposed to malaria (19). Thus, B cell alterations in both HIV-1- and malaria-infected subjects are likely consequences of prolonged inflammatory responses that occur under these conditions, rather than caused by direct pathogen–B cell interactions. The specific B cell alterations described in chronically HIV-1-infected individuals include effects on both antigen-inexperienced cells and antigen-experienced cells as discussed below.

Antigen-Inexperienced Cells

HIV-1-infected individuals display increased frequencies of circulating immature transitional B cells (21). As transitional B cells display increased sensitivity to spontaneous apoptosis, this may lead to a decreased pool of mature naive B cells (22, 23). Altered migratory capacity of immature transitional B cells was also observed, which could affect the distribution of these cells between blood and secondary lymphoid organs in HIV-1-infected individuals (24). Furthermore, as mentioned earlier, peripheral B cell selection is regulated by BAFF, a B cell growth factor shown to be elevated in both chronic infection and autoimmunity (25, 26). BAFF is regulated by type I interferons (27); thus, increased BAFF levels in HIV-1 infection may result from sustained type I interferon responses due to chronic viremia. A potential consequence of increased BAFF levels is that B cell selection thresholds are altered, which may promote survival of B cells that otherwise would be subject to negative selection such as polyreactive or auto-reactive clonotypes (28, 29). Whether the naive B cell repertoire in HIV-1-infected individuals more frequently display features associated with poly- or self-reactivity is not known but will be important to investigate, especially in relation to the generation of broadly neutralizing antibodies (bNAbs) (30).

Antigen-Experienced Cells

HIV-1-infected individuals also display alterations of the memory B cell compartment. Activated human memory B cells, defined as $CD20^{+}/CD21^{lo}/CD27^{+}$, and tissue-like memory B cells, defined as $CD20^{+}/CD21^{lo}/CD27^{-}$, are increased during persistent HIV-1 infection, whereas resting memory B cells, defined as $CD20^{+}/CD21^{hi}/CD27^{+}$, are decreased in frequency (13, 31). Consequences of these B cell compartment alterations are observed already

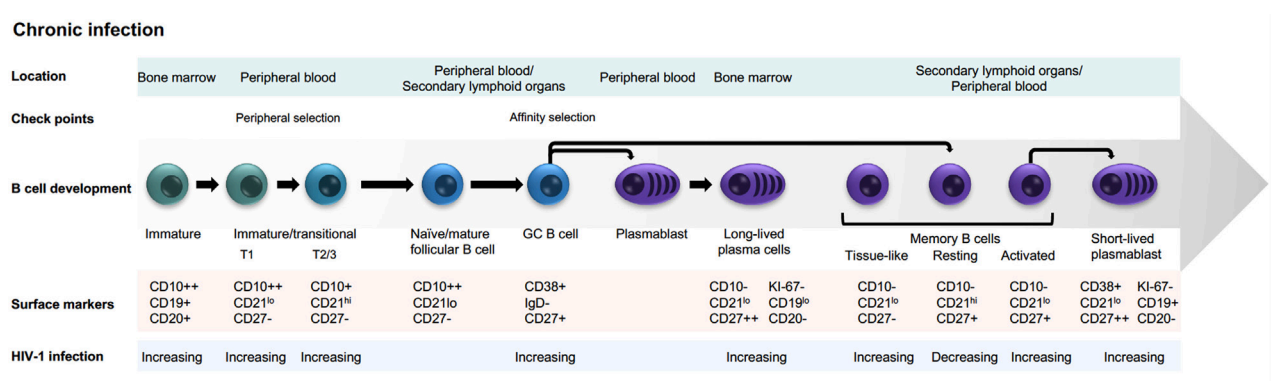

FIGURE 1 | B cell subsets and dysregulation in HIV-1 infection.

early in infection in the form of poor maintenance of serological antibody responses to previous vaccination (i.e., measles, tetanus, and pneumococcus) (17), as well as impaired responses to new vaccinations (32). During the chronic phase of the infection, exhausted B cells also appear. Exhausted B cells are characterized by a decreased capacity to proliferate in response to stimulation (33). The exhausted memory B cell phenotype is reminiscent of that of exhausted T cells with expression of molecules that negatively regulate antigen receptor signaling or homing to sites of inflammation (34, 35). Furthermore, HIV-1-infected individuals display increased frequencies of circulating $CD20^{-/lo}/CD27^{hi}/CD38^{hi}$ plasmablasts (36) consistent with non-antigen-specific differentiation of memory B cells into ASCs resulting in hypergammaglobulinemia and decreased numbers of resting memory B cells. Thus, the immune system in chronically HIV-1-infected individuals is different from that of healthy subjects in several ways, which likely affects the kinds of antibodies that are elicited. Below, we compare and contrast what is known about the induction of neutralizing antibody responses in chronic infection versus in immunization for an improved appreciation of these differences.

THE Env TRIMER AS A NEUTRALIZING ANTIBODY TARGET

The envelope glycoproteins of HIV-1 (Env) are the only virus-encoded antigens exposed on the external surface of the virus particle and thus the sole targets for neutralizing antibodies. The HIV-1 Env spike is composed of a trimer of dimers in a tightly packed infectious entry unit where the external glycoprotein gp120 is non-covalently attached to the transmembrane protein gp41 (37, 38). The native HIV-1 Env trimer complex is meta-stable and readily acquires lower energy forms that are highly immunogenic [reviewed in Ref. (39)]. Antibodies elicited by these non-native forms of Env are non-neutralizing, or only capable of neutralizing sensitive (tier 1) viruses, which are distinctly different from circulating neutralization-resistant (tier 2) virus variants (38).

The functional Env spike is exceptionally well shielded from immune recognition by N-linked glycans that cover most of the Env protein surface (40). The sites for N-linked glycosylation in the primary Env amino acid sequence vary between different virus strains and between different time points of viral evolution of a given strain demonstrating the plasticity of Env. HIV-1 evolves constantly in response to host antibody responses in each chronically infected individual, and neutralization-sensitive viruses are readily eliminated *in vivo* leaving only resistant variants in the circulating pool (41). An interesting recent study demonstrated that currently circulating HIV-1 variants are more neutralization resistant than variants isolated from the beginning of the epidemic, in part due to the acquisition of a denser Env glycan shield over time (42). The intrinsic neutralization resistance of HIV-1 is a major challenge for vaccine development where the goal is to induce antibodies capable of neutralizing a broad range of tier 2 isolates to curb HIV-1 transmissions worldwide.

Neutralizing Antibodies Elicited by Chronic Infection

Env-specific antibodies generated during the first months of HIV-1 infection are non-neutralizing or strain-specific neutralizing. Non-neutralizing antibodies are elicited by highly immunogenic non-functional forms of Env as mentioned earlier. Strain-specific antibodies neutralize the autologous virus that elicited them but not contemporary viruses that arose subsequently as a result of immune escape from the first wave of antibodies (41). About 2–4 years after the acute of infection, approximately 20% of infected individuals develop cross-neutralizing antibodies (**Figure 2**) and 1–2% of infected individuals develop bNAbs, which exhibit exceptionally potent neutralizing capacity against a large proportion of virus isolates (43, 44). Isolation and mapping of bNAbs at the monoclonal antibody level allows definition of their target epitopes, revealing sites of vulnerability on the virus that can be targeted by epitope-focused vaccine approaches (45–52).

Since chronic HIV-1 infection is characterized by an arms race between viral evolution and the adaptive immune response, new epitopes are continuously generated, sequentially driving the B cell repertoire toward the generation of bNAbs (53–55). The extensive antigenic variability in Env results mainly from the error-prone HIV-1 reverse transcriptase, which generates swarms of variants in each infectious cycle from which immune escape variants are selected. Despite the high antigenic variability of HIV-1 Env, some determinants are conserved as mutations in these elements compromise viral fitness. These regions are targets for bNAbs and include the primary receptor binding site, the CD4bs, certain variable region 2 (V2) determinants in the trimer apex, the base of the V3 region, and the gp120–gp41 interface region [reviewed in Ref. (56)]. In the case of bNAbs targeting the V3 base, the surrounding N-glycans are often part of the epitope (57, 58). The glycan reactivity observed in many HIV-1-infected individuals (59) is intriguing since antibodies against N-linked glycans is essentially a response against self-structures, which is uncommon in healthy subjects. Thus, the development of such antibodies in chronic HIV-1 infection may reflect a relaxation of peripheral check-points allowing potentially self-reactive B cells to escape negative selection (60).

Several studies have shown that bNAbs possess a high degree of divergence from their corresponding germline antibody sequences, indicating extensive SHM of the antibody sequences (57, 58, 61). High SHM suggests that multiple rounds of affinity maturation and selection in germinal centers have occurred, which appears to be required to develop features associated with broad HIV-1 neutralization. High levels of SHM are not unique to bNAbs but are generally seen in HIV-1 infection (62), as well as in other chronic infections and some settings of autoimmunity (63). This suggests that extensive SHM is a consequence of prolonged antigen exposure and persistent inflammatory responses, processes that allow selection of B cells over long periods of time. However, it is likely that not all changes introduced by SHM are required for bNAb activity as shown for the bNAb VRC01, where a subset of the amino acid changes that differed between the

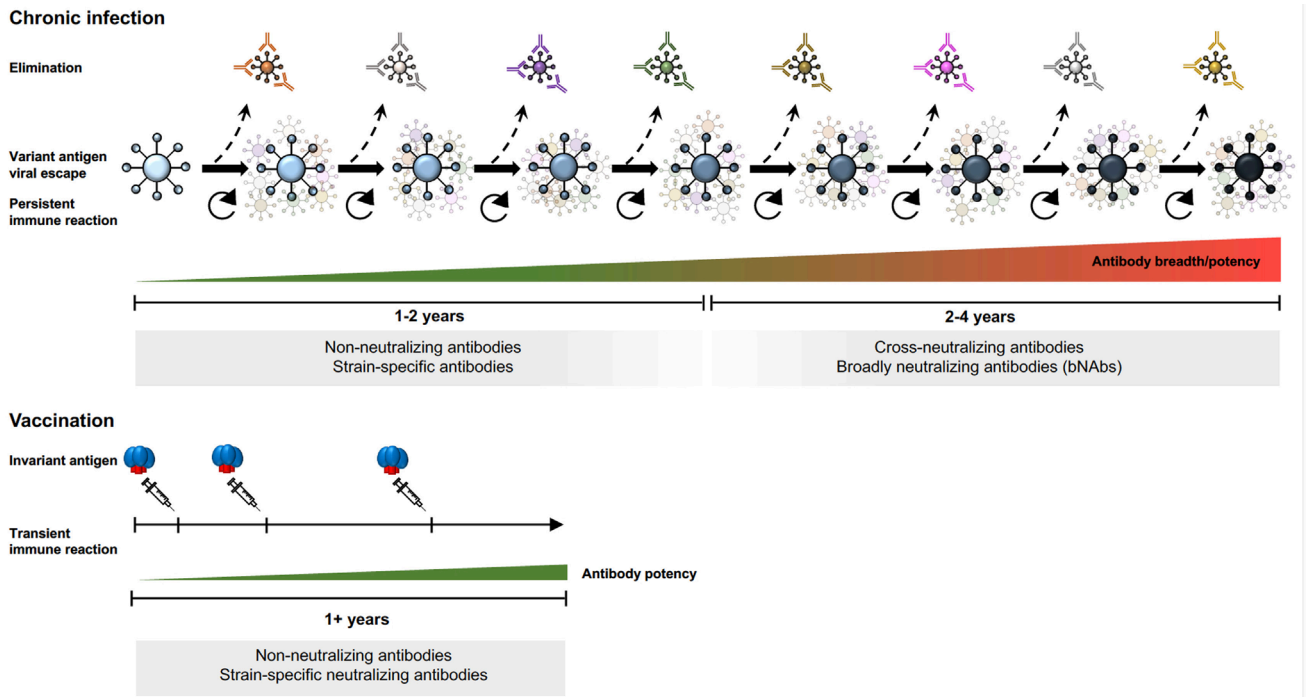

FIGURE 2 | Env-specific antibody responses in chronic HIV-1 infection and after subunit Env vaccination.

mature antibody and the assigned germline VH1-2*02 sequence were sufficient to confer bNAb activity (64).

The high degree of divergence of bNAb sequences from their germline Ig gene segments complicates the process of inferring the unmutated recombined ancestor sequences for these antibodies. Studies of germline-reverted bNAb sequences have shown that they rarely bind Env suggesting that they possess very low initial affinities to the unmutated BCR (65). However, in most cases where this was studied, the Env present in the patient at the time of elicitation of the bNAb lineage was not known. An exception to this is the identification of antibody CH103, which binds the presumed transmitted/founder Env in its germline-reverted form (55). The lack of Env binding to germline-reverted bNAbs may be explained by the fact that some human germline variable (V) alleles are missing in the current databases, which could affect the processes of germline reversion (66). In support of this, it is becoming increasingly clear that there are more human antibody V alleles than previously appreciated (67–70). An improved understanding of human antibody germline genes is therefore needed. We recently reported that next-generation sequencing (NGS) coupled with a new computational tool, IgDiscover, can accelerate the definition of germline-encoded Ig gene segments and allow higher-throughput studies (70).

HIV-1 bNAb sequences stand out not only because of high levels of divergence from their germline sequences in terms of single nucleotide differences but also because they frequently display insertions and deletions (indels) introduced during the process of SHM (71). Indels, which are rarely seen in antibodies elicited in healthy subjects, generate further diversity in infection-induced Env-specific antibody repertoires, an area that is only beginning to be understood. The present increase in NGS-based antibody repertoire analysis provides highly valuable information about how the human B cell response evolves during chronic infections. Another characteristic feature of some classes of HIV-1 bNAbs, such as the apex-targeting antibodies, is their exceptionally long heavy chain complementarity-determining region 3 sequences. B cells encoding BCRs with such long HCDRs are rare in the naive B cell population but appear to be preferentially selected in Env-specific responses, at least in a subset of individuals. This feature is likely required for the antibodies to penetrate the dense glycan shield and bind conserved determinants at the Env trimer apex (72, 73). Collectively, these genetic features demonstrate that HIV-1 antibodies are highly selected and bNAb specificities arise from extensive co-evolution processes between the virus and responding B cells.

Neutralizing Antibodies Elicited by Subunit Env Vaccination

The persistent B cell selection observed during chronic HIV-1 infection is in stark contrast to the transient response that takes place following vaccination with non-replicating subunit vaccines. Highly mutated antibodies are not induced by current immunization regimens but might be achievable by using heterologous Env immunogens administered in a sequential manner to promote responses to common determinants on HIV-1 Env. So far, bNAbs have not been elicited by immunization of primates with natural

immune repertoires. Given that bNAb development in infection depends on extensive B cell selection on a constantly changing pool of virus escape variants, it is not surprising that conventional immunization regimens do not induce bNAb specificities. It is also not known if certain precursor populations are lost during peripheral B cell selection processes, which are known to be under tighter control in healthy vaccine recipients than in chronically infected HIV-1 individuals as mentioned earlier.

Immunization studies using early generation Env trimers provided valuable information about the B cell response elicited in both small animals and in primates. While tier 1-neutralizing antibody responses are readily induced, tier 2-neutralizing responses are mostly limited to autologous tier 2 responses (**Figure 2**) (74, 75). For a detailed understanding of epitope-specific antibody responses induced by vaccination, methods for antibody specificity mapping and isolation of monoclonal antibodies are needed. Such methodologies are under continuous development to facilitate analyses of vaccine-induced responses at a higher level of resolution [reviewed in (76)]. Results from immunized non-human primates demonstrate that Env vaccine-induced responses consist of many different clonotypes, most of which appear to be modestly expanded (77–79). Highly polyclonal B cell responses are also observed in humans vaccinated with tetanus toxoid, another protein subunit-based vaccine, administered using a homologous prime-boost regimen (80, 81). It is perhaps not surprising that vaccine regimens based on homologous boosting result in polyclonal B cell responses with modest levels of SHM where each clonotype has reached an affinity ceiling to the invariant vaccine antigen (82), rather than being driven by a constantly changing antigen that repeatedly resets the affinity threshold for B cell selection, as is the case in HIV-1 infection.

Despite the many contrasts between chronic infection and vaccination, dissection of Env vaccine-induced antibody responses at the monoclonal level has also revealed similarities in terms of the targeted epitopes. For example, antibodies against non-neutralizing epitopes in gp41 as well as against tier 1-neutralizing epitopes in variable region 3 (V3) are readily elicited in both settings suggesting that these specificities are abundant in the naive B cell repertoire in both humans and commonly used animal models as shown by monoclonal antibody isolation (78, 83, 84). Similarly, CD4bs-directed antibodies capable of neutralizing tier 1 viruses, exemplified by the non-broad neutralizing antibody F105, are elicited both in infection (85) and in vaccination of non-human primates (86). The availability of protocols for efficient cloning of antibodies from non-human primates (86, 87) has facilitated such studies and are now widely used to dissect vaccine-induced responses in rhesus macaques. With the exception of one study (88), less is known about epitope-specific antibody responses in immunized rabbits where germline Ig genes so far are insufficiently characterized, currently hampering monoclonal antibody isolation in this model.

While early generation HIV-1 Env vaccine candidates were poor mimics of the functional Env spike, recent work has resulted in immunogens that better mimic the native viral spike. The definition of a native spike structure is that bNAbs epitopes are retained while non-neutralizing Ab epitopes are not. Soluble trimeric Env immunogens that meet these criteria include the BG505 SOSIP trimers and the Native Flexibly Linked (NFL trimers) (89–91) for which high-resolution structures were obtained (92–94). Emerging *in vivo* evaluation of the immunogenicity of these trimers, when used in homologous prime-boost regimens, demonstrates that they elicit autologous tier 2-neutralizing antibody responses but limited neutralization breadth (95). The epitopes mediating strain-specific neutralization may be different for different HIV-1 strains, or in different host species, as exemplified by the finding that antibodies against the V2 region mediate the autologous neutralizing activity induced by clade C 16055 trimers in NHPs (95), while antibodies against the gp120-gp41 interface mediate the autologous neutralizing activity induced by clade A BG505 trimers in rabbits (88). The role played by potential differences in host B cell repertoires in terms of the specificities induced by a given immunogen remains insufficiently understood but will be important to determine to better understand predictability of different animal models for assessment of human vaccine candidates. In this respect, it was shown the same immunogen that elicits potent autologous neutralizing antibodies in rabbits fails to do so in mice (96). Further work is required to define similarities and differences in germline antibody genes and expressed repertoires between commonly used animal models, including small animals, NHPs, and humans.

In conclusion, while much has been learnt from studying the development of bNAbs in chronic HIV-1 infection, focused efforts are now needed to translate these findings to the setting of vaccination. Given the challenge of this goal, achieving this will require coordinated vaccine evaluation trials in both well-chosen animal models and in humans.

AUTHOR CONTRIBUTIONS

MS created the figures. MS and GKH jointly wrote the manuscript.

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REVIEW
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Non-Neutralizing Antibodies Directed against HIV and Their Functions

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B cells produce a plethora of anti-HIV antibodies (Abs) but only few of them exhibit neutralizing activity. This was long considered a profound limitation for the enforcement of humoral immune responses against HIV-1 infection, especially since these neutralizing Abs (nAbs) are extremely difficult to induce. However, increasing evidence shows that additional non-neutralizing Abs play a significant role in decreasing the viral load, leading to partial and sometimes even total protection. Mechanisms suspected to participate in protection are numerous. They involve the Fc domain of Abs as well as their Fab part, and consequently the induced Ab isotype will be determinant for their functions, as well as the quantity and quality of the Fc-receptors (FcRs) expressed on immune cells. Fc-mediated inhibitory functions, such as Ab-dependent cellular cytotoxicity, antibody-dependent cellular phagocytosis, aggregation, and even immune activation have been proposed. However, as for nAbs, the non-neutralizing activities are limited to a subset of anti-HIV Abs. An improved in-depth characterization of the Abs displaying these functional responses is required for the development of new vaccination strategies, which aim to selectively trigger the B cells able to induce the right functional Ab combinations both at the right place and at the right time. This review summarizes our current knowledge on non-neutralizing functional inhibitory Abs and discusses the potential benefit of inducing them *via* vaccination. We also provide new insight into the roles of the FcγR-mediated Ab therapeutics in clinical trials for HIV diseases.

Keywords: HIV-1 infection, non-neutralizing antibodies, antibody functions, antibody-dependent cellular cytotoxicity, Fc-receptor-mediated inhibition

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INTRODUCTION

A strong antibody (Ab) response is mounted following HIV infection but most Abs targeting the HIV have little neutralizing capacity. Upon humoral immune activation *via* infection, B cells undergo somatic hypermutations and isotype switching of the immunoglobulin gene in order to enhance the efficacy of the Ab response against the specific antigen (1). B cells can then differentiate into long-lived plasma cells (2). However, most of the B cells induced are directed against decoyed immune-dominant epitopes that have no or low antiviral function. The targeted epitopes are either useless for antiviral activity (directed against unfolded glycoprotein that are not present on infectious viruses) or against epitopes able to efficiently and quickly mutate to escape from the immune response. Only 10–20% of infected individuals are able to mount a B-cell response leading to the production of broadly neutralizing Abs (bnAbs). These bnAbs represent, therefore, only a minor

amount of the humoral Ab response induced following HIV infection. They have specific characteristics: they are produced from B cells that undergo unusually long maturation steps with extraordinary levels of somatic mutations compared to germline and display long heavy chain complementarity-determining regions 3 to be able to bind masked epitopes. This allows the development of Abs that target specific antigens with high affinity (2).

In addition to germline mutation, the consecutive immunoglobulin class switching will change the Ab isotype (3). This Ab isotype switch is also determinant for its gain of function. The heavy chain constant region determining the Ab isotype will not only impact the neutralization capacity (*via* the Fab domain) but also play a crucial role on the Ab effector functions (*via* the Fc domain). In fact, the heavy chains define the Fc domain that will directly modulate the Fc-mediated inhibitory functions. These functions will greatly influence the further immune response. Interestingly, Fc-mediated inhibitory function was detected not only on neutralizing Abs (nAbs) but also on some specific Abs lacking neutralizing activity, therefore, called non-neutralizing inhibitory Abs (4) [reviewed in Ref. (5–11)].

In vivo, the Fc-mediated functions are now being addressed. It is well documented that the Fc-mediated effector functions contribute to Ab-mediated protection against HIV-1 for bnAbs (5–8, 12, 13). Two recent studies have tracked virus replication after early experimental mucosal infection and passive protective bnAb therapy (14, 15). Liu et al. showed that, in animals pretreated with bnAb PGT121 1 day before challenge with high-dose mucosal SHIV, early viral foci are detected at the distal site of infection before complete virus clearance (14). These results showed that bnAbs are able to eliminate the infected cells if some virus escapes from the neutralization of infection. Moreover, Hessell et al. found that early short-term post-exposure treatment with a cocktail of bnAbs VRC07-523 and PGT121 in newborn macaques, 1 day after oral $SHIV_{SF162P3}$ challenge can intercept replicating viral foci established by day 1 (15). This study demonstrates that passive immunotherapy by Ab can eliminate viral foci and thereby prevent the establishment of viral reservoirs (14, 15). These two studies exploring early virus replication in the presence of nAbs clearly demonstrate that HIV escaping from neutralization can infect cells at a distal site of virus inoculation and be subsequently eliminated by bnAbs. They reveal that the complete lack of infection is not mandatory to obtain protection by nAbs. The discovery that nAbs can eliminate a few foci of infected cells is extremely useful for the vaccine field as this type of activity cannot be referred to as neutralization. The mechanism by which Abs ensure clearance of infected cells is not known but obviously these additional observations reinforce the potential role of Fc-mediated functions in the protective mechanisms of bnAbs. These results open a complete new area of research for the development of protective Ab responses. Additional experiments are now required to define the mechanism of infected cell clearance. In particular, increased analysis of the Ab protection at very early time points following challenge will help to identify the multiple inhibitory functions displayed by bnAbs.

The role of Fc-mediated functions of Abs lacking the broadly neutralizing capacity in HIV protection is still a matter of debate. Importantly, specific Fc-mediated functions of non-neutralizing Abs (non-nAbs) are the only correlates of protection against infection observed in the RV144 vaccine trial conducted in Thailand (16–19). Still, how non-nAbs have contributed to protection remains unclear. In the non-human macaque model, the non-nAbs have shown some trends of decreased viral load or decreased number of transmitted founder viruses (20, 21). The exact mechanisms leading to this lower infection rate is not known, but again indicated that non-nAbs may participate in protection. Active immunization with HIV-1 vaccine candidates suggests that weakly neutralizing or non-nAbs protect by using Fc-mediated effector functions, albeit with a much lower dynamic range as for passive immunization with bnAbs (22). New tools, such as knockout mice or Abs engineered to abrogate or enhance certain functions, were recently developed. These technologies recently paved way for the demonstration of the role of Fc-mediated functions (23). Treatment with a non-nAb directed against the principal immunodominant domain of gp41 allowed for the selection of a recurring HIV mutation within the CD4 binding site in a totally Fc-dependent manner (23). These data are consistent with the hypothesis that a high titer polyclonal anti-envelope (env) non-nAb response may be sufficient to reach low levels of protection against HIV. Future directions need to more precisely characterize the functions and Ab characteristics needed to achieve such protection.

The identification of these additional non-neutralizing inhibitory Abs opens a whole new area of research. Functions involving the Fc domain of Abs can occur simultaneously, sequentially, and can sometimes be conflicting with other Ab functions. They were shown to contribute to the overall protective effect of Abs and to an efficient humoral immune response (5, 8–10, 12, 13, 20, 21, 23–25). This review will discuss the opportunity, difficulties, limitations, and parameters influencing these Fc-mediated Ab functions.

FUNCTIONAL ACTIVITIES OF Abs CAPTURING INFECTIOUS HIV PARTICLES

HIV-specific Abs are directed against numerous epitopes of the HIV glycoprotein, but only few are accessible as a quaternary structure of the functional trimeric envelope. Among them, five hotspot epitopes were shown to be involved in HIV neutralization (26, 27). Even so, Abs to additional epitopes were shown to bind to infectious viruses either by targeting additional epitopes on the trimeric env or non-functional env spikes expressed on HIV particles. These additional Abs, although not neutralizing, are able to bind and capture infectious virus, form immune complexes and/or virus/Ab aggregates, therefore leading to additional inhibitory functions.

HIV INHIBITION BY AGGREGATION

Formation of virus aggregates is a very basic mechanism of inhibition leading to the decrease of virus infectivity (28–32). The aggregates are formed by a network of Ab/virus interactions, where the virus is trapped. This leads to virus inactivation by

limiting the distribution and accessibility of available pathogens, decreasing their motility or disrupting their integrity. This mechanism applies to Abs binding to numerous epitopes exposed at the surface of the virus particle. Aggregation more likely occurs with polymeric IgA that are able to dimerize *via* their Fc domain and IgM displaying pentameric forms. Indeed, inhibition by aggregation was proposed for the exceptional protective effect observed with IgA1 (33). In this study, a correlation was observed between the binding capacity of the anti-HIV IgA1 subclass Abs and the protective effect on rectal experimental challenge (33). For IgG, aggregation occurs by the recognition of two distinct epitopes/virions entities. This activity, therefore, usually has a dome-shaped relationship to the Ab concentration, declining at higher occupancies when it becomes improbable that a free paratope of an Ab molecule already bound to one virion can find a free epitope on a second virion. In the female reproductive tract containing cervical mucus, HIV aggregates will be trapped more efficiently as free virus particles (34). Moreover, the immune complexes formed may be retained efficiently in the mucus by their binding to MUC16 *via* the Fc domain of IgG Abs (24). In addition to this mechanic inhibition of HIV by aggregate formation, more complex mechanisms involving a further binding of the Abs to the Fc-receptor (FcR) expressed on the surface may take place.

THE ROLE OF FcRs

Fc-mediated inhibitory activity is entirely dependent on the capacity of Abs to trigger FcRs. These FcRs have to interact with the Fc domain of the Abs to trigger the Fc-mediated functions. Based on their homology, three classes of FcγRs have been described (FcγRI, II, and III). The distinct family members, including FcγRI, FcγRIIa, FcγRIIb, FcγRIIIa, and FcγRIIIb, are differentially expressed on the surface of immune cells, such as B cells, dendritic cells (DCs), NK cells, macrophages, neutrophils, eosinophils, and basophiles (35–39). They differ in their Ab affinities, favoring certain IgG subtypes depending on their amino acid sequences. This differential binding capacity, depending on the Ab isotype and the FcR genotype and its expression on the cell modulates the Ab activities and their capacity to activate or inhibit FcR-expressing cells. Therefore, the different FcR polymorphisms of the host need to be taken into consideration when analyzing FcR-mediated functions of Abs.

Single-nucleotide polymorphisms (SNPs) have been described to occur in FcγRIIa, FcγRIIIa, and FcγRIIb at protein positions 131, 158, and 232, respectively, while human FcγRI was not found to be polymorphic. Since these SNPs affect FcR expression and IgG isotype binding leading to distinct effector functions, they can influence HIV vaccine efficacy, infection risk, and disease progression. For example, specific polymorphisms at the FcγRIIa (change from H to R at position 131) and the FcγRIIIa (change from V to F at position 158) gene loci have been associated with an HIV vaccine benefit (40). Li et al. described that subjects carrying a SNP in FcγRIIc (126C>T) were associated with a significant prevention of infection with an AE HIV-1 strain in the RV144 vaccine clinical trial (41). On the contrary, a small study that compared the FcγRIIa and FcγRIIIa genotype profiles of 73 patients that were able to control HIV with progressor patients did not find any difference in genotype frequency (42). The role of the different FcR polymorphisms and how it will impact on the overall HIV immune response is not known. Therefore, future research will need to assess in more details the role of FcR polymorphisms of the host on HIV infection and HIV vaccine development.

ANTIBODY-DEPENDENT CELLULAR PHAGOCYTOSIS (ADCP)

Antibody-dependent cellular phagocytosis, which relies on phagocytes to internalize and degrade Ab-opsonized pathogens, is a well-described immune process. Abs coated to pathogens *via* their Fab domain will bind with their Fc domain to the FcR expressed on monocytes, macrophages, and neutrophils to increase rapid elimination of the microorganisms. In the case of HIV, phagocytosis of immune complexes *via* the Fc domain of the nAbs was found to be associated with protective activity in the macaque model (43–45) and, recently, phagocytosis by macrophages or activated neutrophils was proposed to play a significant role in human tissues, even though it is yet unknown how exactly this inhibition occurs (46). Interestingly, this activity was also described for non-nAbs able to form immune complexes. It was shown that for some HIV-specific Abs, the binding *via* the Fab domain, on the one hand, and the binding to an antigen-presenting cell (APC) *via* the Fc domain, on the other hand, leads to efficient inhibition of HIV replication of the APCs (4). Phagocytosis by cell lines was shown using different HIV-specific Abs and gp120-coated beads (47) and when these cell lines were engineered to express different FcRs, the FcR-mediated inhibitory function of Abs was partially recovered. This type of activity relies on multiple Abs, able to form immune complexes and especially for Abs directed to the HIV gp41 epitope (33). Although HIV inhibition by phagocytosis of the immune complex could not be demonstrated using this FcR-expressing cell line, it was proposed that immune complex binding of FcγRI provides a kinetic advantage for gp41 nAbs against partially cryptic epitopes (33). An alternative mechanism may be proposed based on the observation that virus co-localizes with Abs and FcRs at the surface of APCs for a prolonged period. In this case, HIV captured at the cell surface *via* FcRs is deviated from the infection process, which requires binding to receptor/co-receptor for fusion with the cell membrane.

IMMUNOLOGICAL Ab FUNCTION

Antigen-presenting cells are specialized cells devoted to phagocyte immune complexes *via* their FcRs. This phagocytic process is much more efficient than the direct phagocytosis of pathogen by endocytosis. This mechanism of Fc-mediated phagocytosis of immune complexes will lead to an optimized induction of the adaptive immune response by APCs. In this regard, Abs forming the immune complexes may directly participate in the induction of the adaptive immune responses required for prolonged protection. The contribution of Abs in the development of an adaptive

immune response was first described in the cancer field (48). Abs targeting tumor antigens were shown to interact with immune cells through Fc-dependent mechanisms to induce adaptive immune responses (49–51).

Increasing body of evidence suggests that this mechanism may also apply following HIV infection. Noteworthy, *in vitro*, the presence of HIV/Ab immune complexes induces the maturation of human DCs, supporting immune activation (52–54). The stimulation of the adaptive immune response was also observed following nAb therapy in infected macaques (45, 55). An increase of specific B-cell responses following passive nAb transfer in a non-human primate (NHP) model was described by Haigwoog's team (56). The immune complexes were able to activate T-cell immunity (57). More recently, human clinical data described the elicitation of host humoral responses in viremic subjects after a single injection of the potent anti-HIV nAb 3BNC117 (58). 3BNC117 immunotherapy was found to accelerate the level of neutralization breadth. Overall, these studies attribute an "immunogenic" role to Abs in that they may be able to induce primary and memory responses more efficiently than free viral particles or infected cells. Accordingly, Abs without neutralizing potency but able to form immune complexes may also lead to immune activation. Further investigations will be necessary to characterize the Abs involved in the implementation of an adaptive antiviral response, paving the way to new fields of applications.

FcR-MEDIATED INHIBITION OF CELL-TO-CELL HIV-1 TRANSMISSION

Noteworthy, APCs have been described as "Trojan horses" that, in addition to their capacity to mount an efficient immune response, will also facilitate the spread of HIV by efficient HIV transmission and dissemination to the surrounding CD4 T lymphocytes. Indeed, spread of HIV-1 infection through direct cell-to-cell HIV-1 transmission has been shown to be 100- to 1,000-fold more efficient than infection by cell-free virus, making a large and efficient contribution to HIV propagation and dissemination through the body (10, 59–61). Therefore, preventing cell-to-cell transmission of HIV-1 by specific Abs is crucial for inhibiting HIV-1 propagation. However, most *in vitro* neutralization assays and *in vivo* nAb protection experiments have been performed by using cell-free virus.

Studies analyzing the inhibition of cell-to-cell HIV-1 transmission by nAbs used diverse models of HIV-1 transmission, with different donor and target cells, various viral strains, and Ab and different readout for cell-to-cell transmission. Consequently, the results are divergent and controversial, some studied showing decreased Ab potential when HIV is directly transmitted to a target cell compared to inhibition of cell-free virus (62–72), whereas other studies showing similar inhibitory potential for cell-free versus cell-to-cell transmission (52–54, 73). Noteworthy, in comparative studies where the experimental design is normalized for the same replication capacity between cell-free or cell-associated virus and where the same primary target cells were used, identical Ab inhibitory activities were observed (52–54, 74). Under these conditions, cell-to-cell HIV-1 transmission from DCs/macrophages to CD4 T cells was inhibited to a similar extent as cell-free virus particles. Interestingly, similar results were described for antiviral compounds after normalization for virus replication and target cells (73, 75). These findings highlight the potential role of bnAb in protection from early HIV-1 transmission and rapid dissemination at mucosal frontlines if locally present early after sexual transmission.

As HIV-1 Abs can bind FcRs, Abs may inhibit HIV-1 transmission *via* FcR-mediated inhibitory activity. It was shown that non-neutralizing inhibitory Abs such as 246-D do not directly affect HIV-1 transmission from infected DCs to autologous CD4 T cells (54). Therefore, non-neutralizing inhibitory Abs were proposed to have no direct effect on HIV transmission. However, such Abs were shown to significantly reduce the percentage of infected DCs in DC-T cell co-cultures (54). For these non-neutralizing inhibitory Abs, a strong association was found between the FcγR-specific binding capacity, the inhibition of HIV-1 replication and the DC maturation. This suggests that the binding of these Abs to DCs triggers the maturation of these cells, resulting in lower levels of R5 virus replication (10, 54). Moreover, IgG-opsonized HIV-1 has been showed to impair provirus formation, p24 production and to decrease the long-term transmission rate to autologous non-stimulated CD4 T cells (76). These unconventional mechanisms of HIV inhibition detected in DCs but not in CD4 T lymphocytes may explain the lower levels of infection in the co-culture in the presence of non-nAbs. Therefore, these Fc-mediated inhibitory activities of Abs in DCs may participate in the overall diminution of HIV replication in DC–T cell HIV-1 transmission.

Altogether, the multiple Ab inhibitory activities should be taken into consideration for the study of the inhibition of cell-to-cell HIV-1 transmission. A better understanding of this FcR-mediated inhibition of HIV transmission is needed for future Ab-based therapeutics and protection strategies.

ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY (ADCC)

Antibody-dependent cellular cytotoxicity, a complex and potent Fc-mediated effector function, requires the linking of an HIV-infected target cell to an immune effector cell *via* HIV-specific Abs. In this regard, Abs have to bind to HIV env, which is expressed on the surface of infected cells, *via* their Fab part and use their Fc domain to interact with FcRs expressed on the surface of effector cells, such as NK cells. This double interaction triggers the release of cytotoxic granules containing perforin and granzymes from the effector cells, leading to the death of the Ab-bound infected target cells.

Antibody-dependent cellular cytotoxicity as well as non-neutralizing anti-V1/V2 Ab induction was shown to correlate with reduced HIV-1 infection risk in the human vaccine trial RV144 and in several NHPs studies (16, 18, 77–79). The data strongly suggest for ADCC to be a significant mechanism of protection against HIV-1 *in vivo* (7, 10, 22, 80). Interestingly, non-neutralizing anti-V2 monoclonal Abs elicited in HIV-1-infected patients recently showed strong cross-reactive ADCC

activity using different primary subtype B and C isolates as well as subtype B Transmitted/Founder viruses *in vitro* (81). This study reinforces the potential role of V2-specific Abs. However, as ADCC is a complex and multilayered activity, questions remain about which ADCC assay best reflects the biology of protection and shows the best correlation with *in vivo* studies. *In vitro* assays are difficult to carry out and the variability obtained between different ADCC assays developed in the HIV field is alarmingly high, due to different assay formats and readouts circulating in the field.

As the HIV env is conformationally highly dynamic and as different epitopes are exposed during the different phases of infection because of structural rearrangements, the window of opportunity for Abs to bind to their specific epitope in order to mediate ADCC might only be a few hours, during the viral entry and budding phases (7, 8, 82). Furthermore, different env forms (such as intact env, non-trimeric env, gp41 stumps, env peptides presented by the MHC, and so on) are expressed on infected cells depending on the Nef and Vpu accessory proteins present in the chosen virus type (primary virus, pseudovirus, infectious molecular clone). Also, HIV was shown to prevent the accumulation of env at the surface of target cells *via* a Vpu-mediated BST-2 antagonism (83). Noteworthy, the epitopes tackled by Abs with potential ADCC functions may differ from that involved in neutralization, opening the possibility of additional, enlarged, and distinct pattern of functional Abs. As a result, depending on the different env conformations, the recognition of specific epitopes will be influenced and have an impact on the ADCC results (84).

As ADCC relies on the capacity of the Ab to target infected cells, it could be proposed that by extension, Abs directed to all type of markers specifically expressed on infected cells may make the job. Therefore, targeting infected cells with Abs directed to FcRIIa, a marker recently identified on HIV cells reservoirs (85) or to specific markers of cell stress induced following infection (as NKG2D or MHC-E) may also participate in infected cell clearance. Another factor influencing ADCC outcomes and, thus, HIV disease progression are the target cells that carry out the lysis, which are predominantly NK cells. Their maturation and activation status as well as their subset distribution can vary widely in different tissues and according to the individual. Therefore, the activation of the ADCC target cells may be envisaged to enhance ADCC efficiency. Also, different polymorphisms on FcγRIIIa, expressed on NK cells, can impact their activation and ADCC activity (86).

ANTIBODY-MEDIATED COMPLEMENT ACTIVATION

The complement system is an integral part of the innate immune system which has multiple effects, including opsonization, recruitment of inflammatory cells, and cell lysis/virolysis. Complement activation can occur through three distinct pathways: classical, alternative, and lectin, and is vital for both innate and adaptive immune responses (87–91). Complement activation results in the generation of C3 and C5 convertase complexes, which cleave C3 and C5, respectively, to generate the anaphylatoxin components C3a and C5a as well as the opsonin C3b, membrane attack complexes initiator C5b and, finally, to perforate the viral surface causing disruption and, thus, complement-mediated lysis (87, 88, 91).

Antibody-mediated complement activation by HIV has been widely studied over the years. The initially published studies on complement and HIV were conflicting (92, 93). Some reports said that the virus did not bind human serum complement unless Ab to the virus was present. Others suggested that the virus activated and bound complement spontaneously, even in the absence of Ab. The current knowledge, however, concludes that HIV has developed a sophisticated defense that protects the virus by failing to bind complement proteins. Indeed, virions bind complement poorly (especially the gp120 that is refractory to complement binding) (94). Moreover, HIV incorporates the human cell membrane complement down-regulatory molecules CD46, CD55, and CD59 during budding, thereby inhibiting complement-mediated damage to the virus. For this reason, the use of primary isolates produced by primary cells is absolutely mandatory for the study of complement-mediated effects. HIV also captures serum factor H to downregulate complement binding (95–97). On the other hand, HIV has evolved several mechanisms to exploit the complement system to facilitate the binding of HIV to target cells *via* CR2 or CD21 proteins, therefore leading to the enhancement of viral infectivity and the formation of virus reservoirs at different stages (98–104). For example, complement-mediated enhancement of HIV-1 by autologous non-nAbs obtained during acute HIV-1 infection was recently illustrated in *in vitro* studies (95, 98).

Interestingly, the role of complement activity of the Fc domain of nAb b12 evaluated in the non-human macaque challenge model revealed that a b12 Fc mutant defective for C1q binding and complement activation exhibited comparable activity to that of wild-type b12 (13). This indicates that complement is not required for optimal *in vivo* Ab protection against SHIV infection (13). Nonetheless, complement activation by V1V2-specific Abs was stronger and detected more frequently in RV144 with a reduced risk of HIV-1 infection than in two related trials, VAX003 and VAX004, for which no significant protection was observed (105). These results suggest that a certain level of Ab-dependent complement activity may have contributed in part to a modest protection against the acquisition of HIV-1 infection in the phase III RV144 HIV-1 vaccine trial. Together, complement can mediate a variety of biological functions, the relative contribution of virus lysis and enhancement in the tissue and in the periphery may differ and needs to be further investigated. Additional studies will be needed to define the role of complement activation and regulation in HIV infection and to unravel whether the beneficial or the detrimental effects of complement and Ab dominate *in vivo*. A possible balance of Ab-mediated immune responses, including complement activation, may be the key for the induction of *in vivo* protection against HIV.

CONCLUSION

The plethora of additional Ab functions listed below demonstrates the extremely large potential of functional Abs.

Therefore, there is no single mechanism or assay that has come to the front to predict vaccine efficacy. This is a major issue confronting researchers in the HIV field and it is also important for other cases of Ab-mediated protection against infectious diseases.

The Abs will be produced by B cell following an interplay of somatic hypermutations and isotype switching. The successive modifications leading to the maturation of the immune response is still poorly understand. Recently, the frequency of HIV-env-specific memory B cells correlated positively with the neutralization breadth in HLA-B*57+ HIV elite controllers but not in HLA-B*57-elite controllers (ECs), suggesting a very specific induction or preservation of HIV-specific memory B cells in these patients (106). However, the factors allowing the establishment of this efficient humoral response is not known.

The long-lasting persistence of HIV following infection demonstrated that the sole repetitive contact with an antigen is not sufficient to mount a humoral response able to generate functional Abs. What are the additional component necessary to induce the rearrangement necessary to obtain B cells producing Abs with the Fab domain that recognize the right epitope and the Fc domain with the best functionality? Even more enigmatic, which immunization protocol can trigger such a response? The in-depth characterization of the different Ab functionality is the first step toward the understanding on how to trigger such an efficient B-cell response.

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Increasing the Clinical Potential and Applications of Anti-HIV Antibodies

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Preclinical and early human clinical studies of broadly neutralizing antibodies (bNAbs) to prevent and treat HIV infection support the clinical utility and potential of bNAbs for prevention, postexposure prophylaxis, and treatment of acute and chronic infection. Observed and potential limitations of bNAbs from these recent studies include the selection of resistant viral populations, immunogenicity resulting in the development of antidrug (Ab) responses, and the potentially toxic elimination of reservoir cells in regeneration-limited tissues. Here, we review opportunities to improve the clinical utility of HIV Abs to address these challenges and further accomplish functional targets for anti-HIV Ab therapy at various stages of exposure/infection. Before exposure, bNAbs' ability to serve as prophylaxis by neutralization may be improved by increasing serum half-life to necessitate less frequent administration, delivering genes for durable *in vivo* expression, and targeting bNAbs to sites of exposure. After exposure and/or in the setting of acute infection, bNAb use to prevent/reduce viral reservoir establishment and spread may be enhanced by increasing the potency with which autologous adaptive immune responses are stimulated, clearing acutely infected cells, and preventing cell–cell transmission of virus. In the setting of chronic infection, bNAbs may better mediate viral remission or "cure" in combination with antiretroviral therapy and/or latency reversing agents, by targeting additional markers of tissue reservoirs or infected cell types, or by serving as targeting moieties in engineered cell therapy. While the clinical use of HIV Abs has never been closer, remaining studies to precisely define, model, and understand the complex roles and dynamics of HIV Abs and viral evolution in the context of the human immune system and anatomical compartmentalization will be critical to both optimize their clinical use in combination with existing agents and define further strategies with which to enhance their clinical safety and efficacy.

Keywords: HIV antibodies, virus neutralization, passive immunotherapy, antibody prophylaxis, antibody engineering

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INTRODUCTION

Antibody (Ab)-based therapies have a robust history of therapeutic utility in the setting of infectious diseases, first serving as serum therapy in the 1800s to treat diphtheria and most recently, as monoclonal antibody (mAb) preparations developed to combat emergent outbreaks such as Ebola. Endogenous antibodies raised within the context of HIV infection have similarly demonstrated antiviral activity (1), but typically arise too late in the natural history of infection to prevent disease progression (2). Within infected individuals, viral populations consistently outpace host immune responses in a coevolutionary race to gain functionally favorable mutations contributing to immune evasion or viral

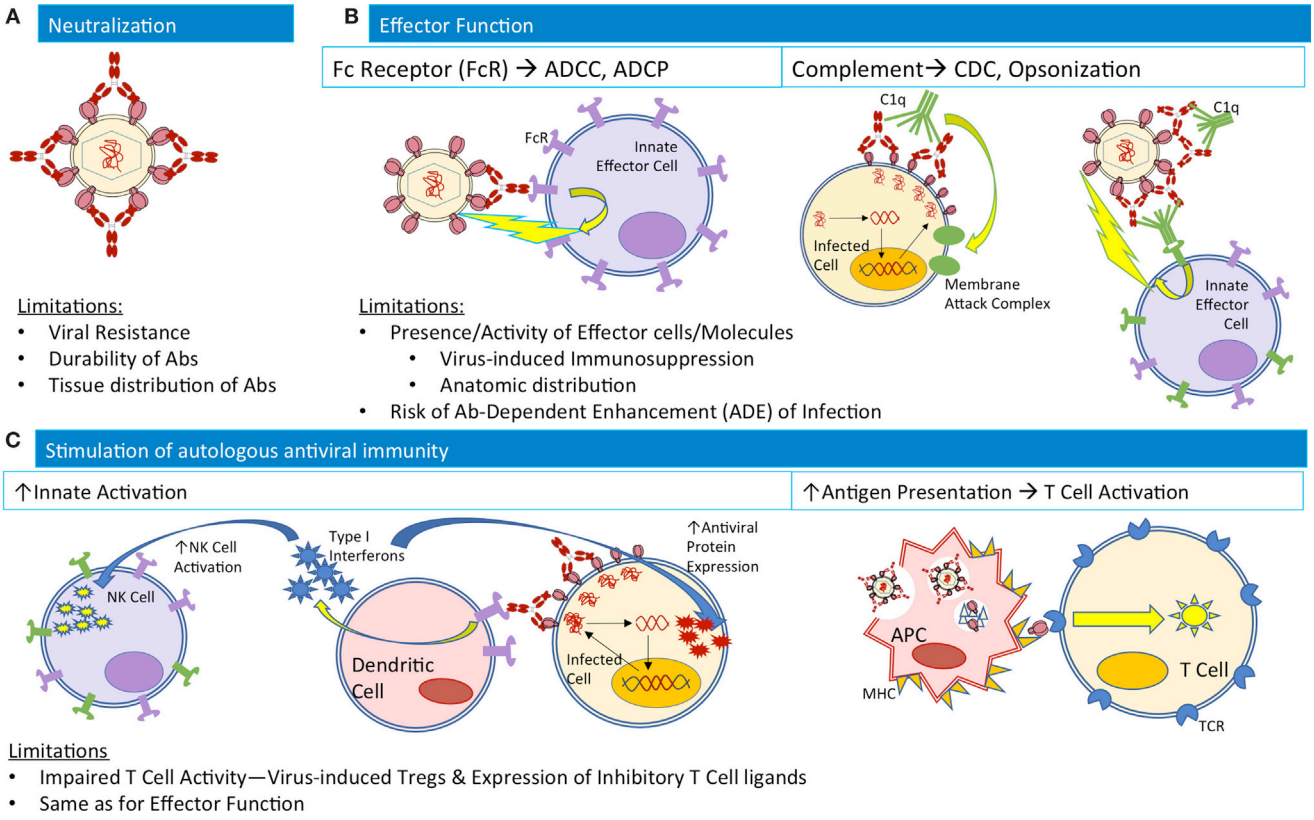


FIGURE 1 | Native Ab functions contributing to antiviral activity and their limitations in the natural course of infection. **(A)** For neutralization, Abs (red) bind viral envelope proteins to block interactions with cellular receptors**. (B)** For effector functions, Abs bound to both viruses and infected cells may engage innate effector cells (purple) to mediate ADCC or ADCP, or complement component C1q (green) to mediate CDC or interactions with complement receptors on innate effector cells for opsonization-based phagocytosis. **(C)** To stimulate autologous antiviral immunity, Ab-bound infected cells may interact with dendritic cells to release type I interferons (blue stars) to stimulate NK cell activation and expression of antiviral proteins within infected cells. Alternatively, antigen presenting cells may phagocytose Ab-virus immune complexes and process viral antigens for presentation to T-cells to mediate cellular immune responses. Abbreviations: ADCC, Ab-dependent cellular cytotoxicity; ADCP, Ab-dependence cellular phagocytosis; CDC, complement dependent cytotoxicity; FcR, Fc receptor; MHC, major histocompatibility complex; TCR, T-cell receptor.

neutralization/suppression, respectively. However, heterologous administration of particularly potent and broad antibodies prior to exposure or to acutely infected individuals has demonstrated therapeutic utility in humanized mice (3–7), macaques (8–13), and humans (14–19).

Several reviews have described the activity and potential of broadly neutralizing antibodies (bNAbs) for HIV prevention and therapy (20–27). Building upon a recent comprehensive review of engineering opportunities to extend the functional capacity and antiviral activity of bNAbs (28), this review incorporates findings from more recently published macaque and human bNAb clinical trials to explore both observed and potential challenges to successful bNAb implementation at various stages of exposure/disease to prevent infection, minimize viral spread, suppress viral growth, and eliminate viral populations.

Promise/Potential: bNAbs in Human Clinical and Macaque Preclinical Trials

The abundance of studies supporting the antiviral activity and potential of bNAbs to mediate protection from and control of HIV infection in animal models have renewed hope and interest in bNAbs for clinical use. Antibodies can exert antiviral activity through a combination of (1) virus neutralization, preventing initial infection, and viral spread, (2) Fc-mediated effector functions, contributing to the clearance of infected cells, and (3) enhancement of endogenous host antiviral immune responses (**Figure 1**). In the last 2 years alone, promising human clinical studies to investigate therapeutic benefit in postinfection settings (14–19) and additional preclinical studies to investigate protective efficacy in preexposure/infection settings (29, 30) have clarified the mechanisms of action and efficacy of bNAb administration.

Human clinical studies of VRC01 (14, 17, 31), 3BNC117 (15, 18, 19, 32), and 10-1074 (16) have demonstrated the antiviral activity of bNAbs, offering therapeutic utility in both acute and chronic infection settings. Beyond safety and tolerability, all three bNAbs reduced viral load (15–17) during administration and two, VRC01 and 3BNC117, successfully delayed viral rebound upon discontinuation of antiretroviral therapy (ART) (14, 18). Treatment dosing regimens remain to be optimized and may differ among Abs, dependent upon both the usual considerations of individual mAb pharmacokinetic and pharmacodynamic properties, but also each mAb's HIV-specific pharmacodynamic

properties, such as the slope and completeness of neutralization (33), susceptibility to viral evasion, and propensity to mediate viral (or antigen) trafficking/processing/presentation. In addition, characteristics of individual subjects, such as viral load, diversity, and sensitivity to select bNAb(s) at time of treatment may be considered for more individualized regimens.

Concurrently with direct antiviral activity, treatment with 3BNC117 stimulated and enhanced endogenous antiviral immune responses: in 14/15 viremic individuals treated with 3BNC117, sera from week 24, well after serum levels of 3BNC117 had dropped below detection limits, demonstrated increased breadth and/or potency against a pseudovirus panel as compared to week 0 (19). Interestingly, the increase in neutralization capacity of week 24 sera from ART-treated individuals receiving 3BNC117 was less pronounced than in untreated individuals receiving 3BNC117, suggesting that viral replication and activity contributes to the development of heterologous neutralization (19). Previous studies have also demonstrated the enhancement (13, 34, 35) and importance (36) of autologous humoral and T-cell responses in response to bNAb therapy in macaque models of SHIV [reviewed in Ref. (37)].

The use of HIV Abs in preclinical animal models have similarly demonstrated the potential of mAbs to provide pre- or postexposure prophylaxis, similarly to the early use of immunoglobulins to protect against infection by RSV and Hepatitis A [reviewed in Ref. (38)]. Protection against SHIV acquisition has been demonstrated for multiple bNAbs (9–11, 39–41) with protection dependent upon SHIV strain, bNAb dosage, and bNAb serum concentrations at time of challenge. In models of high-dose SHIV challenge, treatment with ≥5 mg/kg 3BNC117 or 10-1074 successfully blocked SHIV acquisition after a single intrarectal challenge of 1,000 times the 50% tissue culture infectious dose (TCID50), or approximately three times the half-maximal animal infectious dose (42). In a larger study (60 challenged animals vs. 4), the same group determined that serum titers of bNAbs as low as 1:100 were sufficient to prevent SHIV acquisition in ~50% of macaques receiving a single intrarectal challenge at 1,000 TCID50 (8). More recently, the same three bNAbs studied in human clinical trials, VRC01, 3BNC117, and 10-1074, have been tested in preclinical macaque models of repeated low-dose SHIV exposure with impressive results (29). A single infusion of 3BNC117 successfully prevented virus acquisition in models of repeated low-dose intrarectal challenges for up to 23 weekly intrarectal challenges at 10 times the TCID50, whereas control animals acquired infection after two to six challenges. Across the three bNAbs evaluated, the length of protection correlated with Ab potency and half-life. Similarly, in humanized mouse models of HIV acquisition, passive transfer of the bNAb PGT126 demonstrated sterilizing protection against multiple vaginal HIV challenges (30).

Role of Non-Neutralizing Abs (nnAbs)

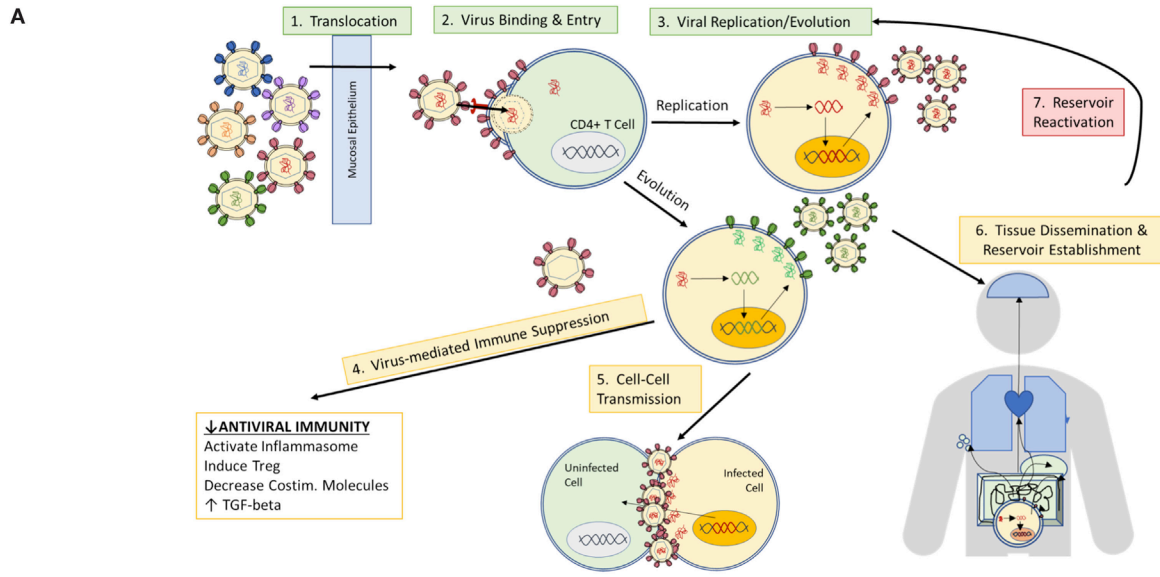
As opposed to neutralizing Abs which bind epitopes on functional trimeric Env to prevent cell receptor engagement, nnAbs bind epitopes exposed in non-infective conformations adopted by the unstable Env antigen, such as open Envelope trimers, gp140 monomers, and dissociated gp41 stumps (due to instability or induced by binding to cell receptors). nnAb responses have demonstrated protection through Fc-mediated effector functions and by exerting additional selective pressure and evolutionary constraints upon remaining viruses in humanized mice (43, 44). In a recent study, Horwitz et al. demonstrated the capacity of nnAbs to modulate the course of HIV infection in humanized mice *via* Fc-mediated effector functions in two nnAb cases: (1) using anti-HA Abs in humanized mice challenged with a newly developed recombinant indicator HIV strain containing an HA-tag-, (HIVivoHA) or HIVivoHA-infected cells and (2) using a patient-derived nnAb 246D (45) targeting a linear gp41 epitope in humanized mice challenged with HIV-1_{YU2} virus or HIV-1_{YU2}-infected cells (44). In both cases, passive transfer of nnAbs mediated modest protection from viral challenge, reduced viral load in established infection, cleared virus-infected cells, and exerted selective pressure for escape mutations that ultimately deleted or concealed the targeted epitope, all in an Fc-dependent manner that was diminished or absent in passive transfer of the same nnAbs modified with mutations that abrogated binding to activating Fc-receptors (44). Older studies in macaques have suggested that nnAbs may decrease the number of transmitted/founder variants and the viral load in acute viremia, but ultimately did not protect from infection (46–48). Thus while the efficacy of nAbs has been linked to Fc-dependent mechanisms (40) the sufficiency of these antibody activities to drive protection from infection among nnAbs has not been established in NHP. Similarly, the protective capacity of non-neutralizing HIV Abs in humans has been suggested by mother-to-child-transmission studies [reviewed in Ref. (49)] and by the association of V1/V2 nnAbs with protection in the RV144 HIV-1 vaccine trial (50, 51), but remains to be demonstrated.

Therapeutic Applications and Goals by Stage of Infection

Based on the established roles of mAbs in various infectious diseases, autologous Abs in the natural history of HIV infection, and HIV Abs in clinical and preclinical trials, anti-HIV mAbs find multiple indications for clinical use with therapeutic goals defined by the stage of HIV exposure and disease (**Figure 2**). Before viral establishment, mAbs could be used either prior to exposure to prevent viral acquisition or postexposure to prevent or limit viral establishment. After viral acquisition in chronic infection settings, therapeutic goals extend to include viral suppression to stabilize and prevent progression of disease, and viral eradication to cure patients entirely of infection. This review investigates the current limitations of and engineering strategies with which to improve the utility of bNAbs at each stage of infection/disease to (1) prevent infection, (2) limit viral establishment/spread, and (3) treat chronic infection *via* suppression of viral growth and reduction/elimination of viral reservoirs (summarized in **Table 1**).

ENHANCING PREEXPOSURE PROPHYLACTIC POTENTIAL: PREVENTING VIRAL INFECTION

Development of durable protection against HIV has remained a challenge due to the great diversity of HIV species and their



B

	Viral Translocation	Virus Binding & Entry	Viral Replication & Evolution	Immune Suppression	Cell-cell transmission	Migration & Reservoir Establishment	Reservoir Reactivation & Spread
Pre-Exposure Prophylaxis							
Post-Exposure/Acute Infection							
Chronic infection							

FIGURE 2 | Clinical goals for the use of anti-HIV Abs vary according to **(A)** mechanisms of viral exposure/infection at the time of administration, and **(B)** the viral events which therapeutic Abs seek to inhibit among indicated use prior to exposure (green), as postexposure prophylaxis or treatment of acute infection (yellow), and for treatment of chronic infection (red).

adaptive capacity to evade immune-mediated pressure. Viral strains can be described by clade or subtype with viral diversity profiles varying by geographic location, or by neutralization sensitivity designated as very high (tier 1A), above-average (1B), moderate (2), or low (3) sensitivity to Ab-mediated neutralization (pooled plasma samples from four to six clade-matched infected individuals) (52). Clade-matched viral variants are often more sensitive to neutralization by plasma/NAbs from individuals infected by the same clade (52). Thus, the profiling of viral variants endemic to geographical regions could inform the selection of NAbs offering the greatest breadth and potency of neutralization. Ab-based vaccines may function to protect from infection in two ways: (1) neutralization to prevent viral infection in the first place and (2) rapid clearance of virus or virus-infected cells, which will be expanded upon in Section "Enhancing Prophylactic and Therapeutic Potential in Acute Infection: Preventing Viral Reservoir Establishment/Spread." To offer sterilizing immunity, Abs must offer durable protection with sufficient targeting to anatomic sites of exposure to neutralize viruses and prevent infection. To clear virus and virus-infected cells, Abs must be both readily available at therapeutic concentrations and broadly reactive to maintain efficacy against the diversity of viral strains to which an individual might be exposed. Thus, current and potential limitations to the prophylactic use of bNAbs include: (1) development of viral resistance, (2) requirement for strict regimen adherence, (3) anatomical distribution to sites of exposure, and (4) risk of Ab-dependent enhancement (ADE) of infection.

Viral Resistance

The arsenal of bNAbs available today targets epitopes spanning a significant portion of the surface of the trimeric HIV Envelope gp140 protein including the V1/V2 loops at the trimer apex, V3 loop glycans, CD4 binding site (CD4bs), gp120-g41 interface, and membrane-proximal external region (MPER) [reviewed in Ref. (53)]. Individual bNAbs vary in neutralization breadth and potency, with some CD4bs targeting bNAbs able to neutralize >90% of global circulating HIV-1 strains at low concentrations (54). However, resistance can develop to even the most potent of bNAbs and has indeed been observed in human clinical trials of all three bNAbs tested thus far (14–17). Even among bNAbs targeting the same epitope, different barriers to resistance development may exist from individual to individual and may arise in part from preexisting bNAb-resistant viral strains. Engineering strategies to combat the development of viral resistance reviewed previously (28) include (1) structure-based modifications to increase the breadth, potency (both neutralization and effector function), and half-life of individual bNAbs, (2) combinations of

TABLE 1 | Summary table of strategies for the improvement of anti-HIV Ab therapy.

Indication	Goal	Mechanism	Limitation		Improvement Strategies
Vaccine	Block viral entry	Neutralization	Viral resistance	↑ Breadth and potency	Structure-based modifications to ↑ binding Broadly neutralizing antibody (bNAb) cocktails Bispecific and trispecific bNAbs
			Strict requirement for adherence to dosing schedule	↑ t1/2	FC engineering
					Glycan "masking" Carrier proteins, peptides, RBCs
				Continuous Ab expression (adeno-associated virus)	↓ Immunogenicity to ↓ anti-bNAb responses Targeting multiple tissues for comprehensive protection Enable evolution of delivered Abs: B Cell engineering
			Anatomical distribution	↑ Targeting to sites of exposure	Topical gel delivery ↑ Binding to mucosal transporters Targeted gene delivery
			Risk of Ab-dependent enhancement	↑ Breadth and potency	See above
				Maintain protective concentrations of Abs	Dosing schedule or gene delivery
Postexposure prophylaxis and acute infection	Prevent reservoir establishment	Stimulate autologous antiviral immunity	Insufficient protection after bNAb levels decay	↑ Viral processing and presentation	Coadministration of virus/infected cells (immune complex)
				Counter virus-mediated immunosuppression	Coadministration of immunostimulatory drugs/Abs targeting characterized mechanisms
				Further restrict viral evolutionary space	Identify Abs targeting "non-survivor" epitopes
	Clear acutely infected cells	Ab-mediated Effector functions	Low potency?	Fc engineering for FcR/complement binding Add toxic payload	Protein/glycoengineering, subclass switching Immunotoxin, Ab-drug conjugate
	Prevent cell–cell transmission	Unclear	limited understanding of mechanism	Elucidate mechanism, especially role of Env conformational changes to define "neutralizing" epitopes for cell–cell transmission	
Chronic infection	Suppress viral replication	All of the above (AOTA)	Resistance	Combine with antiretroviral therapy (ART) to suppress replication and opportunities tot evolution	
	Target virat reservoirs	AOTA	Tissue distribution or Abs and reservoir accessibility	Tissue-targeted delivery	Ex: liposomal delivery to central nervous system (CNS)
				Cover diverse populations in compartmentalized tissue	Combine w/additional Abs, ART, latency-reversing agents
			Low Env expression in chronic infection	Target Env epitopes of chronic infection	
				Target non-viral surface markers	All potential reservoir cells, including uninfected (e.g., CD52), or upregulated on infected cells (e.g., CD32a)
				Reactivate reservoirs	Add LRAs
	Long-term clearance of reservoir cells	Autologous T-cell-mediated response	Low cytotoxic T-lymphocyte (CTL) response due to immune suppression	bNAb-based chimeric antigen receptors (CARs)	↑ Clinical safety (↓ risk of CAR mediating infection, synthetic biology "switch" on/off/homing strategies)
			CTL trafficking limitations	Investigate/improve bnAb access to CTL sanctuaries	
	Virol eradication	AOTA	Costs of eliminating reservoir cells in certain tissues (e.g., CNS)	Pair with gene editing strategics so infected cells may survive	

*Overlap of therapeutic goals for listed indications (see **Figure 2**) are not shown in this table. Goals which are targets for multiple indications are grouped under the indication for which they are the primary focus.*

Abs in cocktail therapies, (3) modifying bNAbs to become bispecific, to carry toxic payloads, or to redirect cells in bNAb-based therapies, and (4) altering delivery strategies.

Since the previous review, three additional studies of newly isolated neutralizing Abs have further supported the importance of structural Ab-Env interactions to neutralization breadth and viral evasion. Demonstrating the importance of Ab binding modes to development of viral resistance, N6, a new bNAb targeting the CD4bs with a novel mode of recognition, does so with amino acid features similar to previously identified mutations to increase the potency of VRC01-class Abs, and demonstrated near-pan neutralization breadth of 98% of HIV isolates tested, including many isolates resistant to other CD4bs antibodies (55). Defining a new neutralizing epitope, the recently isolated/characterized bNAb N123-VRC34.01 recognizes a unique trimer-specific, cleavage-dependent epitope at the N terminus of the gp41 fusion peptide (56). Finally, two recently isolated V2-specific Abs, PGDM1400, and CAP256-VRC26.25, demonstrated unprecedented neutralization potency, protecting against high-dose SHIV challenge at serum Ab concentrations <0.75 μg/mL for CAP256-VRC26.25-LS (57). In addition, these V2-specific bNAbs exhibited neutralization breadth complementary to that of V3-specific bNAb PGT121 against Clade C viruses, ultimately resulting in >90% coverage when used in combination (57).

Recent studies have investigated optimal strategies for combining bNAbs in cocktail therapies (3, 58–61), bispecific formats (62, 63), and novel tri-specific molecules (64). A combination of only three bNAbs targeting different epitopes has been suggested to be sufficient to cover transmitted viral diversity and evolution based on a study conducted in humanized mice (58) and predictive *in silico* models of neutralization breadth and potency (59). In an alternative form of combining epitope specificities, the most potent and broad bispecific Ab to date, 10E8v2.0/iMab, demonstrated 100% neutralization breadth across a 118-member pseudotyped panel with mean inhibitory concentration of 0.002 μg/mL and prevented HIV acquisition in humanized mouse models of infection, demonstrating the synergistic potential of bispecific Abs targeting distinct epitopes (63). In another study, a novel bispecific Ab hinge engineering strategy employing the IgG3 hinge to increase Fab domain flexibility for bivalent binding and to maintain IgG1-Fc function enhanced the *in vivo* therapeutic activity of bispecific bNAbs (62), emphasizing the synergistic avidity-enhancing effect of intratrimeric, heterobivalent crosslinking of Fab arms to increase Ab potency (65). In another novel approach, trispecific Ab molecules containing bNAb specificities against the V1V2 loop trimer apex (PGDM1400), CD4bs (VRC01 and N6), and MPER (10E8v4) were found to mediate increased breadth and potency compared to individual parental bNAbs both *in vitro* and in SHIV challenge models (64). The authors speculated that the tri-specific bNAb may have decreased risk of viral resistance compared to cocktail strategies where differences in component bNAb half-lives may decrease selective pressure (64). However, whether these trimeric molecules engage multiple epitopes simultaneously and/or otherwise confer added benefit over a cocktail consisting of the same three bNAbs remains to be determined.

Beyond development of viral resistance within an individual to bNAb therapy, implications of widespread use of bNAbs as prevention may influence the composition and evolutionary dynamics of worldwide HIV strains. HIV drug resistance is increasingly observed due to poor patient adherence enabling the development of resistance, and subsequent transmission of newly developed drug-resistant strains (66). Similar potential for the development of bNAb-resistant "super-strains" of HIV exists, as bNAb-resistant strains often coexist or arise within individuals from whom bNAbs were isolated. Trade-offs between viral evasion and fitness costs incurred by some resistance mutations (67–70) may mitigate these concerns. However, resistance mutations without fitness costs (70, 71) and the development of compensatory mutations to restore fitness have also been described (67), and antibodies vary with respect to sensitivity to evasion and ease of compensation. Combination strategies such as the cocktails or multispecific molecules described above may best prevent the development of "super-strains" of HIV by further restricting the viral evolutionary landscape. Thus, strategies to optimize bNAb administration and pharmacokinetics to make treatment regimens manageable and supportive of strong treatment adherence will be critical to avoid the development of bNAb-resistance on a more global scale.

Alleviating Requirements for Regimen Adherence

Because viral rebound quickly occurs upon bNAb decay and renewed replication enables opportunities for viral evolution, protective bNAb dosing schedules must be strictly followed to prevent both viremia and viral resistance. Two methods to decrease the frequency of dosing are (1) increasing the serum half-life of bNAbs and (2) bNAb gene delivery for continuous *in vivo* expression.

Increasing Serum Half-Life of bNAbs

Interestingly, bNAb levels decayed more quickly in HIV(+) individuals as compared to controls in human clinical trials, potentially due to the formation of Ab-virus immune complexes in infected individuals that are more rapidly cleared from circulation. For bNAbs to offer prevention potential, and to avoid the development of resistance, serum half-life would need to be long enough to maintain protective concentrations at reasonable dosing schedules. Fc engineering strategies to increase the half-life of bNAbs have been described [reviewed in Ref. (28, 72)], including studies of the VRC01-LS variant which demonstrated a threefold longer serum half-life and increased translocation to mucosal tissues, ultimately leading to improved potency and protection against high-dose rectal challenge in non-human primates (29, 73, 74). VRC01-LS (M428L and N434S) (29, 74) has now advanced into Phase I clinical trials (NCT02797171, NCT02840474, NCT02599896, NCT02256631).

Continuous Protection *via* Gene Delivery: *In Vivo* Expression of bNAbs

In an indirect way to extend the lifetime of bNAb therapy, gene delivery has been increasingly explored to achieve durable Ab

concentrations, most prominently by adeno-associated virus (AAV) vectors [reviewed in Ref. (75)]. Historically, AAV delivery-based gene therapy has demonstrated safety and efficacy in both macaques (76–79) and humans (80–85) for a variety of diseases, and has become the first clinically and government-approved gene therapy in Europe (86, 87). Within the realm of HIV, AAV-delivered HIV-specific bNAbs and Ab-like molecules such as CD4-Ig have demonstrated sterilizing and durable protection against SIV/SHIV infection in macaques (73, 88–90) and HIV infection in humanized mice (4, 91), and are now undergoing Phase I human clinical trials to evaluate safety, deliverability, and potential efficacy in England (NCT01937455).

Current limitations to bNAb gene delivery include the development of anti-bNAb responses and the virus independence of bNAb expression. First, several studies of AAV-delivered bNAbs to macaques have demonstrated the development of anti-bNAb responses (73, 88, 90, 92), despite "rhesus-ization" of bNAbs and addition of immunosuppressive therapy, potentially due to immune-stimulating effects of the AAV itself which can trigger innate pattern recognition receptors and toll-like receptors or engage preexisting cellular (93) or humoral (94) immunity. Side-by-side comparisons of anti-bNAb responses in passively transferred bNAbs vs. AAV-delivered bNAb treatment have been proposed to delineate immunogenic contributions from AAV vs. Ab (75). Engineering strategies to decrease the immunogenicity of AAV capsids and coadministration of immunosuppressive agents (cyclosporine, T-cell inhibition, IVIG, corticosteroid) have been proposed and shown promise (75). However, immunosuppressive agents may also decrease bNAb Fc-mediated effector function and the development of autologous antiviral responses, placing the bulk of protection on neutralization. Thus, studies to determine the costs and benefits of adding immunosuppressive agents to AAV-delivery regimens are warranted.

Second, current AAV-delivery of bNAbs results in bNAb expression independent of viral trafficking, replication, and evolution, and therefore (1) may not be ideally distributed for prevention of infection/reservoir establishment and (2) cannot respond to changes in the viral population. Intramuscular delivery of vectored gene therapy to skeletal muscle is most extensively studied thanks to muscle tissue's amenability to long-term gene expression, abundant vascular supply for quick transport to the systemic circulation, and ease of accessibility (95). However, vectored gene delivery to additional tissues including the liver, brain, spinal canal, skin, and eyes have been described (95). Targeted gene delivery to these tissues may be especially useful if protective Ab concentrations in these tissues are not possible from circulation alone.

However, such bNAb-expressing tissues are unable to respond to viral evolution, and may become less useful as viral populations develop resistance to the administered Ab. Thus, strategic delivery of bNAb genes to B-cells for integration at native BCR loci (gene targeting into the *Igh* locus) under the normal regulation of heavy-chain expression, Ab class-switching, and somatic mutation may offer the added benefit of coevolution with viral populations. A similar technology of *in vivo* bNAb-as-BCR evolution has been used in HIV Env immunogen studies in transgenic knock-in mice containing B-cells expressing germline heavy chain variants of VRC01-class Abs (96–98), which were successfully activated/expanded and underwent somatic hypermutation in response to various Env immunogen regimens. Viral challenge of similarly generated knock-in mice containing genes for mature bNAbs as BCRs may demonstrate proof-of-concept for bNAb-based BCR engineering. Clinical translation of such a strategy could parallel chimeric antigen receptor (CAR) T-cell procedures, whereby B-cells could be extracted from a patient and engineered *ex vivo* to expressed bNAb-based BCRs prior to reinfusion. Investigations into efficient and targeted IgH knock-in would be critical to this approach and increased understanding of B-cell differentiation and subtypes, BCR editing, and tolerance checkpoints would be beneficial. Additionally, switchable gene expression may be desired to prevent unchecked expansion/growth. While this ability to coevolve may not ultimately provide any benefit, natural infection histories provide both reasons for optimism and pessimism. In favor of the optimistic possibilities, the ability of bnAbs to improve autologous antibody neutralization potency, and their ability to collaborate with other lineages for beneficial outcomes suggests that the ability to adapt over time could be advantageous.

Targeting Anatomical Sites of Exposure

One probit analysis of bNAb-treated macaques suggested that a serum level of 100 times the bNAb IC50 affords 50% protection against intrarectal infection (41), a level that is estimated to be attainable by biannual passive Ab injections given the serum Ab levels and half-lives of VRC01 and 3BNC117 in human clinical trials (25). In an SHIV macaque study, IV infusion of 2 mg/kg PGT121 completely protected subjects from intravaginal challenge with 5×10^4 TCID50 SHIV-SF162P3, with no detectable viral RNA or DNA found in distal tissue sites by day 10 after challenge (99). However, concentrating Abs at the sites of viral exposure may allow even lower doses to be protective. Because viral exposure often occurs at mucous membranes including the rectal and vaginal tracts, the presence of bNAbs at mucosal sites to mediate immune exclusion may improve protection. Therapeutic administration and Ab engineering strategies to improve bNAb use for mucosal immunity were described previously (28) and included topical gel delivery, Fc engineering to enhance binding to FcRn and pIgR at mucosal sites, and designing IgA and chimeric IgGA variants of bNAbs. In addition, some of the strategies described above such as targeted AAV-delivery of bNAb genes to specific tissue sites or BCR engineering to express class-switched IgA versions of bNAbs may be beneficial. Studies have found contrasting evidence for (100–103) and against (104) a role for bNAbs, formatted as various isotypes, in preventing transepithelial migration. The reason for this discrepancy is unknown but may be related to the utilization of older-generation or less potent bNAbs in the prior studies (2F5, 2G12, 4E10), whereas the most recent studies investigate newer-generation, more potent bNAbs. In that study of bNAbs targeting a wide range of epitopes, bNAbs did not block the transcytosis of either cell-free or cell-associated HIV-1 *in vitro* and instead relied upon neutralization to decrease the infectivity of transcytosed viruses (105). Thus, increasing the local concentration and

neutralization breadth and potency of bNAbs at mucosal sites may enhance protection against mucosal infection.

Potential Risks: ADE of Infection

Thus far, ADE of HIV infection has only been observed *in vitro* and grouped into complement- (106–108), Fc Receptor (FcR)- (109–112), and conformationally mediated (113, 114) mechanisms which ultimately facilitate virus internalization or receptor-independent virus-cell membrane fusion [reviewed in Ref. (115, 116)]. In addition, antibody-virus immune complexes could increase trafficking of infectious virions to lymph nodes, thereby amplifying rates of viral infection and replication. While debate exists over whether ADE occurs in natural HIV infection, the presence of enhancing Abs have been correlated with disease progression in some studies of sera from HIV-infected individuals (117, 118) [but not others (119)] and suggested to explain increased rates of infection in individuals with relatively low Ab responses in vaccine trials (120) and correlations of particular FcR genotypes characterized by stronger Fc-binding affinities with higher infection risk (121, 122). Both nnAb and neutralizing Ab at subneutralizing concentrations can enhance infection *in vitro* (109), and epitope specificity does not necessarily determine an Ab's potential for ADE (115). Thus, maintaining protective concentrations of bNAbs *via* repeated dosing or continuous expression (AAV) may be especially critical to decrease the risk of ADE.

ENHANCING PROPHYLACTIC AND THERAPEUTIC POTENTIAL IN ACUTE INFECTION: PREVENTING VIRAL RESERVOIR ESTABLISHMENT/SPREAD

After exposure, bNAbs may be used as prophylaxis to prevent the establishment and spread of viral reservoirs [reviewed in Ref. (123, 124)]. Successful elicitation or administration of HIV-specific Abs in macaque models of acute SHIV challenge and infection have correlated with reduced acute viremia and limited reservoir seeding (46, 125, 126). The window for postexposure prophylaxis has been estimated to be as short as 24 h to block infection by cell-free virus in macaque models of SHIV infection (127, 128) and within the first 9–10 days to limit viral reservoir seeding and spread (129). Resistance continues to be a major concern for all of the described bNAb indications in this review, but may be especially relevant in postexposure settings where autologous viral populations may be screened for preexisting resistance to bNAbs. Mucosal barriers and/or autologous immune responses often limit the diversity of transmitted/founder (T/F) strains; in one study, 80% of individuals infected through heterosexual and 60% through homosexual contact were found to have a single founder virus strain (130). Thus, the low viral diversity present in acute postexposure settings render it a particularly useful time at which to screen viral populations to inform the choice of bNAb(s) therapy. Efforts to adequately sample viral diversity later during chronic infection become more difficult as latent reservoirs are established and thus viral sensitivity screening may be less useful at later time points.

In addition to the previously described goals to neutralize virus to prevent initial infection, postexposure prophylactic use of Abs additionally seeks to limit reservoir seeding and spread. Enhancing the ability of mAb therapies to (1) increase autologous immune responses and (2) target acutely infected cells represent two strategies by which to accomplish this goal.

Increasing Protection by Influencing the Autologous Adaptive Immune Response

Both neutralizing Ab and nnAbs depend upon Fc-mediated effector functions for antiviral activity *in vivo* (131, 132). Through the Fc portion, elicitation of even nnAb responses offers therapeutic utility, demonstrating protective effects in both vaccination (121, 133) and passive transfer studies (44, 134, 135). Stimulation of autologous Ab responses, whether neutralizing or not, thus remains a promising means by which to generate durable effects from Ab therapy.

Broadly neutralizing antibody therapy has been associated with enhanced autologous antiviral immune responses in both human (19) and macaque (13, 34, 35) studies. Proposed mechanisms for this observed effect include (1) facilitation of viral processing and presentation, (2) potential immune-stimulating effects in an otherwise suppressed adaptive immune background conferred by HIV infection, and (3) restriction of viral evolutionary space by both administered bNAbs and elicited autologous Abs. Efforts to increase autologous Ab responses may thus focus upon enhancing each of these mechanisms.

Enhancing Viral Processing and Presentation

Increasing the effector function capacity of anti-HIV Abs by Fc engineering to skew binding toward particular Fc receptors represents one mechanism by which to engage and stimulate endogenous immunity, and has been previously reviewed in Ref. (28, 72). Beyond engineering bNAb molecules, adjunctive coadministration of envelope, virus or infected cells with Abs in immune complexes can engage FcγRs on antigen-presenting cells to facilitate antigen internalization and enhance APC activation and presentation, ultimately "boosting" endogenous antiviral immunity [reviewed in Ref. (136)]. Although one study found that opsonization of HIV-1 with polyclonal anti-HIV IgGs was associated with decreased dendritic cell activity (137), further investigations of Abs of varying neutralization potency formatted as different isotypes have been proposed to clarify the generalizability of that study (136). In another study, administration of HIV-1 gp120 Env and a CD4bs mAb resulted in enhanced neutralization potency of elicited humoral responses in mice (138). Notably, Fab-mediated effects that resulted in greater presentation of particular epitopes in the Ab-bound immune complex were determined to be at least partially responsible for the increased neutralization potency of the elicited antibody response (139–141). Additional parameters to be investigated in the use of immune complexes to stimulate endogenous antiviral immunity include antigen format (soluble vs. virus vs. infected cell), Ab format (neutralization capacity, isotype, Fc variants), ideal ratios of Ab:Ag to form complexes, and routes of administration (136).

Combatting Viral-Mediated Suppression of the Antiviral Immune Response during Acute HIV Infection

Acute HIV infection is characterized by early suppression of antiviral immune responses to support viral growth and spread. Mechanisms for this antiviral-specific immunosuppression include increased activation of the NLRX1 inflammasome (129, 142), which negatively regulates interferon-stimulated antiviral genes, and increased secretion of TGF-beta (129) that inhibits adaptive immune responses. In addition, viral interactions can induce early activation of regulatory T-cells (143, 144), and increase the expression of inhibitory T-cell markers PD-1 and CTLA-4 (145, 146). The effect of these immunosuppressive mechanisms on Ab-mediated effector function remains to be determined (124), but likely decreases the efficiency with which Ab-mediated stimulation of autologous immune responses arise. Combination with immunostimulatory drugs and antibodies targeting these specific mechanisms of immunosuppression may thereby increase the development of autologous antiviral immune responses, but may be a double-edged sword as there is a concomitantly increased risk of enhancing the development of anti-bNAb responses or the pool of CD4+ T-cells available for infection. *In vivo* studies of such approaches will be especially critical to determine the utility and/or feasibility of this approach.

Identifying Abs Targeting "Non-Survivor" Epitopes: Limiting Viral Evolution

Finally, autologous Ab responses may have antiviral effects by limiting the space for viral evolution through the targeting of "non-survivor" epitopes, regions in which resistance mutations incur survival costs or complete lethality [reviewed in Ref. (43)]. These epitopes can be distinct from epitopes recognized by bNAbs, against which resistance mutations commonly develop and are often contemporaneous with the presence of the bNAb in individuals from which they are isolated. Thus neutralizing epitopes identified thus far are largely "survivor" epitopes and a recent review has raised the concern of "survivor bias" in present studies of protective humoral responses (43). Potential non-survivor epitopes include functionally critical regions targeted by non-neutralizing epitopes that become exposed upon conformational changes including CD4-inducible epitopes (147) and gp41 epitopes like the fusion peptide (46, 56): passive transfer of nnAbs targeting these regions successfully decreased the number of transmitted/founder viruses from high-dose SHIV challenge in macaques (46).

Clearing Acutely Infected Cells

Acutely infected cells must be cleared early to prevent the establishment of reservoirs. Toward this goal, Abs can engage innate effector cells through the Fc portion to stimulate Ab-dependent cellular cytotoxicity (ADCC), Ab-dependent cellular phagocytosis (ADCP), or complement-dependent cytotoxicity (CDC). To further improve Abs' capacity for cell-clearance, bNAbs may be engineered for enhanced Fc-mediated effector functions [described previously in Ref. (28, 72)] or modified through the conjugation of toxic payloads [reviewed in Ref. (148)].

Enhancing Ab Effector Function

Engineering strategies to augment Fc-mediated effector functions of HIV Abs were described in detail previously (28, 72), including IgG subclass switching and protein/glycoengineering to bias Fc receptor/complement component binding profiles. Multiple Fc-engineered mAbs have now entered and/or demonstrated safety and efficacy in various phases of clinical trials as well [reviewed in Ref. (149)]. The results of these studies will inform the capacity of *in vitro* and animal models of Fc-engineered Ab function to predict effector function in humans. They may further help to model the relationships between changes in Ab-Ag binding affinity, Fc-Fc receptor binding affinities, and clinically significant differences in effector functions in humans as has been described in animal models (150–152), and to determine whether there is an optimal Fc receptor binding affinity profile to elicit particular effector functions.

Immunotoxins

In acute infection, potent, transient cytotoxicity may be sufficient to inhibit reservoir establishment. Thus, conjugation of Abs with more toxic payloads such as bacterial exotoxins may be tolerable as a short-term solution to ensure rapid and complete cytotoxicity in place of or in addition to Fc-mediated effector functions to treat acute infection. In contrast, Ab-based immunotherapies that are more amenable to long-term use with more durable effects will be discussed in Section "Enhancing Therapeutic Potential for Chronic Infection" to treat chronic infection. In addition, viral Env has been suggested to be more highly expressed during early infection (153), making viral Env-targeting Abs potentially more useful as targeting agents during this period.

In one study, HIV-specific recombinant immunotoxin (RIT) employing Pseudomonas exotoxin A, 3B3-PE38, in combination with ART significantly decreased the number of HIV RNA-producing cells compared to ART alone in BLT humanized mouse models of HIV infection (154), although a potential for toxin immunogenicity and viral resistance were cited as limitations to chronic use of the immunotoxin. In a recent study testing a panel of HIV-specific mAbs as RITs, epitope specificity was found to correlate most with cytotoxicity against H9/NL4-2 cells (HIV Env expressing cell line), as compared to binding/neutralization potency (155). The most effective RIT employed mAbs targeting a non-neutralizing epitope in the gp41 loop region, which lies close to the plasma membrane and may thus allow the toxin to enter the cell more effectively (155, 156). Combination with soluble CD4 (sCD4) further increased the cytotoxicity of gp41 loop-targeting RITs, likely due to increased exposure of the gp41 epitope after sCD4 binding induced conformational changes in Env and increased internalization of Env-bound RITs in the presence of sCD4 (155, 157).

In vivo studies of another gp41-specific RIT employing a Ricin A chain (RAC) toxin, 7B2-RAC, also demonstrated efficacy in SHIV-infected macaques prior to the development of antidrug Abs after 2–3 weeks due to RIT immunogenicity (158). In the same study, to combat this observed immunogenicity, the authors PEGylated RITs prior to use in mouse models of HIV, which resulted in lower antidrug Ab levels in a subset of mice (158).

However, additional methods to decrease RIT immunogenicity [reviewed in Ref. (159)] may be required. In addition, cytotoxic payloads with decreased immunogenicity may be used instead of protein toxins to make antibody-drug conjugates (ADCs). In the SHIV macaque study of 7B2-RAC, ADCs employing existing small molecule cytotoxic drugs were also tested but were less efficacious than the RIT, likely because their drug toxicities were 1-log less potent than the RAC toxin (158). Thus, ADCs may become more competitive as more potent cytotoxic small molecule drugs are developed to rival recombinant toxins.

Preventing Cell–Cell Transmission

In addition to infection by free HIV, cells may become infected by horizontal transmission from other infected cells [reviewed in Ref. (160)]. The frequency with which cell–cell transmission occurs *in vivo* is unknown, but infection by cell-associated virus has been demonstrated in Macaque models of infection by SHIV-infected splenocytes (161), and suggested by studies of mother-to-child transmission of HIV during pregnancy, labor, and delivery [reviewed in Ref. (162)] and by spatial segregation of viral sequences (163). In addition, cell–cell transmission of virus was found to be more efficient than infection by free virus *in vitro* (164) and could lead to multiple infections of a single cell (165). A recent study found that different bNAbs exhibited Ab- and viral strain-dependent capacities to inhibit cell–cell transmission: for non-CD4bs-epitope targeting Abs, mAbs with increased potency of free virus neutralization exhibited greater losses in neutralization activity of cell–cell transmission, suggesting that optimal binding characteristics for free virus neutralization differ from those for cell–cell transmission neutralization (166). In another recent macaque study, bNAb PGT121 administered at protective concentrations against cell-free virus were only partially efficacious (3/6 macaques) at protecting from SHIV-infected splenocyte challenge (161). Studies to elucidate the mechanisms by which cell–cell transmission occurs and conformational differences in Env structure during transmission (167) would be beneficial to defining a strategy to improve this type of neutralization.

ENHANCING THERAPEUTIC POTENTIAL FOR CHRONIC INFECTION

Current therapy for chronic infection aims to suppress viremia to prevent symptoms from virus-stimulated immune activation and to prevent the growth/spread of viral reservoirs to preserve CD4+ T-cells. Today, ART largely accomplishes these goals to maintain low viral loads by blocking viral replication, but its use is limited by long-term end-organ drug toxicities, a strict requirement for treatment regimen adherence, and the development of viral resistance (168). In addition, persistent low-level viremia can remain even under ART treatment (169–171), potentially from cells infected prior to therapy initiation or in tissues with poor drug penetration (172) or residual virus replication in latently infected cells (169, 173, 174). Thus, therapeutic alternatives for chronic HIV infection that may lessen the burden or address limitations of ART are desired.

Encouraging results for the utility of bNAbs as treatment for chronic infection [reviewed in Ref. (26, 168)] from recent human clinical trials include effective suppression of circulating free virus in individuals harboring bNAb-sensitive strains (15, 17), delayed viral rebound after ART treatment interruption (14, 19) suggesting reduction of cell-associated virus or viral reservoir size (32), elicitation of host immune responses (19), and suppression of HIV replication in reservoir cells (175). Most of these results were found in a subset of treated individuals, dependent upon the preexisting resistance of circulating/reservoir strains, and in all cases viremia rapidly rebounded upon bNAb decay or cessation. Thus, strategies to combat both preexisting and *de novo* development of viral resistance remain a target of Ab therapy for chronic infection.

Combination with ARTs

Given the relative success of existing ART in treating chronic HIV infection, the comparison between bNAb therapy vs. ART or the benefit of adding bNAb therapy to ART has garnered interest. The potential for bNAbs to enhance the effects of ART lies in the ability to address residual sources of viral replication and further limit the development of viral resistance. One study found that the combination of bNAbs with ART was no better than treatment with ART alone in macaque models of SHIV infection (126), likely due to the already low level of viral replication and in some cases undetectable viremia of subjects undergoing ART alone in the observed period. On the other hand, ART significantly limits, but may not completely prevent, viral evolution of both circulating and tissue reservoir populations (176, 177). Thus bNAbs may be especially useful in combination with ART, which removes the major limitation of evolving resistance. In addition, the tissue distribution of ART and bNAbs or bNAb-based therapies may complement each other, with bNAbs "cleaning up" after persistent viral replication from virus-infected cells in tissue compartments receiving subtherapeutic levels of ART, such as lymph node germinal centers which may be more readily accessible to Ab- or Ab-based bispecific molecules interacting with APCs or T-cells (168). On the other hand, ART-mediated suppression of viral replication decreases the expression of Env epitopes on the surface of infected cells, and may thereby require more potent bNAbs or Abs targeting non-Env markers of infection.

Targeting Viral Reservoirs: Accessing Tissues and Identifying Cell Targets

Distinguishing which tissues and cell types can support viral reactivation and/or contribute to AIDS progression is critical to defining the extent of viral eradication desired/needed and the development of strategies with which to target cellular reservoirs. For viral remission, accepting persistent viral latency in some reservoirs with low reactivation potential and/or high costs of cellular/tissue damage may be acceptable. Multiple studies have suggested that decreasing the size of the viral reservoir delays viral rebound after ART is stopped (178–180), with one modeling study suggesting that a four-log reduction of the simulated 3×10^5 member reservoir size comparable to observed reservoirs of 10^5–10^7 (181) could prevent viral rebound after ART altogether (182).

Tissue Reservoirs: Distribution and Accessibility

Viral reservoirs may establish in multiple tissue sites (183) and cell types (184), making sufficient access to and efficacy in reservoir tissue sites and identification of target cells key components of combatting latent HIV infection. The primary site for viral replication occurs in central lymphoid tissues (18, 19), with lymph nodes, spleen, and GI tract lymphoid tissue harboring the largest numbers of HIV-infected cells (183). Unfortunately, these secondary lymphoid organs can act as pharmacologic sanctuaries limiting ART concentrations and viral suppression: lower concentrations of ART in lymph nodes (vs. blood) have been associated with persistent viral replication within lymph nodes (185). However, viral RNA/DNA has been found in nearly all tissues, including immune-privileged sites such as the central nervous system (CNS), testes, and placenta (183). Mixed evidence for compartmentalization, or differences in viral populations among different tissues and in circulation, exists (183) and may indicate a need for combination therapy with additional Abs, ART, or latency reversing agents (LRAs) with wider tissue penetration or more tissue-specific administration/targeting, such as liposomal delivery of drugs to the CNS [reviewed in Ref. (186)].

Reservoir Cell Types: Surface Markers of Infection

Within individual tissues, CD4+ T-cells comprise the majority of cell types harboring latent virus but viral DNA has been found in non-CD4+ T-cells [reviewed in Ref. (187)], including CD4−/CD8− T-cells (188), macrophages [reviewed in Ref. (189)], monocytes, tissue macrophages (190), and follicular dendritic cells (191, 192). Identifying reservoir cells can be challenging due to their relative quiescence and transient expression of low levels of viral antigens. Expression of HIV Env may additionally be different in latent cells as compared to cells with active viral replication. Given the instability of trimeric Env, non-neutralizing epitopes accessible on monomeric gp140 or gp41 stumps have been suggested to be displayed on the surface of infected cells over time (193). Thus, epitope targets of therapeutic HIV mAbs for chronic infection may vary significantly from those for the acute postexposure setting, reflective of the differing goals of targeting latent cells vs. active virus.

One strategy to combat this challenge is to identify non-viral surface markers that are expressed, or preferably upregulated, on infected cells. In an extreme example, CD52 expression on a wide breadth of immune cells capable of serving as reservoirs during HIV infection—nearly all T-cells, B-cells, and plasmacytoid dendritic cells—may be targeted by anti-CD52 Abs to deplete reservoir cells (194, 195), but uninfected immune cells may also be affected. Instead, Abs recognizing markers suggested to be upregulated by infection (196) may preferentially target reservoir cells and ameliorate some of the side effects expected from more general immune depletion strategies. In addition, these Abs may be used to guide the delivery of more toxic payloads in Ab-based therapies such as immunotoxins, bispecific T-cell engagers, or CARs in cellular therapy.

In another approach, LRAs may be used to re-activate cells and increase expression of viral antigens. However, the reactivation of virus increases the production of viral particles and risk of increasing cellular infection rates, and therefore must be balanced with potent elimination therapy, including bNAbs, in "shock and kill" strategies to quickly and efficiently eliminate reactivated cells. Coadministration of bNAbs with three viral inducers in humanized mice reduced the proportion of mice with viral rebound after Ab levels decayed, whereas Abs alone or combinations of bNAbs with a single inducer failed to affect viral rebound rates (132). Thus, strategies to optimize the combinations of Abs and inducers (25, 197) or to increase the potency or long-term effects (e.g., autologous immune responses) of Abs as elimination therapy may be necessary to maintain viral suppression after the decay of therapeutic Ab.

Long-term Clearance of Infected Reservoir Cells: Cellular Therapy

Natural Abs rely upon Fc-mediated effector function to clear infected cells. However, Ab-mediated effector functions may be less active or unavailable in infected tissue reservoirs with immunosuppressed or immune-privileged microenvironments. Thus, an alternative strategy to increase the potency with which Abs may destroy infected cells focuses upon addressing the limitations of T-cell-mediated responses. Effective cytotoxic T-cell responses have been associated with viral control in studies of relatively rare long-term non-progressors (198, 199) and HIV-exposed seronegative individuals (200). Similarly, persistent viral suppression after Ab therapy in a subset of SHIV-infected macaques (3 out of 18) was associated with improved host virus-specific cytotoxic T-lymphocyte (CTL) responses (13). Thus, anti-HIV Abs may be used to augment or complement cellular immune responses for long-term term viral control.

Engineering for Enhanced Cytotoxic Responses: CAR Cells

Rather than relying upon the natural development of host CTL responses, an alternative strategy employs HIV-specific Abs to re-direct T-cells toward HIV-infected cells. Promising bispecific T-cell engaging molecules (201, 202) and CAR T-cells (203, 204) have been previously reviewed (28) and are increasingly viable given the recent advent of the FDA's first recommendation for clinical approval of a CAR T-cell therapy (Novartis CTL019). Strategies with which to enhance the cytotoxic activity of bispecific T-cell engaging molecules and HIV-specific CAR T-cell approaches were described previously (28). This review thus focuses upon strategies with which to improve the clinical safety and efficacy of CAR therapies for HIV infection.

One concern is that HIV-binding CARs may render T-cells more susceptible to infection, especially CD4ζ-based CARs (205). Thus, strategies to protect anti-HIV CAR-modified cells include the cotransduction/expression of fusion inhibitors (206, 207), and knock-out/knock-down of CCR5 expression (208–211). A second concern is that the necessary expansion of engineered T-cells can lead to exhaustion and loss of activity (205), compounded by the fact that T-cells often already express inhibitory markers associated with exhaustion during chronic HIV infection (145, 146). To combat this predisposition for T-cell exhaustion, stem/progenitor cells may be modified with

CARs instead with the added benefits of the generation of more durable and potentially diversified cell types bearing the CAR, as well as the built-in thymic immune tolerance checkpoints through which T-cells developing from stem/progenitor cells must proceed (205). Hematopoietic stem/progenitor cells modified with a CD4ζ-CAR in humanized mouse models of HIV infection successfully differentiated and maintained CAR expression in multiple cell types, including T-cells and NK-cells, and reduced viral loads in treated animals (204).

More general concerns with the clinical use of cellular therapies as a class have been reviewed (212), and include the potential for cytokine storm from mass T-cell activation and cytotoxicity (213, 214), cellular transformation from genomic integration of viral vectors due to insertional mutagenesis (215), and autoreactivity (216). Strategies to mitigate these risks employ synthetic biology tools [reviewed in Ref. (217)] such as inducible suicide or "switch" strategies to induce apoptosis of CAR T-cells (218, 219), feedback-based "pause" switches (220), and preferential homing/activation based on "logic gate" requirements for engagement of multiple antigens (221–225).

Complementing Autologous T-Cell Responses: Access to T-Cell Sanctuaries

Cytotoxic T-lymphocyte trafficking patterns may limit their ability to access all viral reservoir sites (226). In one macaque study of SIV infection, the viral reservoir population of elite controllers was found to differ from that of progressors: elite controller macaques largely harbored virus in follicular helper T-cells (T_{FH}) whereas progressor monkeys harbored virus across a wider breadth of T-cell subtypes (226), suggesting that protective CTL responses may not be able to access T_{FH} reservoir cells. Thus the ability of bNAbs (or other anti-HIV Abs) to access and clear reservoir cells from CTL sanctuaries (such as T_{FH}s in B-cell follicles) is of particular interest (25).

Potential for a True "Cure": Viral Eradication vs. Reservoir Eradication

A true HIV "cure" would entail the complete eradication of virus from an infected individual, including all latent reservoir cells. By this definition, an extremely potent form of "shock-and-kill" strategies would likely be necessary to expose and eliminate all reservoir cells using HIV mAbs. In addition, the tangled link between viral eradication and tissue reservoir cell eradication poses a potential cost to these types of immunotherapy, especially in cases such as CNS reservoirs, where cells have limited regeneration capacity but make vital functional contributions to quality of life (186). Thus, alternative gene-editing approaches to specifically excise integrated viral DNA from infected cells (227) may be needed in combination with mAb-based approaches to achieve such a "cure."

In an alternative definition, a "cure" may be functionally described as undetectable levels of virus in the absence of additional therapy. Such a "functional cure" may be more feasible by the Ab-based strategies described above, with particular emphasis on the life-long delivery of immunotherapy (gene or cellular therapy) or the stimulation of sufficiently broad and potent autologous immune responses for life-long immune surveillance.

CONCLUSION

Preclinical studies of bNAbs to prevent and treat SHIV infection in macaques and Phase I human clinical trials demonstrating reduction of viral load and even reservoir size support the clinical utility and potential of bNAbs for prevention, postexposure prophylaxis, and therapy of acute and chronic infection. Observed and potential limitations of bNAbs noted thus far in these recent studies include the selection of resistant viral populations, immunogenicity resulting in the development of antidrug (Ab) responses, and the potentially toxic elimination of reservoir cells in regeneration-limited tissues. Opportunities to improve the utility of HIV Abs address these challenges and build upon each other as the timing/stage of infection progresses. Before exposure, bNAbs' ability to prevent infection by neutralization may be improved by increasing serum half-life to necessitate less frequent administration, delivering genes for durable *in vivo* expression, and targeting bNAbs to sites of exposure. After exposure and/or in the setting of acute infection, bNAb use to prevent/reduce viral reservoir establishment and spread may be enhanced by increasing the potency with which autologous adaptive immune responses are stimulated, clearing acutely infected cells, and preventing cell–cell transmission of virus. In the setting of chronic infection, bNAbs may better mediate viral remission in combination with ARTs and/or LRAs, by targeting additional markers of tissue reservoirs or infected cell types, or by serving as targeting moieties in engineered cell therapy. Finally, various combinations of the described bNAb applications may play a role in the development of a true "cure" for HIV to eradicate HIV entirely, although the risk of eliminating certain reservoir tissue cells may encourage the use of alternative strategies to eliminate viral DNA from latent cells without eradicating the cells. In conclusion, bNAbs are potent and promising agents for HIV prevention and treatment at various stages of infection. Their sole use as therapy faces challenges of viral evasion, immunogenicity, and reservoir latency, which can be combatted by employing various, often complementary strategies in combination with each other and/or existing ART regimens. While the clinical use of HIV Abs has never been closer, remaining studies to precisely define, model, and understand the complex roles and dynamics of HIV Abs and viral evolution in the context of the human immune system and anatomical compartmentalization will be critical to optimizing their clinical safety and efficacy.

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Immunologic Insights on the Membrane Proximal External Region: A Major Human Immunodeficiency Virus Type-1 Vaccine Target

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Broadly neutralizing antibodies (bNAbs) targeting conserved regions within the human immunodeficiency virus type-1 (HIV-1) envelope glycoprotein (Env) can be generated by the human immune system and their elicitation by vaccination will be a key point to protect against the wide range of viral diversity. The membrane proximal external region (MPER) is a highly conserved region within the Env gp41 subunit, plays a major role in membrane fusion and is targeted by naturally induced bNAbs. Therefore, the MPER is considered as an attractive vaccine target. However, despite many attempts to design MPER-based immunogens, further study is still needed to understand its structural complexity, its amphiphilic feature, and its limited accessibility by steric hindrance. These particular features compromise the development of MPER-specific neutralizing responses during natural infection and limit the number of bNAbs isolated against this region, as compared with other HIV-1 vulnerability sites, and represent additional hurdles for immunogen development. Nevertheless, the analysis of MPER humoral responses elicited during natural infection as well as the MPER bNAbs isolated to date highlight that the human immune system is capable of generating MPER protective antibodies. Here, we discuss the recent advances describing the immunologic and biochemical features that make the MPER a unique HIV-1 vulnerability site, the different strategies to generate MPER-neutralizing antibodies in immunization protocols and point the importance of extending our knowledge toward new MPER epitopes by the isolation of novel monoclonal antibodies. This will be crucial for the redesign of immunogens able to skip non-neutralizing MPER determinants.

Keywords: human immunodeficiency virus type-1, broadly neutralizing antibodies, membrane proximal external region, B-cells, polyreactivity, membrane interaction, immunization, immunogens

INTRODUCTION

An Apparently Easy Vaccine Target

The human immunodeficiency virus type-1 (HIV-1) envelope glycoprotein (Env) is the sole viral antigen exposed on the virion surface. Env is synthetized as a precursor gp160 glycoprotein that will yield after cleavage a mature complex constituted by the non-covalent association of three gp120 (surface) and three gp41 (transmembrane) subunits, resulting in a heavily glycosylated trimer of heterodimers (1–5). Env determines the process of HIV-1 entry into the target cell that

will lead to the fusion of the viral and host cell membranes (6). This process initiates with the high affinity interaction between gp120 and the CD4 molecule on the surface of target cells. This interaction promotes a series of conformational changes that transiently expose the gp120 coreceptor binding site allowing the gp120 attachment to the CCR5 or CXCR4 chemokine receptors (7–9). Coreceptor ligation triggers structural rearrangements in gp41 that permit the initiation of viral fusion. The gp41 fusion peptide (FP) inserts into the target cell membrane accounting for a short-life prehairpin fusion intermediate in which both cellular and viral membranes are connected by an extended conformation of gp41. Next, alpha-helical domains HR1 and HR2 of each gp41 monomer are folded back together to generate a 6-helix bundle conformation that brings both target cell and viral membranes closer to finally produce the membrane merge (10, 11). During this process both FP and the membrane proximal external region (MPER) play a crucial role in membrane destabilization (12).

Given its exposure on the virion surface and its role in viral infectivity, Env is the main target of HIV-1 protective humoral responses. The elicitation of Env broadly neutralizing antibodies (bNAbs), defined as those capable of neutralize the wide viral diversity, is one of the main goals for a successful HIV-1 vaccine (13). The notion that the human immune system is capable of producing HIV-1 bNAbs has been established by two pieces of evidence: (i) the identification of such immune responses in sera from HIV-1 infected individuals and (ii) the isolation of monoclonal bNAbs from these individuals (14, 15). These naturally induced bNAbs allowed the identification of conserved Env regions that helped researchers to delineate an HIV-1 Env vulnerability map. The study of bNAbs and the epitopes targeted by them are contributing enormously to our understanding of the HIV-1 humoral response as well as to the rational design of immunogen candidates (14, 16).

Whereas a big collection of bNAbs against gp120 Env subunit has been generated, a limited number has been also isolated against the less exposed gp41 subunit (17). Although neutralizing antibodies targeting the HR1 alpha-helical region have been described (18–20), the MPER is the major gp41 neutralizing determinant (21, 22). This highly conserved and unusual tryptophan-rich motif is located adjacent to the viral membrane, covering the last C-terminal residues of the gp41 ectodomain (aa 660–683, HXB2 numbering) and connects the extracellular portion of Env with the TM domain (23, 24). The importance of the MPER on Env functionality was highlighted by analysis of mutant viruses containing deletions, insertions or substitutions within this region (24–26). Substitution of the five MPER conserved tryptophan residues dramatically compromised the incorporation of gp41 into virions and, thus, blocked viral entry (24). Moreover, simple deletion of the W_{666}-I_{682} spanning sequence completely abolished syncytium formation (27). These observations indicated that the MPER plays a major role in the HIV-1 Env-mediated fusion and viral infectivity, which is consistent with the high level of sequence conservation (23). The functional implications in viral infectivity, the high level of conservation and the lack of N-linked glycosylated residues, together with the discovery of potent and/or bNAbs targeting linear MPER sequences (2F5, 4E10, 10E8), all able to protect against viral challenge in non-human primates (NHP) (28–30), points that the elicitation of MPER-specific neutralizing responses by immunogen candidates is highly desirable (21, 22, 31). In addition, the MPER has a role in HIV-1 CD4-independent viral transcytosis at the epithelial barrier (32), where the conserved $_{662}$ELDKWA$_{667}$ gp41 sequence interacts with galactosyl ceramide receptors (33). Secretory IgA from cervicovaginal secretions of HIV-1 infected individuals are capable of blocking viral transcytosis via $_{662}$ELDKWA$_{667}$ sequence binding (34).

The MPER presents some immunological, physical, and structural, properties that impact directly on its immunogenicity, explaining the lower MPER neutralizing response of HIV-1 infected individuals comparing with other Env vulnerability regions (35, 36). Those include steric hindrance by gp120 and high hydrophobicity that makes the MPER to be partially embedded within the viral membrane (37). Structurally, the information regarding the native conformation of the MPER within the Env trimer is still limited (5), adding the challenge of developing an immunogen against a structurally ambiguous epitope. Finally, MPER-specific bNAbs show reactivity against self-antigens and host tolerance mechanisms have been suggested to influence the elicitation of MPER neutralizing responses (38).

Here, we discuss the properties that make the MPER both a unique as well as a challenging HIV-1 vaccine target; we review the MPER immune response during natural infection, the particular features of MPER bNAbs isolated and the different attempts to generate MPER-specific neutralizing antibodies by immunization within the last years. Although the results reflect a generalized failure, new insights into our knowledge have been achieved. The fact that other Env vulnerability sites have followed a similar path supports the notion that the MPER is still an HIV-1 vaccine target worth exploring (31).

ISOLATION OF MPER NEUTRALIZING ANTIBODIES

The strongest evidence supporting that the human immune system can develop a potent neutralizing MPER-specific response results from the isolation of monoclonal antibodies from HIV-infected individuals. From the naturally induced 2F5, 4E10, 10E8, z13, m66.6, and CH12 antibodies identified, three of them (2F5, 4E10, and 10E8) display a broadly neutralizing activity (28, 39–46). 2F5 and 4E10 are among the first HIV-1 bNAbs discovered. They were generated by electrofusion of peripheral blood mononuclear cells mixtures from different HIV-1 infected individuals (47). 2F5 targets the linear sequence $_{662}$ELDKWA$_{667}$ (39) within the N-terminal moiety of the MPER, where the central core $_{664}$DKW$_{666}$ is essential for neutralization, as demonstrated by alanine-scanning mutagenesis assays (48). 2F5 has a relatively high potency and was found to neutralize 57–67% of the viral isolates tested with an IC50 below 50 μg/mL (42, 49). However, HIV-1 subtype C viruses are usually 2F5-resistant due to a mutation in the central core epitope (DSW instead of DKW) (49–51). 4E10 targets the distal conserved tryptophan rich moiety located C-terminal to the 2F5 epitope which includes

the sequence $_{671}$NWFDIT$_{676}$ and is extended toward C-terminal residues, where W672, F673, I675, T676, L679, and W680 have the most significant contacts with the antibody (43). Although presenting a moderate potency, 4E10 displays a remarkable breadth against 98–100% of the viral isolates, depending of the panel tested, with an IC50 below 50 μg/mL (49, 52). Further characterization of 2F5 and 4E10 antibodies has shown reduced potency of both antibodies, against transmitted-founder viruses (T/F IMC) or against replicating viruses obtained from primary lymphocytes when compared with pseudovirus obtained in 293 T cells (53–56). Despite these potential limitations, both 2F5 and 4E10 were shown to protect against viral challenge in NHP (28, 29) and their administration into human recipients showed no major clinical complications (57).

In order to delineate a complete map of HIV-1 neutralizing determinants, starting in 2009, a substantial effort has been made on the isolation of new bNAbs. The development of high-throughput analysis of single memory B cells and the use of fluorescently labeled Env-based protein probes to isolate antigen specific B cells (58–60) contributed enormously to the discovery of new HIV-1 neutralizing antibodies. In this context, the discovery in 2012 of the monoclonal antibody 10E8 recovered the interest toward the MPER region as a major vaccine target (42). 10E8 neutralized 98% of a panel of 181 pseudovirus with an IC50 below 50 μg/mL, showing a mean IC50 for sensitive viruses of 0.25 μg/mL, whereas mean IC50 values for 4E10 and 2F5 were 1.3 and 1.92 μg/mL, respectively (42). Interestingly, 72% of the panel was neutralized by 10E8 with an IC50 below 1 μg/mL, comparing with 37 and 16% for 4E10 and 2F5, respectively (42). Therefore, 10E8 could neutralize with a far greater potency and breadth than previously discovered anti-MPER bNAbs 2F5 and 4E10, and was comparable with some of the most potent HIV-1 bNAbs like VRC01 or PG9/PG16 (15). Notably, 10E8 was also reported to protect against viral challenge *in vivo* (30).

Interestingly, 2F5, 4E10, and 10E8 antibodies are IgG3 (42, 61); however, the role of this IgG subclass in the neutralizing properties of these antibodies, if any, remains elusive. Although IgG1 and IgG3 are the predominant antibodies elicited against viral antigens (62), both subclasses show important differences. IgG3 shows higher affinity for Fcγ receptors than IgG1, a shorter half-life and a long highly flexible hinge region which has been suggested to be crucial to facilitate the access of these antibodies to the MPER and mediate their neutralizing activity (63, 64). However, it is still unclear whether an IgG3 background is absolutely required, since anti-MPER neutralizing responses have been identified in the non-IgG3 fraction of some HIV-infected individuals (65), and a change to IgG1 did not affect the neutralizing activity of 2F5 and 4E10 antibodies (61, 66). In this context, anti-MPER bNAbs could have been specifically generated from germline precursors preferentially undergoing IgG3 class switching (67) and, in some cases, after affinity maturation and antigen selection by somatic hypermutation, switching to a more downstream IgG subclasses, such as IgG1, by sequential class switching recombination (68). Because IgG3 is one of the less represented IgG subclasses, with the shortest half-life in plasma and IgG3-dominant humoral responses are uncommon (63), elucidating whether this IgG subclass is required for the development of anti-MPER bNAbs, might be crucial to define immunization strategies aimed to generate effective long-lasting anti-MPER responses.

Independently of their origin, all these antibodies are the result of a long process of affinity maturation and are highly mutated with an unusually long and hydrophobic IgH complementary determining region 3 (CDR H3) (42, 69, 70). Notably, these antibodies share a common neutralization mechanism in which the interaction of the hydrophobic CDR H3 apex with the membrane seems to be essential (see next section) (71, 72). Accordingly, autoreactivity/polyreactivity are odd characteristics of 2F5 and 4E10 antibodies. Initially, 10E8 was reported to be non-polyreactive but subsequent studies suggested that 10E8 needs to bind membrane lipids, especially cholesterol, to mediate neutralization (42, 73, 74).

Depending on the bound antibody, the MPER can acquire a particular conformation. Crystal structures of 2F5 in complex with an MPER peptide showed that the core motif DKW forms a type 1 β-turn structure (75). Contrary, the MPER in complex with 4E10 was found to form an α-helical conformation from D674 to K683 (70, 76). Recently, the crystal structure of 10E8 bound to an scaffolded MPER construct revealed that the full epitope of 10E8 is composed of both MPER and lipids (74). Encouragingly, the frequency of 10E8-like antibodies in HIV-infected individuals seemed to be superior to 2F5 or 4E10 specificities in the cohort where 10E8 was isolated (42).

Very recently, a new lineage of distal MPER-specific bNAbs, designated as DH511, was isolated from memory B-cells and plasma of an HIV-infected donor (67). DH511 lineage presented long CDR H3 loops of 23 to 24 aminoacids, an VH and VL somatic mutation rate of 15–22 and 14–18%, respectively, and was derived from the same heavy chain germline gene family as 10E8 (VH 3–15). Similarly to 2F5, 4E10, and 10E8, DH511 clonal lineage presented an IgG3 isotype. Interestingly, the most potent mAb of this clonal lineage, DH511.2, neutralized 206 out of 208 pseudovirus of a geographically and genetically diverse panel with a median IC50 of 1 μg/mL, being slightly more broad but less potent than 10E8 (67).

LIPID BINDING AND THE CONCERN OF POLYREACTIVITY

MPER and Lipids

Biophysical models suggest that the MPER acquires an alpha-helical conformation partially embedded into the viral membrane, constituted by two independent domains separated by a flexible hinge (37, 77). These two segments showed to present different membrane-interacting properties. The C-terminal domain remains embedded into the membrane, whereas the N-terminal domain is more exposed (37, 77–79). The high tryptophan content is likely responsible of the MPER potential to interact with and destabilize lipid membranes (80, 81). According to its amphiphilic characteristics, hydrophobic residues remain buried into the membrane whereas the most polar ones are solvent-exposed (37). Of note, the MPER topology depends on the membrane context where it is presented (82, 83) and membrane

lipids such as cholesterol and sphingomyelin can modulate the capacity of the MPER to destabilize membranes (82, 83). MPER and cholesterol interactions are further supported by the existence of the sequence 679-LWYIK-683 located at the C-terminus which was identified as a cholesterol recognition amino acid consensus motif (84). This motif seems to play an important role during the incorporation of Env into the virion, stabilizing the trimer complex (22).

Neutralization Mechanisms and the Importance of Membrane Interaction

Antibody binding to a precise peptide sequence is necessary but not sufficient to achieve MPER-dependent antibody neutralization. Accordingly, z13e1 or 13H11 antibodies overlap the sequences bound by 4E10 and 2F5 respectively with similar affinities but displaying a far low neutralization potency (44, 85). MPER bNAbs show an enrichment of their long CDR H3 loops in hydrophobic residues that seem to be important for their neutralization capability (48, 86, 87). Whereas some residues of the CDRs are important for binding to the peptidic epitope, the most hydrophobic loops interact directly with membrane lipids (71, 72, 87). SPR-based studies demonstrated that whereas anti-MPER bNAbs bind to a peptide sequence following a Langmuir curve model, binding against peptide-membrane complexes follow a two steps (encounter-docking) model. First, the antibody attaches to the lipid membrane through its long hydrophobic CDR H3 and concentrates within the proximity of the MPER epitope to subsequently bind to the prehairpin intermediate of gp41, once the conformational change takes place (71, 72). This mechanism facilitates the accessibility of the antibody to its epitope, overcoming the poor exposure of the MPER and takes advantage of its close proximity to the viral membrane. Of note, upon binding, 2F5 or 4E10 promote an MPER conformational change, due to the extraction of the membrane-embedded epitope (37, 77).

Interestingly, the 2F5 antibody was predicted to bind lipids via CDRL1 and CDRH3 (88) and lipid binding sites were recently determined for 4E10 and 10E8 by X-ray crystallography (74, 89). 4E10 was shown to interact specifically with phosphatidic acid, phosphatidylglicerol and glycerol phosphate by using the CDR H1 and CDR H3 loops to bind polar head and hydrophobic tail groups respectively (89). In a second study, 10E8 lipid binding site was identified at the proximity of CDR L1 and CDR H3 loops (74). Therefore, the full epitope of MPER bNAbs is constituted by both peptide residues and membrane lipids. Notably, neutralizing activity of an anti-MPER single-chain bivalent llama antibody induced by immunization was also dependent of the hydrophobic CDR H3 apex without being involved in peptide recognition (87). Membrane interaction, thus, seems to play a major role in the neutralization mechanism of MPER bNAbs (26, 37, 72, 73, 77, 86).

The widely described importance of the membrane in MPER structure and functionality of the specific bNAbs suggest a role of lipids as a natural scaffold shaping the MPER structure. In this regard it is likely that lipids participate in the selection of germline precursors of bNAbs, pointing their relevance for immunogen design. Therefore, the generation of neutralizing anti-MPER responses may require its presentation within a membrane environment to properly present neutralizing determinants and to implement lipid cross-reactivity. The role of membrane lipids over MPER immunogenicity is, thus, a relevant issue currently being evaluated in immunization studies.

Binding to Self-Antigens: A Major Roadblock for MPER Neutralizing Antibodies?

Reactivity with self-antigens was suggested to explain the failure of generating MPER neutralizing antibodies by immunization as well as their low frequencies during natural infection (38, 90, 91). Gp41 antibodies generated during acute infection are usually derived from polyreactive antibodies whose precursors cross-react with antigens from intestinal microbiota (92–94). In 2005, polyspecific binding of 4E10 and 2F5 mAbs to cardiolipin and other anionic phospholipids was reported (90). Furthermore, conserved host antigens bound by 2F5, 4E10 and 10E8 have been also identified (95, 96). 2F5 binds to the enzyme kinureninase (KYNU), which contains the identical sequence (ELDKWA) of the 2F5 epitope, and is highly conserved between different mammal species. 4E10 binds to splicing factor-3b subunit-3 and type I inositol triphosphate (IP_3R1) (95) and, although initially described as non-autoreactive, 10E8 recognize the FAM84A protein (96). Collectively, these findings suggested that immunological tolerance might be involved in HIV-1 evasion of immune responses since autoreactive B-cells that cross-react with MPER sequences might be impaired in the naive repertoire (91, 97).

This hypothesis was tested by monitoring B-cell development in knock-in (KI) mice models carrying the same V(D)J rearrangements as mature bNAbs 2F5 and 4E10. These models showed a normal early B cell development but exhibited a blockade in the transition of pre-B to immature IgM+ B cells, which is defined by the first tolerance checkpoint (98–101). B-cell central tolerance takes place in the bone marrow (BM) and abrogates the development of autoreactive B-cells by several mechanisms such as clonal deletion or receptor edition (102). After that, some autoreactive B-cells can still egress from BM as anergic cells, which show a hyporesponder status and a reduced lifespan. However, in special circumstances anergic B-cells can be activated and differentiate to antibody-producing cells (103). In accordance with this, immunization of 2F5 KI mice with MPER peptide-liposome immunogens could rescue anergic B-cells to produce specific neutralizing antibodies (104, 105). More recently, a 2F5 germline KI mouse model showed 2F5 precursors deletion while the remaining anergic B cells could be also activated by germ-line mimicking immunogens (106). These outcomes indicated that the generation of 2F5 and 4E10 antibodies is likely controlled by immunological tolerance mechanisms and launched the hypothesis that HIV-1 host mimicry is an evolutionary strategy of pathogens and not particularly restricted to HIV-1 (95, 96). However, it is important to highlight that HIV-1 epitope mimicry does not impair the functionality of the host enzyme kynureninase, bound by 2F5 (107), and infusion of 2F5 or 4E10 in human recipients showed no major clinical complications (57), supporting

the safety of eliciting MPER protective antibodies by vaccination (57, 107).

THE MPER RESPONSE DURING NATURAL INFECTION AND BALANCE BETWEEN NEUTRALIZING AND NON-NEUTRALIZING ANTIBODIES

The whole gp41 is mostly occluded by gp120 within the native viral spike, being the MPER transiently exposed during the fusion process (25). In consequence, B-cells accessibility to gp41 and native MPER may be compromised. Despite this, a strong antibody response is generated against the gp41 subunit in the course of HIV-1 infection probably due to gp120 shedding, non-functional forms of Env or transient epitope exposure during viral entry (108). Interestingly, the anti-gp41 humoral response can be detected two weeks after HIV-1 acquisition (108). This response, typically non-neutralizing and highly cross-reactive to gut commensal bacteria (92–94), is mainly focused against more exposed regions of gp41 such as the immunodominant disulfide loop, different from the MPER (108, 109).

Whereas MPER antibodies can be easily detected by ELISA, the analysis of their contribution to neutralizing activity of human plasma samples was found to be challenging. With this purpose chimeric SIV or HIV-2 viruses engrafted with HIV-1 MPER sequences or peptide-coated beads adsorption assays were developed (110–112). Accordingly, the presence of anti-MPER antibodies and the evaluation of their neutralizing capacity have been reported (35, 36, 65, 111, 113–116). The characterization of different cohorts in Europe, the United States, and South Africa indicated that MPER-specific neutralizing responses are less represented during natural infection comparing with other neutralizing specificities. For example, in a South African cohort of 156 HIV-1 infected individuals, only three showed higher titers of anti-MPER antibodies (65). Depletion of these antibodies resulted in loss of the neutralization breadth but the antibody specificities were found to be targeting a distinct epitope from those recognized by previously identified neutralizing epitopes (bound by 2F5 and 4E10 bNAbs), highlighting the existence of additional neutralizing specificities within the MPER (65). A recent study of the Protocol C cohort analyzed the neutralization profile of 439 plasma samples showing a far great less prevalence of MPER-specific antibodies when comparing with other specificities, mainly V3 N332-dependent glycan supersite (36). Remarkably, 27% of HIV-1 infected patients from an American cohort presented MPER-specific neutralizing activity (42). We previously showed that 66% of ART-naive chronically HIV-1 infected subjects presented MPER antibodies that were stable, at least for 1 year, and with an heterogeneous neutralizing capacity, highlighting the coexistence of neutralizing and non-neutralizing antibodies targeting the MPER (117). Moreover, anti-MPER antibodies correlate with the total anti-Env humoral response (117) and neutralization breadth (113, 118) and have been identified in HIV-infected individuals at different stages of the infection (119). Therefore, this landscape highlights that regardless of the cohort of study, anti-MPER antibodies (neutralizing and non-neutralizing) are present in HIV-1 infected subjects but their prevalence seems to be highly heterogeneous and probably strongly dependent on the methodology used (42, 65, 114, 117–119). Thus, the optimization of the current methodology for the quantification of MPER antibodies is highly desirable in order to establish their real prevalence. Human studies characterizing the MPER-specific neutralizing response are summarized in **Table 1**.

The results obtained from these studies also point out that the MPER is sufficiently immunogenic to generate a humoral response and that no specific constraints limit antibody generation against this region. However, the relatively low prevalence

TABLE 1 | Human studies detecting MPER-specific neutralizing responses.

Year published	Number of participants	Main findings	Reference
2006	96	One individual with 4E10-like neutralizing activity. No epitope competition	(110)
2007	3	No MPER-specific neutralizing activity	(112)
2007	14	4 individuals with MPER-specific neutralizing activity. 2 of them within the 6 months after seroconversion. No correlation with breadth	(111)
2009	156	3 individuals high MPER titer, associated with breadth. Distinct epitope from 4E10, 2F5, or z13	(65)
2009	70	MPER titer correlated with breadth. 4E10-like. Anti-cardiolipin antibodies correlated with breadth and MPER titer	(113)
2009	32	MPER-specific neutralization in 4 individuals	(114)
2010	19	Modest MPER-specific neutralization in 6 individuals	(35)
2011	308	4 out of 9 breadth neutralizers displayed MPER-specific neutralization (17–30% contribution)	(116)
2011	40	7 individuals > 40% breadth. MPER cross-neutralizing antibodies	(115)
2012	78	21 MPER-specific neutralizing activity. 8 out of 21 displayed 10E8 neutralization pattern	(42)
2014	35	8 individuals showed ID50 > 400 against chimeric HIV-2/MPER viruses whereas 66% had detectable MPER titers in ELISA and flow cytometry	(117)
2015	177	19% of the cohort showed MPER-specific neutralizing titers (ID50 > 1,000) against chimeric HIV-2/MPER viruses	(118)
2016	439	One individual with potent MPER-specific neutralizing activity	(36)

of MPER-neutralizing responses identified to date indicates that some hurdles are involved in the generation of this type of antibodies. The low accessibility of this region, which may compromise the affinity maturation process, as well as other mechanisms such as lipid cross-reactivity, might be determinant for the establishment of a balance between neutralizing and non-neutralizing MPER antibodies. Therefore, this balance is a relevant issue with important implications for vaccine design, where immunogens exposing native MPER neutralizing determinants should be implemented.

ELICITING ANTI-MPER ANTIBODIES BY IMMUNIZATION

The particular features of the MPER described above, mainly low accessibility, close proximity to the membrane and subsequent hydrophobicity add additional hurdles for immunogen design against this vulnerability site. Moreover, the scarcity of MPER bNAbs isolated to date, comparing with other Env specificities does not contribute to enlarge our knowledge regarding the MPER complexity and the functional epitopes that should be targeted.

Initial approaches to induce 2F5 or 4E10-like antibodies attempted to introduce their corresponding binding sequences into chimeric viruses, fusion proteins or peptide-based vaccines (21). Although MPER-specific antibodies were elicited, neutralizing responses were not. Therefore, it became clear that additional variables beyond the recognition of specific peptidic sequences within the MPER should be considered. The common characteristics revealed later by MPER bNAbs, such as membrane cross-reactivity and binding to the gp41 prehairpin intermediate (72, 120), suggested that similar antibodies could be obtained by presenting MPER-based antigens in such precise conformational states in a membrane-like environment. In accordance, there are two major standpoints that are currently being addressed in MPER-based vaccinology: (i) what are the relevant structures that most likely mimic the native-bound form of MPER bNAbs and (ii) which is the role of membrane lipids over the MPER immunogenicity, including the precise lipid components and adjuvant systems. A summary of the most recent (since 2010) strategies followed in immunization protocols are listed in **Table 2**.

Conformational states bound by anti-MPER bNAbs have been approached (121, 122, 129, 133). The use of computational methods permitted the design of scaffolds consisting in unrelated

TABLE 2 | Selection of recent immunization studies to elicit MPER neutralizing antibodies.

Immunogen	Animal model	Major findings	Reference
Prime/boost gp140 oligomer/MPER-peptide liposome	Guinea pig Rhesus macaque	Binding to the prefusion intermediate and the DKW 2F5 core	(121)
Liposomes containing a trimeric gp41-based protein	Llama	Bivalent single chain neutralizing antibody dependent of hydrophobic CDRH3	(87)
Fusion intermediate conformation of gp41 convalently linked to liposomes	Guinea pig	Gp41-specific antibodies binding to the gp41 fusion intermediate. Modest neutralization activity against 5 tier-1 and 2 tier-2 pseudovirus	(122)
Liposomes containing an MPER peptide, molecular adjuvants and encapsulated T-helper epitopes	Balb/c mouse	Superior antibody titers with MPER antigens anchored to liposomes comparing with oil-based emulsions	(123)
Proteoliposomes of diverse composition containing a gp41-based miniprotein	C57BL/6 mouse	Superior antibody titers of proteoliposomes based on lipids overrepresented on the viral membrane. Immunodominance against a 2F5 overlapping epitope	(124)
Recombinant Norovirus P particles (NoV PP) engrafted with the 4E10/10E8 epitopes emulsified with Freund's adjuvant	Guinea Pigs Balb/c mouse	MPER-specific antibody titers and modest neutralization against SF162 isolate	(125)
MPER engrafted between the trimeric core structure and the trimeric domain of influenza A virus	Guinea pig	Induction of low MPER-specific titers	(126)
Bovine papilomavirus VLPs engrafted with the extended epitopes of 2F5 and 4E10, or the full MPER	Balb/c mouse	Epitope-specific IgG and mucosal secretory IgA	(127)
Engineered replication-competent reovirus vectors displaying the MPER sequence	Rabbit Balb/c mouse	No elicitation of MPER antibodies	(128)
Epitope-engrafted scaffold mimicking the 2F5-bound form of gp41	Guinea pigs Balb/c mouse	Isolation of antibodies resembling the 2F5 structure-specific recognition of gp41	(129)
Tandem peptide containing four copies of the 10E8 epitope with Freund's Adjuvant	Rabbit	Modest neutralizing antiboy titers against tier-1 and tier-2 strains	(130)
Live attenuated *Salmonella* presenting the 10E8 epitope in the frimbriae	Balb/c mouse	MPER-specific antibodies and stimulated B-cell differentiation	(131)
Gp41 peptide grafted on virosomes	Rhesus macaque	Protection against SHIV challenge was correlated with the induction of vaginal gp41-specific IgA with transcytosis-blocking properties	(132)

protein structures selected from database but able to accommodate the neutralizing 2F5 binding sequence in a conformation close to the peptide-bound crystal structure. Such scaffolds induced polyclonal responses mimicking a 2F5-like binding profile in immunized animals (129). Crystallographic analysis confirmed that monoclonal antibodies isolated from immunized animals mimicked the conformation of 2F5 in a flexible gp41 peptide, high affinity to the same sequence and similar angle of epitope approach (129, 134). Same outcomes were obtained with scaffolds targeting the 4E10 (135) and z13e1 (136) binding motifs. In spite of such structural mimicry, neutralizing activity was not achieved, likely because additional features such as membrane binding were not addressed in the design of these scaffolds.

Due to the importance for neutralization and their implication in a substantial portion of the free energy of 2F5, 4E10, and 10E8 binding, lipid-containing immunogen are important platforms being explored (71, 87, 88). Given that the complete epitope of anti-MPER bNAbs includes membrane components (74, 89) and that lipid recognition by CDR H3 impacts into their functionality (69, 72, 73, 86, 87), their potential for contributing to MPER-specific neutralizing responses by immunization is worth exploring. In this regard, membrane-mimicking platforms including viral-like particles (VLP) (137, 138) or liposomes (122–124) have been approached. It has been shown that membrane lipids can modulate the MPER structure likely by promoting a native-like conformation and demonstrated to improve immunogenicity (123, 124). In particular, we previously demonstrated that those lipids overrepresented in the viral membrane such as cholesterol and sphingomyelin have the potential to induce stronger antibody titers comparing with simple POPC lipids (124). Interestingly, MPER-specific antibodies from long-lived Bone marrow plasma cells from mice immunized with antigen-coupled liposomes have been also reported. Those antibodies showed that were shaped under selective pressure promoted by the MPER in the context of lipids and did not display any polyreactive feature (139).

Whereas the implementation of lipid-based platforms achieved MPER-specific antibodies, modest neutralizing titers have been reported by a few studies. For example, liposome-peptide antigens in combination with MPLA molecular adjuvant led to the isolation of two MPER-specific IgM antibodies showing lipid cross-reactivity but limited neutralizing capacity (140). The use of an HA/gp41 fusion protein in viral like particles induced modest 4E10-like neutralizating titers (141). One study by Dennison and colleagues obtained MPER-specific antibodies in NHP which bound preferentially to the gp41 prehairpin fusion intermediate rather than a recombinant gp41 construct by using a gp140 oligomer prime boosted with liposomes exposing an MPER peptide regimen. Such preferential binding was thought to be primarily due to structural modifications induced by the liposomes where the antigen was presented (121). Furthermore, the response mapped specifically the 2F5 DKW neutralizing core (121). In spite of these promising results, neutralizing activity was not achieved. Mimicking the gp41 prehairpin intermediate has been also approached by the design of a gp41 immunogen formulated in proteoliposomes. Immunization of guinea pigs showed modest neutralizing titers against tier 1 viruses, although the specificities responsible for such neutralization were not delineated (122). Finally, the role of non-neutralizing antibodies in protection has been shown in some studies. The presence of vaginal IgA with ADCC and transcytosis-bocking properties induced by gp41-grafted virosomes was associated with protection of NHP against SHIV challenge (132). Such vaccine platform was also evaluated in a Phase I clinical trial in healthy women. Vaginal secretions of vaccinated subjects were found to present transcytosis-blocking properties *in vitro* (142).

REMARK

In spite of the recent advances into the MPER physical and immunological properties, we still lack a full roadmap to generate a neutralizing response against this HIV-1 Env vulnerability site. The outcomes derived from MPER immunization studies clearly demonstrate that lipid cross-reactivity, binding to certain neutralizing epitopes or binding to gp41 native structures like the prehairpin intermediate are achievable. Although the implementation of these features will have a crucial role they will be likely insufficient to achieve the full properties of MPER-specific bNAbs in immunization protocols. In contrast, the selection of MPER non-neutralizing antibodies whose B-cell precursors may compete for the antigen presented cannot be excluded. While the knowledge gained from other Env vulnerability regions has advanced from the higher number of bNAbs isolated, to date only the potent 10E8 as well as 2F5 and 4E10 antibodies have been isolated. This fact highlights the need of the isolation of additional MPER bNAbs in order to bypass these gaps of our knowledge, improving immunogen design, while avoiding immunodominant non-neutralizing epitopes.

AUTHOR CONTRIBUTIONS

LM-A drafted the manuscript, JC reviewed the manuscript and JB and BC made substantial, direct, and intellectual contribution to he work. All authors approved it for publication.

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Glutaraldehyde Cross-linking of HIV-1 Env Trimers Skews the Antibody Subclass Response in Mice

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Well-ordered soluble HIV-1 envelope glycoprotein (Env) spike mimetics such as Native Flexibly Linked (NFL) trimers display high homogeneity, desired antigenicity, and high *in vitro* stability compared to previous generation soluble HIV-1 Env trimers. Glutaraldehyde (GLA) cross-linking was shown to further increase the thermostability of clade C 16055 NFL trimers and enhance the induction of tier 2 autologous neutralizing antibodies in guinea pigs. Here, we investigated if GLA fixation affected other aspects of the Env-specific immune response by performing a comparative immunogenicity study in C57BL/6 mice with non-fixed and GLA-fixed 16055 NFL trimers administered in AbISCO-100 adjuvant. We detected lower Env-specific binding antibody titers and increased skewing toward Th2 responses in mice immunized with GLA-fixed trimers compared to mice immunized with unfixed trimers, as shown by a higher Env-specific IgG1:IgG2b antibody subclass ratio. These results suggest that the presence of GLA adducts on Env influences the quality of the induced antibody response.

Keywords: HIV-1 env, gluteraldehyde, cross-linking, immunogenicity, mice, vaccine responses, antibody isotypes

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INTRODUCTION

Most licensed vaccines mediate protection through the induction of highly specific IgG serum antibodies. Consequently, a central goal for HIV-1 vaccine development is to induce antibody responses that are capable of neutralizing a broad range of circulating HIV-1 strains. Over the past decades, HIV-1 envelope glycoprotein (Env) immunogen design efforts have focused on the generation of recombinant, soluble trimeric Env variants consisting of the exterior glycoprotein, gp120, and the ectodomain of the transmembrane protein, gp41, such as the foldon trimers and the SOS trimers (1, 2). As is now appreciated, these early generation trimers were structurally heterogeneous and suboptimal antigenic mimics of the functional HIV-1 spike.

More recently, new generation trimers such as the BG505 SOSIP.664 trimers (3, 4) and various forms of the native flexibly linked (NFL) trimers (5) were designed. These soluble spikes display superior threefold symmetric order and improved antigenic profiles. The SOSIP trimers were the progenitors, containing an internal cysteine linkage between gp120 and gp41 and an isoleucine (I) to proline (P) change in gp41 (I559P) to disfavor the post-fusion conformation of HIV-1 Env (2). The NFL trimers were constructed by replacing the furin cleavage site that is naturally present

between the exterior glycoprotein gp120 and the transmembrane protein gp41 with a flexible linker composed of two repeats of four glycine and one serine residues $(G_4S)_2$. This modification renders these trimers cleavage- and furin-independent, forming a covalent linkage between gp120 and the ectodomain of gp41 (5–7). Besides the I559P change, further developments of the NFL trimers included the introduction of a set of substitutions selected from the BG505 Env sequence, referred to as trimer-derived (TD), which favor the production of trimers that display ordered symmetry and desired antigenic profiles and can be applied to diverse HIV-1 strains (6). Additional modifications of the NFL TD trimer design performed in the context of the Indian clade C isolate 16055 was the introduction of a cysteine bond between residues I201C and A433C to retain gp120 in the pre-CD4-bound conformation, resulting in the 16055 NFL TD CC trimers (6). A set of glycine substitutions in selected gp41 coil-to-helix transition residues were also introduced to further stabilize the pre-fusion state (8).

In addition to efforts using targeted mutagenesis of Env to improve trimer stability, glutaraldehyde (GLA) cross-linking was shown to improve the thermostability of HIV-1 Env trimers as well as the induction of neutralizing antibody responses (7, 9, 10). Depending on the specific Env construct used, negative or positive selection of the trimers may be required prior to fixation to enrich for conformers with desired antigenicity. While intramolecular protein cross-linking may provide a benefit in terms of increasing the durability of conformationally sensitive neutralizing antibody epitopes *in vivo*, less is known about whether fixation affects other aspects of the Env-specific immune response. In this study, we addressed this issue by immunizing C57BL/6 mice with either fixed or unfixed 16055 NFL TD CC trimers formulated in AbISCO-100 adjuvant. After the first boost, we observed an overall reduction in Env-specific serum-binding antibody titers in mice inoculated with fixed trimers compared to mice inoculated with unfixed trimers, which was primarily detected when coating was performed with unfixed trimers. However, this difference was modest when fixed trimers were used for coating and especially after an additional boost. We further detected a pronounced skewing toward Th2 responses with significantly altered Env-specific IgG1:IgG2b ratios in the sera of mice immunized with GLA-fixed trimers compared to mice immunized with unfixed trimers. A similar effect was detected for the IgG1:IgG2c ratios and a trend toward increased production of Th2 cytokines from stimulated CD4+ T cells was observed in mice immunized with fixed trimers. These results demonstrate that protein cross-linking influences the induced antibody responses at several levels *in vivo.*

MATERIALS AND METHODS

Animals, Immunizations and Reagents

Male C57BL/6 Bom mice were purchased from Taconic, Denmark. Mice were immunized subcutaneously with 10 µg of recombinant 16055 NFL TD CC trimers together with 10 µg AbISCO-100 adjuvant (Isconova/Novavax) or with adjuvant alone. The mice were 7–9 weeks of age at the start of the immunizations, and booster immunizations were performed at 4-week intervals. All mice were kept at the animal facility of the Department of Microbiology, Tumor and Cell Biology at Karolinska Institutet. All animal experiments were performed under approved conditions and standard guidelines prior to the experimental start according to the regulations of the Committee for Animal Ethics (Stockholm, Sweden).

Expression and Purification of Soluble Env Trimers

The 16055 trimers were produced as previously described (5, 6). Briefly, the trimers were expressed in 293F cells and were isolated by lectin-affinity chromatography using GNL (*Galanthus nivalis* lectin-agarose; Vector Labs), purified by size-exclusion chromatography (SEC) using Superdex™ 200 columns (GE Healthcare Life Sciences) to isolate the predominant trimeric fractions and further purified by negative selection affinity chromatography using the non-neutralizing CD4bs-directed mAb, GE136 (11).

Trimer Cross-linking and Gel Analysis

Cross-linking of the purified 16055 trimers was conducted as previously described (7). Briefly, 0.5 mg/ml of trimer was fixed with 5 mM GLA (ACROS Organics) at room temperature (RT) for 5 min and then the reaction was quenched by excess 50 mM glycine, pH 7.5. The fixed trimers were negatively selected by GE136 antibody affinity chromatography and re-isolated by Superdex™ 200 size-exclusion chromatography and then analyzed by SDS-PAGE under reducing and non-reducing conditions and by Blue Native PAGE as described previously (7).

Differential Scanning Calorimetry (DSC) and Negative-Stain Electron Microscopy (EM)

The thermal melting (Tm) of the trimers was determined using a Microcal VP-Capillary DSC (Malvern). Briefly, trimers were diluted in PBS pH 7.4 to 0.25 mg/ml and scanned at a rate of 1°C/min. Data collected were analyzed after buffer correction, normalization, and baseline subtraction using the VP-Capillary DSC Automated data analysis software. For EM analysis, the 16055 NFL unfixed and fixed trimers were negatively stained on glow-discharged carbon-coated copper mesh grids (Electron Microscopy Sciences) for 2 min. Following blotting to remove excess sample, grids were transferred onto droplets of 2% phosphotungstic acid (pH 6.7) for 2 min. Following blotting and drying the grids were analyzed on a Philips CM100 electron microscope and imaged at selected magnifications with a Megaview III charge-coupled-device camera.

ELISA for Antigenic Profiling and Detection of Serological Antibody Responses

To assess binding by selected bNAbs and non-neutralizing mAbs, the 16055 NFL CC TD trimers were captured by their

His-tag using a mouse anti-His antibody coated on the ELISA plate overnight (ON), followed by washing, blocking, and detection using anti-mouse IgG as described below. To detect Env-specific antibody responses in serum, 96-well high-protein-binding MaxiSorp (Nunc) plates were pre-coated with 1 µg/ml *Galanthus nivalis* lectin (Sigma) diluted in PBS and incubated ON at 4°C. Plates were then washed six times in washing buffer (PBS/0.05% Tween-20) followed by addition of 150 µl/well blocking buffer (2% fat-free milk in PBS) and incubated for 1 h at RT. After incubation, the blocking buffer was removed from the plates and 200 ng/well unfixed or fixed NFL Env trimers were added and let to incubate at RT for 2 h. Plates were washed six times in washing buffer and were then incubated in blocking buffer for 1 h. After removing the blocking buffer, sera were added to the plates in threefold serial dilution starting at 1:25 dilution in blocking buffer and incubated for 2 h at RT. After washing the plates six times in washing buffer, secondary antibody diluted in PBS was added to each well. For total, Env-specific IgG ELISA, the secondary antibody goat anti-mouse IgG-horse radish peroxidase (HRP) (Southern Biotech) was used in a dilution of 1:1,000. For subclass-specific Env serum antibody detection, goat anti-mouse IgG1-HRP (Southern Biotech) (1:5,000), goat anti-mouse IgG2b-HRP (Southern Biotech) (1:5,000), goat anti-mouse IgG2c-HRP (Southern Biotech) (1:5,000), or goat anti-mouse IgG3-HRP (Southern Biotech) (1:1,500) were added. Secondary antibodies were incubated at RT for 1 h and removed by washing six times in wash buffer. To develop plates, 100 µl/well of TMB stabilized chromogen substrate (Invitrogen) was added and incubated for 10 min in dark at RT. The reaction was stopped by adding 1 M H_2SO_4. The optical density was measured at 450 nm using an Asys Expert 96 ELISA reader (Biochrom).

Preparation of Single Cell Suspension

The mice were sacrificed by cervical dislocation and spleens were taken out for further analysis. Single cell suspension of splenocytes was obtained by passing the dissociated spleen through a 70-µM nylon cell strainer. Hypotonic ammonium chloride solution was used to lyse the red blood cells. Splenocytes were then collected in complete RPMI 1640 medium (containing 5% FBS, 50 µM 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µM streptomycin), and cell numbers were calculated using the automated cell counter Countess (Invitrogen) for further experiments.

CD4+ T Cell Depletion

To deplete CD4+ T cells, the protocol from EasySep negative selection kit was followed (Stemcell Technologies). Briefly, splenocytes were incubated with normal rat serum (Stemcell Technologies) and biotinylated rat anti-mouse CD4 antibody (clone: RM4-5; BD Pharmingen) for 10 min with mixing every third minute. EasySep Strepativin Rapid Sphere 50001 beads (Stemcell Technologies) were added to the cell mixture at a concentration of 75 µl/ml of cell suspension. CD4+ T cells were separated using an EasySep magnet (Stemcell Technologies) and the negative fraction was collected in a new tube and used for further experiments.

Flow Cytometry

Total splenocytes and CD4-depleted cell fractions were stained on ice for 20 min with the following antibodies: CD3e-PE (145-2C11; eBioscience), CD8a-APC (53-6.7; BD Pharmingen), CD4-FITC (H129.19; BD Pharmingen), and B220-PerCP-Cy5.5 (RA3-6B2; BD Pharmingen). The samples were run on a FACSCalibur cytometer (BD Bioscience), and data were analyzed with FlowJo software version 10 (TreeStar).

T Cell ELISpot Analysis

T cell ELISpot analysis was performed to measure cytokine production after stimulation of total splenocytes. 96-well Multiscreen-IP filter plates (Millipore) were pre-treated with 70% ethanol, washed three times in PBS followed by coating with 5 µg/well (50 µg/ml) of anti-mouse IFNγ (mAb: AN18), anti-mouse IL-2 (mAb: 1A12), or anti-mouse IL-4 (mAb: 11B11), all from Mabtech AB. Plates were incubated ON at 4°C. Before addition of splenocytes, the plates were washed six times with PBS/0.05% Tween-20 and blocked in complete RPMI medium for 2 h at 37°C/5% CO_2 in a humidified incubator. After incubation splenocytes, in triplicates, were added to the wells in three different concentrations (200,000, 100,000, or 50,000 cells) in a final volume of 150 µl and stimulated with one of the following stimuli: ConA (2 µg/ml) (Sigma), unfixed or fixed NFL trimers (6.67 µg/ml), or left unstimulated in medium only. After 20 h stimulation at 37°C/5% CO_2 in a humidified incubator, the cells were removed from the wells and the plates were washed six times with PBS/0.05% Tween-20. Then the following biotinylated secondary antibodies in a concentration of 1 µg/ml (Mabtech AB) were added to the corresponding wells: anti-mouse IFNγ (mAb: R4-6A2), anti-mouse IL-2 (mAb: 5H4), or anti-mouse IL-4 (BV06-24G2). After incubation at RT for 2 h, the plates were washed six times in PBS only and streptavidin-ALP (Mabtech AB) in a 1:1,000 dilution was added to wells and incubated at RT for 45 min. After washing with water, plates were developed with 100 µl/well of BCIP/NBT plus substrate (Mabtech AB) for 10 min at RT. To stop the reaction, wells were emptied and washed extensively in water followed by air-drying. The spots were counted in an ImmunoSpot analyzer (CTL Immunospot).

Flow Cytometric Bead Array (CBA) to Detect Cytokines after *In Vitro* Stimulation

Total splenocytes from mice immunized three times were stimulated *in vitro* for detection of cytokine production. One million splenocytes were used for each stimulation in 48-well plates in a total volume of 500 µl. Each mouse was stimulated with either ConA (2 µg/ml) (Sigma), unfixed or fixed NFL trimers (6.67 µg/ml) or left unstimulated in medium only. The plates were incubated for 20 h at 37°C/5% CO_2 in a humidified incubator. Plates were spun down, and supernatants were collected. To measure the secreted cytokine from each mouse and stimuli, the BD CBA Mouse Enhanced Sensitivity Master Buffer Kit (BD Bioscience) was used. IL-5, IL-10, and IL-13 (BD Bioscience) were measured in all samples according to manufacturer's instruction. Standards were prepared from Top

Standard by threefold dilutions down to 1:729. Each sample was diluted in two different dilutions, 1:2 and 1:20. Diluted samples were mixed and incubated with Capture Beads for 2 h in dark at 4°C. The samples were then washed in FACS Flow for 5 min at 300*g*. Supernatant was flicked off before Mouse Detection Reagent was added and incubated for 2 h in dark at 4°C. After an additional washing step, the Enhanced Sensitivity Detection Reagent was added. After 1 h incubation in dark at 4°C, the samples were washed and ran on FACSVerse (BD Bioscience). Standard curves were generated for each cytokine. The samples were then calculated based on the median fluorescence values. If the value was lower than the standard curve, those samples were considered as 0. Samples higher than the detection limit (based on standard curve) was excluded.

Statistical Analysis

GraphPad Prism software version 8 (San Diego, CA, USA) was used to analyze data by Student's *t*-test. Significance was defined as $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$.

RESULTS

In Vitro Characterization of Unfixed and Fixed Env Trimers

In this study, we used the well-ordered 16055 NFL TD CC Env trimers (**Figure 1A**) to investigate the effect of GLA fixation on Env-specific immune responses in C57BL/6 mice. Following the cross-linking procedure, the fixed trimers were isolated by negative selection and size-exclusion chromatography (SEC). Analysis of the purified GLA-fixed trimers by reducing SDS-PAGE confirmed that cross-linking of the trimers had occurred, by a shift in the apparent molecular weight (MW) relative to the unfixed trimers. GLA cross-linking rendered the trimers resistant to disulfide-directed reduction (**Figure 1B**, left). BN-PAGE analysis, under native conditions, revealed that both the unfixed and fixed trimers migrated with similar MWs (**Figure 1B**, right). Both sets of data indicated that the cross-linking had occurred between protomers within each trimer, but not across individual trimers. These results were consistent with what we had reported previously by these types of analyses (7). To measure the thermal stability of the unfixed and fixed 16055 NFL TD CC Env trimers, we used DSC. The GLA-fixed Env trimers displayed a higher thermostability compared to the unfixed counterpart. The thermal denaturation midpoint temperature (Tm) differed nearly 10°C between the two proteins (**Figure 1C**, left), indicating that GLA cross-linking contributes to the overall stability of the protein. The increased Tm was accompanied by a broadening of the thermal transition profile, indicating some molecular heterogeneity following the GLA cross-linking process. To confirm that the trimers remained as single particles following cross-linking and negative selection, we performed negative-stain EM and observed no marked difference in trimers at this level of resolution comparing unfixed to fixed populations (**Figure 1C**, right). To confirm trimer concentrations and that selected epitopes were minimally affected following fixation, we performed ELISA using the bNAbs VRC01, PGT121, and 2G12. We observed that PGT121 recognition was not greatly affected, indicating that the protein concentrations were accurate, whereas there was some decrease in VRC01 and 2G12 recognition following GLA fixation. We included the non-neutralizing antibodies 19b and GE136 that poorly recognized the unfixed trimers, as expected, whereas recognition of the GLA-fixed trimers by these mAbs was completely eliminated (**Figure 1D**).

Env-Specific Binding Antibody Responses in Mice Immunized with Unfixed or GLA-Fixed Trimers

To evaluate the immunogenicity of the unfixed and GLA-fixed 16055 NFL TD CC trimers C57BL/6 mice were immunized three times at 4-week intervals. Sampling was performed 2 weeks after the second immunization and 8 days after the third immunization (**Figure 2A**). Serological responses were compared by first measuring the total Env-specific IgG binding titers after the immunizations. Following the second immunization, there was a clear difference in the total Env-specific IgG response between the two groups with higher responses detected in mice immunized with unfixed 16055 NFL TD CC Env trimers compared to those immunized with fixed trimers as shown by titration curves of the individual mice (Figure S1A in Supplementary Material) and as group means (**Figure 2B**). While this difference was detectable using both unfixed and GLA-fixed trimers as the antigenic target in the ELISA assay, it was more apparent when the unfixed protein was used for coating. After the third immunization, only a modest difference remained between the groups when the unfixed protein was used for coating the ELISA plates (**Figure 2B**), and an even smaller difference was observed when the fixed protein was used for coating (**Figure 2C**). Control mice injected with adjuvant alone showed no Env-specific binding.

Env-Specific Antibody Subclass Responses in Mice Immunized with Unfixed or GLA-Fixed Trimers

To investigate potential qualitative differences in the response elicited by the unfixed and GLA-fixed 16055 NFL TD CC Env trimers, we assessed the elicited serum IgG subclass response after three immunizations. We observed that mice immunized with fixed Env trimers generated a different subclass pattern compared to the mice immunized with unfixed trimers. Specifically, while the IgG1 responses were similar, mice immunized with fixed trimers displayed lower IgG2b, IgG2c, and IgG3 titers compared to mice immunized with the unfixed trimers, independently of whether unfixed protein (**Figure 3A**) or fixed protein (**Figure 3B**) was used as the binding target on the ELISA plate. This difference was not observed when sera collected after two immunizations were analyzed, likely because the IgG subclass responses were still very low at this time point (Figure S1B in Supplementary Material). We also compared the ratios between the subclasses at a serum dilution of 1:25 and observed differences between the groups, which were significant for both IgG1:IgG2b and IgG1:IgG2c ratios when unfixed protein was used for coating (**Figure 3C**, upper

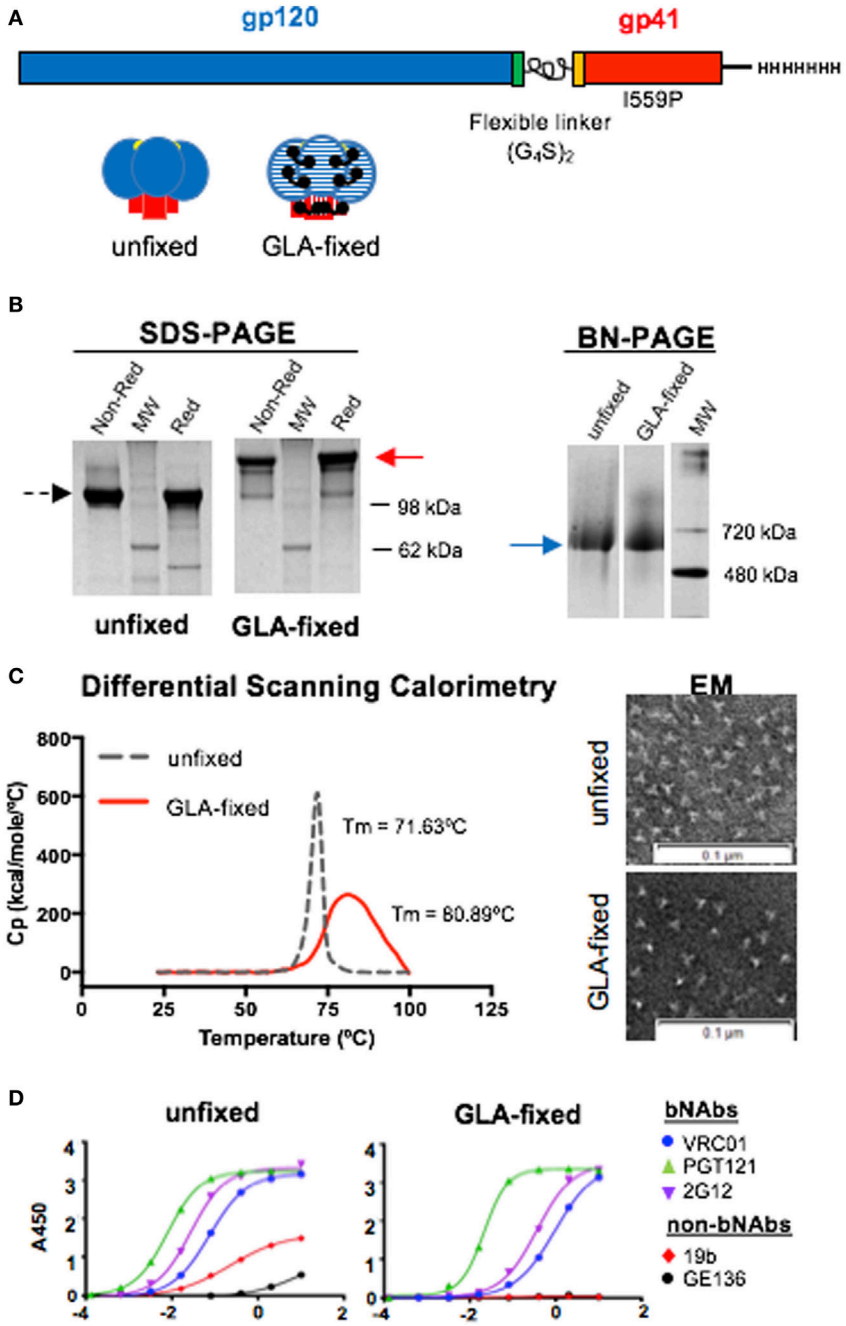

FIGURE 1 | Schematic illustration and *in vitro* characterization of the 16055 native flexibly linked (NFL) trimer-derived (TD) CC Env trimers. **(A)** Linear representation of the NFL TD CC Env trimer sequence with the flexible $(G_4S)_2$ peptide linker indicated between gp120 and gp41 (top) and cartoon of the unfixed and glutaraldehyde (GLA)-fixed Env trimers (bottom). **(B)** Left panel: SDS gel of unfixed trimers under non-reducing conditions and reducing conditions with molecular weight (MW) marker shown in between; middle panel: SDS gel of GLA-fixed under non-reducing conditions and reducing conditions with MW marker shown in between; right panel: blue native gel of unfixed and GLA-fixed trimers. **(C)** Left panel: differential scanning calorimetry curves comparing the *in vitro* stability of unfixed (dashed line) and GLA-fixed (solid line) 16055 NFL TD CC trimers; right panel: negative-stain electron microscopy (EM) images of unfixed and GLA-fixed 16055 NFL TD CC trimers. **(D)** ELISA comparing the antigenic profile using a set of Env-specific monoclonal antibodies of the unfixed (left) and GLA-fixed (right) 16055 NFL TD CC trimers.

panel) and for the IgG1:IgG2b ratio when fixed protein was used for coating (**Figure 3C**, lower panel). These serological results suggested a Th2-shifted response following immunization with the GLA-fixed 16055 NFL TD CC Env trimers. Control mice injected with adjuvant alone showed no Env-specific IgG1, IgG2a, IgG2c, or IgG3.

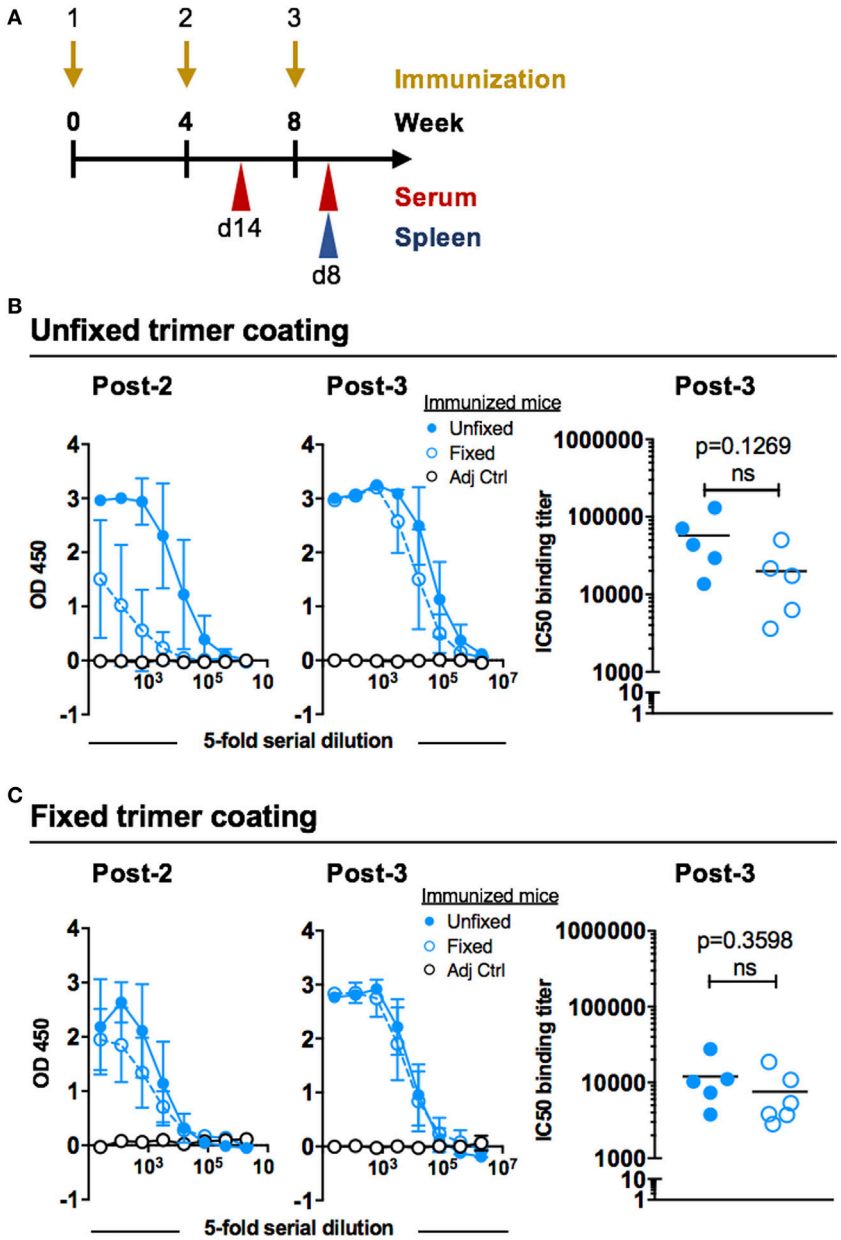

FIGURE 2 | Immunization schedule and Env binding titers after inoculation with 16055 native flexibly linked (NFL) trimer-derived (TD) CC Env trimers. **(A)** C57BL/6 mice (six per group) were immunized at 0, 4, and 8 weeks with 10 μg of unfixed or glutaraldehyde-fixed 16055 NFL TD CC Env trimers together with 10 μg AbISCO-100 adjuvant, or with adjuvant alone ($n = 2$). Serum was collected 14 days following the second immunization and serum and spleens were collected 8 days following the third immunization. **(B)** Env-specific IgG binding titers were measured by ELISA after two or three immunizations using unfixed 16055 NFL TD CC Env trimers for coating: full titration curves (group means) are shown to the left and IC50 binding titers (individual animals) are shown for the post-3 serum to the right. **(C)** Env-specific IgG binding titers were measured by ELISA after two or three immunizations using fixed 16055 NFL TD CC Env trimers for coating: full titration curves are shown to the left (group means) and IC50 binding titers (individual animals) are shown for the post-3 serum to the right. Statistical significance (Student's *t*-test) between post-3 IC50 titers in mice injected with unfixed or fixed trimers immunized mice was tested. Fivefold serial dilution was used for all samples starting at a 1:25 dilution.

To determine if this difference was detected if additional boosts were performed, we performed an independent immunization experiment where mice were injected five sequential times with the unfixed or GLA-fixed 16055 NFL TD CC Env trimers in AbISCO-100. This experiment yielded very similar results with increased IgG1:IgG2b and IgG1:IgG2c ratios in mice immunized with GLA-fixed trimers compared to mice immunized with unfixed trimers. This difference was significant for the IgG1:IgG2b ratio using both unfixed and fixed protein for coating (Figure S2 in Supplementary Material).

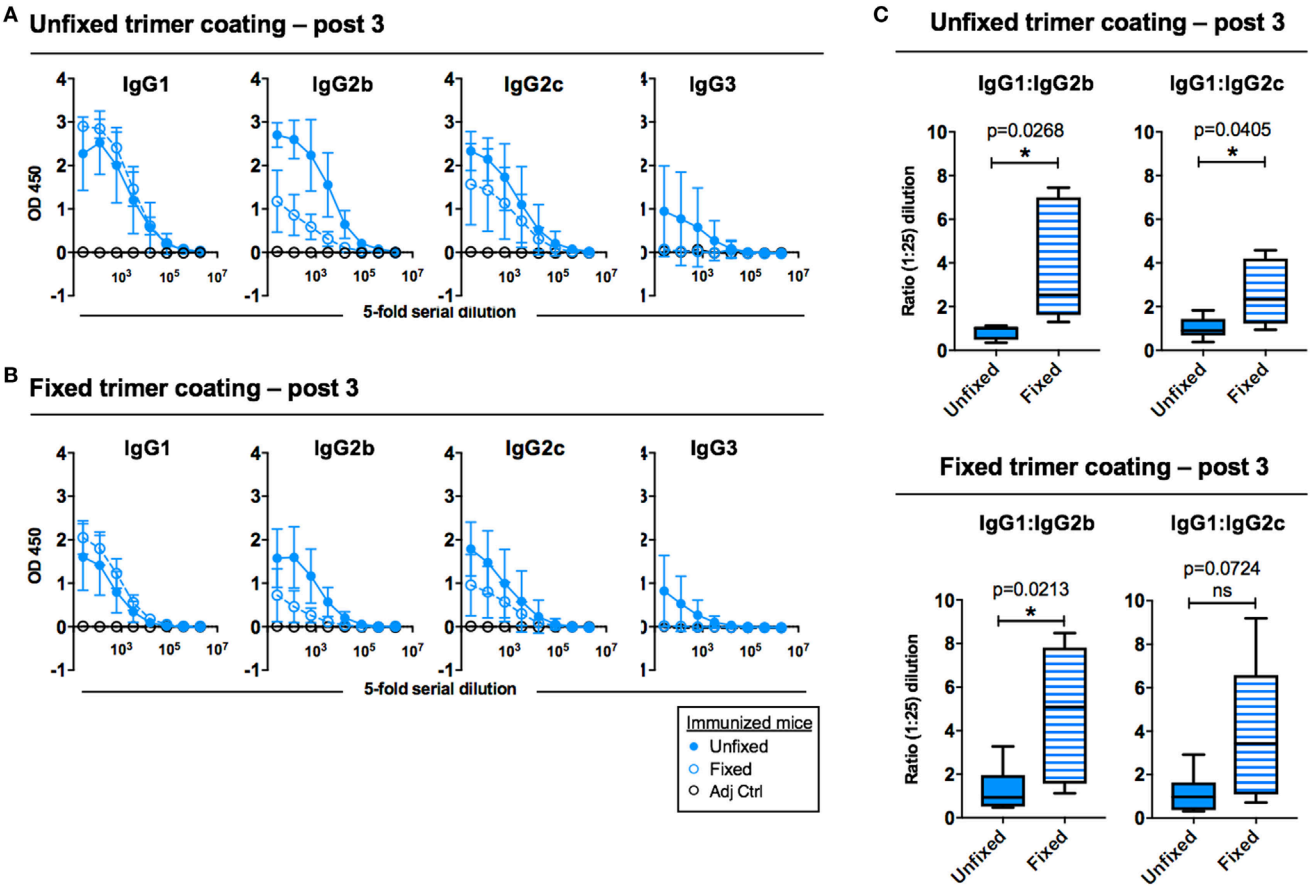

FIGURE 3 | Env-specific antibody subclass responses after three immunizations measured by ELISA. **(A)** ELISA curves (group means) for Env-specific IgG1, IgG2b, IgG2c, and IgG3 using plates coated with unfixed 16055 native flexibly linked (NFL) trimer-derived (TD) CC Env trimers. **(B)** ELISA curves (group means) for Env-specific IgG1, IgG2b, IgG2c, and IgG3 using plates coated with fixed 16055 NFL TD CC Env trimers. **(C)** The ratio of IgG1:IgG2b and IgG1:IgG2c were compared for unfixed protein coating (top) and glutaraldehyde-fixed protein coating (bottom). * and ** indicate statistical significance (Student's *t*-test) between mice immunized with unfixed and fixed 16055 NFL TD CC Env trimers. Fivefold serial dilution was used for all samples starting at a 1:25 dilution.

T Cell Responses Elicited in Mice Immunized with Unfixed or GLA-Fixed Trimers

Having observed that unfixed and fixed 16055 NFL TD CC Env trimers induce qualitatively different IgG subclass responses, we next investigated whether the Env-specific T cells responses also differed between animals in each of the groups. We first used a cytokine ELISpot analysis of splenocytes harvested after the third immunization for this analysis. We evaluated if the response measured by our protein stimulation conditions (20 h at 37°C) resulted from CD4+ T cells by comparing cytokine production in total splenocytes to the CD4+ T cell-depleted splenocytes (Figure S3A in Supplementary Material). This experiment confirmed that both the IFNγ and IL-2 cytokine production measured in response to protein stimulation was CD4+ T cell-dependent as the CD4+ T cell-depleted samples did not secrete cytokine levels that exceeded those of the medium control (Figures S3B,C in Supplementary Material). We next applied this method to analyze spleens harvested from mice immunized three times with unfixed or GLA-fixed 16055 NFL TD CC trimers and detected no significant differences in the number of IFNγ, IL-2, and IL-4 producing T cells upon stimulation with NFL trimers (unfixed or fixed) (**Figure 4A**). We concluded that mice immunized with unfixed or GLA-fixed 16055 NFL TD CC Env trimers had similar numbers of cytokine-producing cells, indicating similar uptake and processing of the GLA-fixed and unfixed trimers by antigen-presenting cells for CD4+ T cell activation. To specifically investigate the presence of T cells producing Th2-associated cytokines, we employed a flow cytometry-based bead assay to detect low levels of cytokines potentially present in supernatants from *in vitro*-stimulated T cells from mice immunized three times with unfixed or GLA-fixed Env trimers. We detected varying levels of IL-5, IL-10, and IL-13 with several mice being under the limit of detection of the assay. However, the mice that did respond with detectable IL-5, IL-10, and IL-13 levels were almost exclusively found among the mice immunized with the GLA-fixed trimers, suggesting a potential connection to the antibody subclass response in these mice (**Figure 4B**).

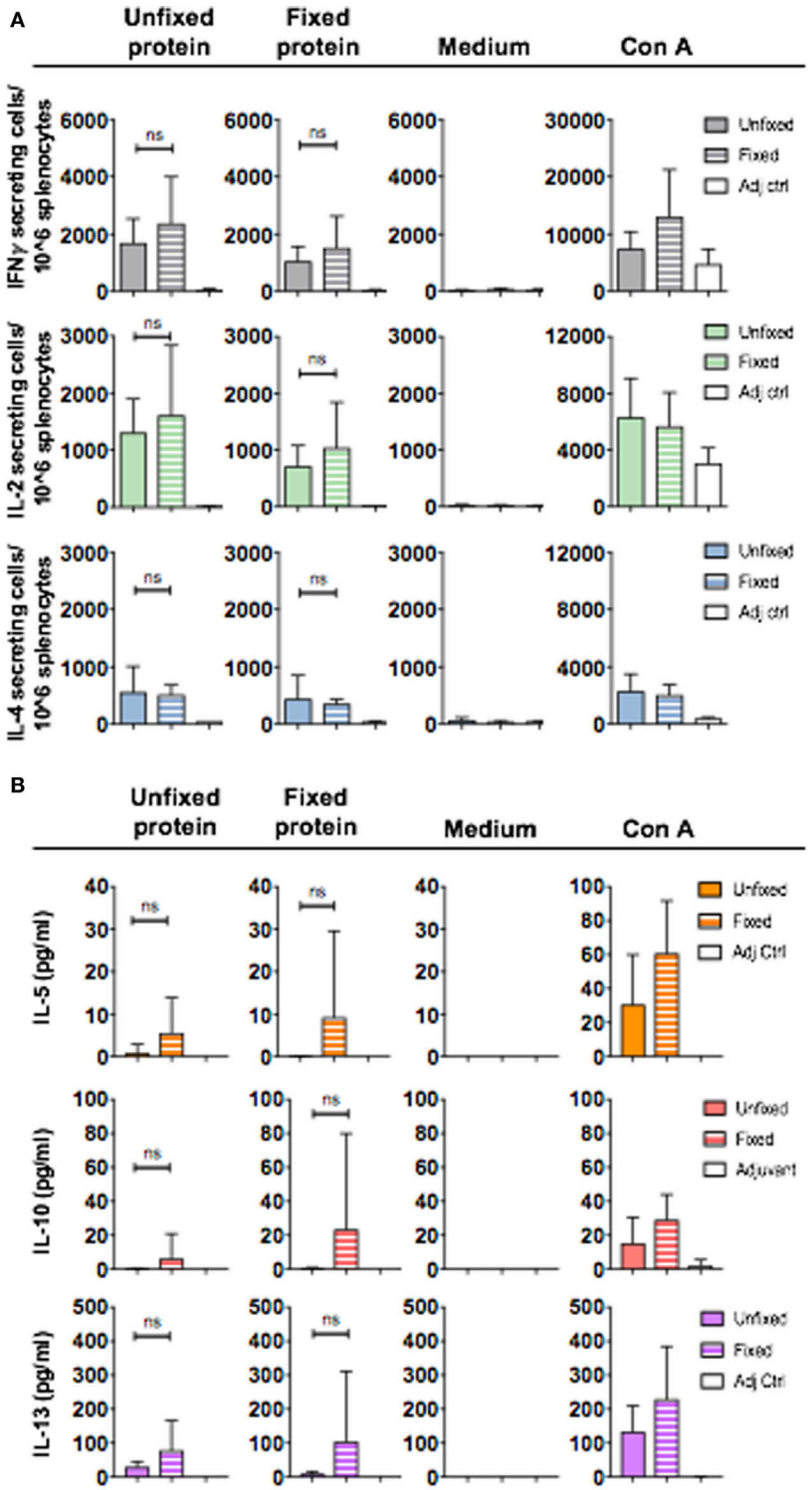

FIGURE 4 | CD4+ T cell cytokine production after *in vitro* stimulation of splenocytes collected after three immunizations with unfixed or fixed 16055 native flexibly linked (NFL) trimer-derived (TD) CC Env trimers or adjuvant only. **(A)** IFNγ, IL-2, and IL-4 cytokine-producing cells (group means) were measured by ELISpot analysis after 20 h stimulation with unfixed or fixed Env trimers or with ConA (positive control) or medium (negative control). Each spot formed in the wells represents one cytokine-producing cell. The average number for cytokine-producing cells for each group was plotted for each stimulus. Statistical significance (Student's *t*-test) between mice immunized with unfixed and fixed protein was tested. **(B)** Detection of IL-5, IL-10, and IL-13 in supernatants following 20 h *in vitro* stimulation with unfixed NFL TD CC trimers, glutaraldehyde-fixed NFL TD CC trimers, Con A (positive control), or medium (negative control) using a flow cytometry-based bead assay. The results were plotted as picograms per milliliter cytokine produced for each condition. The detection limit of cytokines was 0.273 pg/ml.

DISCUSSION

Here, we performed a comparative study in mice to examine the magnitude and quality of the Env-specific immune responses induced by unfixed or GLA-fixed 16055 NFL TD CC trimers. We demonstrate that the GLA-fixed 16055 NFL TD CC trimers displayed increased thermostability *in vitro*, reduced exposure of non-neutralizing antibody epitopes *in vitro* and lower *in vivo* Env-specific IgG antibody responses after two immunizations. However, following three immunizations the difference in Env-specific IgG titers was modest and only detectable when unfixed trimers were used as the target antigen in the ELISA. The difference in magnitude of the response induced by the unfixed trimers compared to the fixed trimers may be because unfixed trimers are more prone to unfolding or dissociation *in vivo*, resulting in exposure of immunogenic but non-neutralizing protein determinants. Thus, a reduced response may be a desired outcome if it means that less antibody responses to irrelevant epitopes. Induction of antibodies against non-desired, non-neutralizing epitopes may register by ELISA when unfixed trimers are used for coating but less so when the fixed protein is used. Thus, the use of both unfixed and fixed trimers as both immunogens and as binding targets in the ELISA plate provides useful information to deduce trends in the elicited antibody specificities *in vivo*. Whether GLA fixation offers an advantage for the quality of neutralizing antibody responses induced by the 16055 NFL TD CC trimers was not addressed in the present study, as this was previously investigated in the guinea pig model (7), as well as in the rabbit model using other well-ordered Env trimer designs (9, 10). We have previously reported tier 1 neutralizing activity in immunized mice (12, 13). However, immunogens that readily elicits tier 2 neutralizing antibody titers in rabbits (9, 10, 14) fail to do so in mice (15, 16).

The most significant observation in this study was that the Env-specific IgG response measured in animals inoculated with GLA-fixed trimers displayed a more Th2-skewed subclass profile than the response elicited in mice inoculated with unfixed trimers. We demonstrated this outcome by measuring Env-specific IgG1, IgG2b, and IgG2c. In mice, IgG1 represents a Th2-skewed response, while IgG2b and IgG2c represent a Th1-skewed response. A previous study in Balb/c mice reported that Env delivered in the form of a DNA vaccine induced a more Th2-biased antibody subclass response profile than did a DNA-based influenza virus hemagglutinin-based vaccine, as detected by an increased IgG1:IgG2a ratio in mice immunized with Env (17). This suggested an intrinsic difference in the type of response induced by the two viral antigens. We previously examined antibody subclass profiles induced by purified HIV-1 Env trimers formulated in the AbISCO-100 adjuvant in a head-to-head comparison between Balb/c mice and C57BL/6 mice and found that balanced Th1/Th2 responses were induced in both strains, with potent Env-specific IgG1, IgG2a, and IgG2b responses detected in Balb/c mice and similarly potent IgG1, IgG2b, and IgG2c responses detected in C57BL/6 mice (13). In the current study, we detected potent IgG1, IgG2b, and IgG2c responses to the unfixed trimers but reduced IgG2b and IgG2c responses to the fixed trimers. This Th2 skewing of the antibody subclass responses was observed in all animals immunized with the GLA-fixed trimers, using either the unfixed or GLA-fixed Env trimers as the antigenic target coated on the ELISA plates and was observed in two independent experiments.

The unfixed and GLA-fixed trimers used here were formulated with the AbISCO-100 adjuvant (also called Matrix-M). We and others have previously shown that this adjuvant induces a balanced Th1/Th2 response also for other protein antigens (18–20). Our results show that the presence of GLA adducts on the trimers influenced the induced immune response in a manner that was not over-ridden by the presence of the adjuvant. We have previously shown that the response induced by protein antigens in AbISCO-100 can be shifted toward a more Th1-driven response by co-administration of a TLR9 agonist (18). Thus, co-stimulation of TLR9 may be one way to balance the Th2 skewing caused by the GLA fixation. Another strategy to direct the Env-specific response away from a Th2-biased profile is to prime with a viral vector expressing Env prior to protein boosting, which we previously showed induced a more Th1-biased response (21). While the assessment of Th1/Th2 skewing by measurements of IgG subclasses may not be readily translatable to other species, our finding may be worthy of further investigation in other models using additional assays of T helper function as different vaccine platforms are under evaluation and prioritization. While formaldehyde treatment of proteins was shown to limit antigen processing by constraining presentation to T cells in one study (22), we did not detect any measurable differences in the magnitude of cytokine-producing profiles of Env-specific CD4+ T cells in our study using unfixed or GLA-fixed protein for *in vitro* stimulation when IFNγ, IL-2, and IL-4 cytokine responses were measured by ELISPOT analysis. When a more sensitive flow cytometry-based bead assay was used to detect additional Th2 cytokines, we observed that the highest IL-5, IL-10, and IL-13 responders were found in the group of mice immunized with the GLA-fixed trimers. This result was non-significant since a majority of the mice were below the level of detection. Nevertheless, it indicated a trend toward an increased Th2 response after immunization with fixed trimers, which may be related to the skewed antibody subclass response.

Chemical fixation of viruses and antigens is used in some commercial vaccines, for example, to inactivate infection by replication-competent whole virus particles. This was successfully done for the polio vaccine but it was less successful for a candidate respiratory syncytial virus (RSV) vaccine (23). In the case of the clinically tested RSV vaccine, formaldehyde was used for fixation. This vaccine worsened clinical symptoms in children exposed to natural RSV infection, triggering its removal from commercial development (24). The negative outcome was associated with a shift in the responses from a Th1 to a Th2 profile, as well as lower levels of neutralizing antibodies (24–26), effects that were suggested to be related to the carbonyl groups on the vaccine antigens (27). These studies, as well as those presented here, indicate the need for an improved understanding of how modifications to protein-based vaccines influence the induced response at multiple levels. For example, protein adducts such as aldehyde groups may interact with scavenger receptors such as CD36 (28), which are expressed by both B cells and professional antigen-presenting cells. Whether such potential effects influence antigen-specific

immune responses to HIV-1 Env or other viral proteins is not known, but may be elucidated by future investigations.

ETHICS STATEMENT

All animal experiments were performed under approved conditions and standard guidelines prior to the experimental start according to the regulations of the Committee for Animal Ethics (Stockholm, Sweden). The ethical permit number is N4/16.

AUTHOR CONTRIBUTIONS

MS: planning and performing *in vivo* experiments, data collection, data analysis, making figures, and writing manuscript. MA: performing *in vivo* experiments, data collection, and reviewing manuscript. JS: planning, performing, data collection and data analysis in T cell in vitro experiments. LY: preparing recombinant proteins for EM and performing EM analysis. YF, KT, RW, and JG: recombinant protein production and characterization, data analysis, making figures, and reviewing manuscript. RTW: planning the study and supervision and reviewing of manuscript. GKH: study planning, supervision, and writing manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/article/10.3389/fimmu.2017.01654/full#supplementary-material.

FIGURE S1 | Total Env-specific IgG and subclass-specific responses measured by ELISA. **(A)** ELISA curves for Env-specific IgG for individual mice (post-2 and post-3) using unfixed 16055 native flexibly linked (NFL) trimer-derived (TD) CC Env trimers (left) or fixed 16055 NFL TD CC Env trimers (right) as the target antigen. **(B)** ELISA curves (group means) for Env-specific IgG1, IgG2b, IgG2c, and IgG3 from post-2 sera using fixed 16055 NFL TD CC Env trimers as the target antigen. Fivefold serial dilution was used for all samples starting at a 1:25 dilution.

FIGURE S2 | Env-specific antibody subclass responses after five immunizations measured by ELISA. **(A)** ELISA curves (group means) for Env-specific IgG1, IgG2b, and IgG2c using unfixed 16055 native flexibly linked (NFL) trimer-derived (TD) CC Env trimers as target antigen. **(B)** ELISA curves (group means) for Env-specific IgG1, IgG2b, and IgG2c using fixed 16055 NFL TD CC Env trimers as target antigen. **(C)** Ratios of IgG1:IgG2b and IgG1:IgG2c calculated using either unfixed and glutaraldehyde-fixed protein coated on the ELISA plates. The serum samples were added to the plates in fivefold serial dilutions starting at 1:50. * and ** indicate statistical significance (Student's *t*-test) between mice immunized with unfixed and fixed 16055 NFL TD CC Env trimers.

FIGURE S3 | CD4+ T cell depletion of splenocytes. **(A)** Assessment of the purity after CD4+ T cell depletion. The FACS plots show the changes in CD4, CD8, and B220 cell populations before and after CD4 depletion. IFNγ **(B)** and IL-2 **(C)** cytokine-producing cells were measured by ELISpot 20 h after stimulation with unfixed 16055 NFL TD CC trimers or with Con A (positive control) or medium (negative control) of splenocytes with or without CD4+ T cell depletion.

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Regulation of Subunit-Specific Germinal Center B Cell Responses to the HIV-1 Envelope Glycoproteins by Antibody-Mediated Feedback

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The regulation of germinal center (GC) B cell responses to single epitopes is well investigated. How monoclonal B cells are regulated within the polyclonal B cell response to protein antigens is less so. Here, we investigate the primary GC B cell response after injection of mice with HIV-1 envelope glycoproteins. We demonstrate that single GCs are seeded by a diverse number of B cell clones shortly after a single immunization and that the presence of Env-specific antibodies can inhibit the development of early GC B cells. Importantly, the suppression was dependent on the GC B cells and the infused antibodies to target the same subunit of the injected HIV-1 envelope glycoproteins. An affinity-dependent antibody feedback has previously been shown to regulate GC B cell development. Here, we propose that this antibody-based feedback acts on GC B cells only if they target the same or overlapping epitopes. This study provides important basic information of GC B cell regulation, and for future vaccine designs with aim to elicit neutralizing antibodies against HIV-1.

Keywords: epitope-specific antibodies, regulation of germinal centers, HIV-1, envelope glycoproteins, epitope-specific B cells

INTRODUCTION

There is abundant evidence that some HIV-1-infected patients develop broadly neutralizing antibodies (bNabs) at the chronic stage of the infection (1, 2). This demonstrates that the human immune system is, under certain circumstances, capable to produce antibodies that may be useful if they could be re-elicited by vaccination. Being the only virally derived component on the outside of the virion, it is not surprising that known bNabs target the HIV-1 envelope glycoproteins (Env) (3). It has been postulated that humoral immune responses to immunodominant regions of Env may suppress responses to less immunogenic regions, and that this could explain why bNabs are infrequently elicited during infection and has, to date, not been elicited by vaccination. Clearly, a better understanding of the regulatory processes for epitope-specific regulation and maturation of B cell responses is of great importance for the development of improved vaccine strategies.

Immunization with recombinant proteins in adjuvant generates T-dependent humoral immune responses that are characterized by the formation of germinal centers (GCs). In GCs, antigen-specific B cells undergo affinity maturation and differentiation into memory B cells and

Ab-secreting plasma cells [reviewed in Ref. (4)]. The resulting polyclonal Ab response comprises a number of different antibodies that each target a distinct epitope surface on the injected protein antigen (5). In the GC, B cell clones that target the same epitope on model antigens are competitively regulated and there is a bias for survival of high-affinity clones (6–8). It was demonstrated that B cell clones with a high-affinity BCR are better at presenting antigenic peptides to Tfh than are B cells with low affinity, and therefore gain a competitive advantage (9), and the importance of robust Tfh responses for the generation of neutralizing antibodies against HIV-1 has been extensively discussed elsewhere (10). However, even within single GCs a wide range of intra- and inter-clonal affinity maturation of B cells occur (11, 12). It is therefore possible that regulatory mechanisms exist to allow for clonal expansion and maturation of B cells with different epitope specificity after challenge with physiologically relevant multi-epitope proteins, such as HIV-1 Env. By dampening the ability of B cells to recognize the immunodominant V3-region on Env, we have previously shown that antibody and plasma cell responses to distinctly different epitope regions were independently regulated after repeated immunizations with recombinant soluble HIV-1 Env in mice (13). Similar results were subsequently found when instead immunosilencing the trimerization domain of Env (14). These findings were not unique to Env, as similar observations had previously been described for a number of therapeutic proteins, including *Pseudomonas* exotoxin A [reviewed in Ref. (15)]. Immunodominance may therefore be driven by a mechanism that is largely independent of inter-clonal competition and additional regulatory mechanisms might play a significant role for the regulation of B cell clones with distinct BCR specificities within the polyclonal response after immunization.

For decades, it has been known that IgG can feedback regulate the humoral immune response, and that this is dependent on the nature of the antigen and subclass [reviewed in Ref. (16)]. It was demonstrated that IgM could mediate inhibition of GC B cell responses by direct binding to antigen, thereby occluding it from recognition by antigen-specific BCRs on B cells (17). Since IgM is readily elicited early during the development of T cell-dependent GC B cell responses, it is unlikely to provide a strong inhibitory effect on GC B cells under physiological conditions. However, an antibody-mediated feedback mechanism that is dependent on the binding specificity of IgG could potentially explain our results where independent expansion of epitope-specific plasma cell responses to HIV-1 Env was observed (13).

A single injection with Env in adjuvant was not sufficient to induce potent Env-specific IgG-secreting plasma cells in mice, rabbits, and non-human primates (13, 18, 19). If antigen-specific GC B cells had been developed at the same time point, this would allow us to investigate how Env-specific GC B cell responses develop without the interference of endogenously produced antigen-specific antibodies. According to this rationale, we set out to define the characteristics of the GC B cell response after one injection of Balb/C mice with Env, and then to address if an antibody-mediated feedback had potential to regulate GC B cell responses in an epitope-specific manner.

MATERIALS AND METHODS

Recombinant Proteins

The design and cloning of trimeric soluble recombinant envelope glycoproteins Env and monomeric gp120 for injection, and trimeric Env, gp120, and gp120ΔV3 for site-specific biotinylation has been previously described (20, 21). All recombinant proteins were produced by using the FreeStyle™ 293 Expression system (Invitrogen) and purified by sequential lectin and his-tag affinity chromatograph (22). Site-specific biotinylation was performed by treating AviTagged recombinant Env and gp120 with biotin-protein ligase (GeneCopoeia, Rockville, MD, USA) (20).

Immunizations

For injections, 10 µg of Env or gp120 was emulsified in Imject™ Alum adjuvant (Thermo Fischer Scientific) and 7- to 10-week-old BALB/c mice were injected *via* the intraperitoneal route. To generate immune serum to Env or gp120, groups of six mice were injected with recombinant Env or gp120 in Imject™ Alum adjuvant two times at a 2-week interval, and serum was collected 2 weeks after the last injection. Serum from mice injected with Adjuvant alone was used as control. Mice were kept at the animal facility at Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet or at the Umeå Center for Comparative Biology, Umeå University, Sweden.

Immunohistochemistry and Laser Microdissection

For immunohistochemistry and laser capture microdissection of GC structures, 8 µm sections of OCT embedded spleens were fixed on super frost plus glass slides (Thermo Scientific) or on PPS membrane slides (MicroDissect GmbH), and fixed using ice-cold acetone. For subsequent laser microdissection, we chose the mid section of a three consecutive 8 µm sections that all demonstrated a GC structure of same shape and relative location in the spleen. To inhibit non-specific binding, sections were treated with 5% goat serum (Dako) and subsequently treated with Avidin/Biotin blocking kit. Slides were then stained with FITC-conjugated anti-IgD (BD Pharmingen) and biotinylated peanut agglutinin (PNA) followed by Alexa555-conjugated streptavidin (Thermo Fisher Scientific). Confocal microscopy was performed on the glass slides with a DM IRBE system (Leica). Laser microdissection was performed on PPS membrane slides in a LMD7000 system (Leica). Single GC structures were defined as PNA^+, IgD^- areas inside splenic follicles (IgD^+, PNA^-) in the center section of each spleen, and collected in RLT buffer for subsequent mRNA extraction.

Flow Cytometry and Cell Sorting

Single-cell suspension of splenocytes was achieved by passing spleen through a 70-µm nylon mesh. RBCs were subsequently lysed with hypotonic ammonium chloride solution for 1 min, and the remaining cells were washed and resuspended in complete RPMI 1640 medium (Sigma) containing 5% FBS, 50 µM 2-ME, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µM streptomycin. Where applicable, splenocytes were enumerated by flow

cytometry using AccuCheck Counting Beads (Life Technologies). The amount of live cells in samples was determined by using a Live/Dead aqua viability kit (Thermo Fischer Scientific). Antibodies used for stainings were FITC-conjugated anti-GL7 antigen, PerCP.Cy5.5-conjugated anti-IgD, PE-conjugated anti-CD95, and Pacific Blue-conjugated anti-B220 (all BioLegend). To determine antigen specificity, cells were incubated with 10 µg/ml biotinylated Env trimers, gp120 trimers, or gp120ΔV3 trimers and subsequently with APC-conjugated streptavidin. Data were collected on a BD LSRFortessa™ X20, and cell sorting was performed on a BD Facs Jazz™ (All BD Biosciences). Analysis of flow cytometric data was performed using FlowJo (FlowJo, LLC).

B Cell Receptor Fragment Analysis

The B-cell repertoire was assessed by spectratyping of VDJ regions of heavy chain families 1, 2, 3, 5, 6, and 7. Briefly, mRNA from tissues was extracted with an RNAeasy kit (Invitrogen), and corresponding cDNA was then generated using iScript (BioRad), according to the manufacturer's instructions. Previously published primers for amplification of the VDJ region (with focus on the uniqueness of the CDR3) of the variable region heavy chain (Vh) families 1 and 2 of mice were used to amplify the target regions [Vh1 forward: TCCAGCACAGCCTACATGCAGCTC; Vh2 forward: CAGGTGCAGCTGAAGGAGTCAGG; and Jrev (common primer in the JH-region): CTTACCTGAGGAGACGGTGA] (23, 24). The amplifications were performed in a total volume of 20 µL, using 2× GoTaq (Promega), 2 µL (1 µM final) of each primer, and 2 µL of cDNA. After 1 min at 95°C, amplification was performed for 40 cycles as follows: 30 s at 95°C, 30 s at 55°C, and 1 min 30 s at 72°C, and ended with a step of 10 min at 72°C. To label the amplified fragments, 5 µL of each P CR product was mixed with 0.5 µM 6-fluorescein amidite (FAM)-labeled Jrev-primer and 5 µL GoTaq and subjected to 10 runoff cycles as follows: 2 min at 95°C, 2 min at 55°C, and 20 min at 72°C, and ended with a 10-min step at 72°C. FAM-labeled products were then processed on an ABI3130 Genetic analyzer (Applied Biosystems). Data were analyzed using PeakScanner v1.0 software (Applied Biosystems). Each peak in the resulting histogram represents one or many B cell clones with identical nucleotide length of the VDJ region of a certain Vh family. For an approximation of a distinct number of clones present in a single GC, a stringent cutoff of 1,000 response units (RUs) was applied to select for dominant clones. The relative dominance of the single fragment with the highest RU value in a GC was calculated with respect to the sum of RUs of all detected fragments in the same ($\%\text{dominance} = RU_{\text{dominant fragment}} \times 100/\Sigma RU_{\text{all fragments}}$).

Enzyme-Linked Immunosorbent Assay

High-protein-binding MaxiSorp plates (Nunc) were coated with 100 or 200 ng/well of recombinant Env or gp120 at 4°C overnight. The coated plates were blocked with 2% fat-free milk in PBS. After washing (PBS, 0.05% Tween-20), serum was added at different concentrations. The wells were then incubated with HRP-conjugated anti-mouse IgG or IgM (Southern Biotech). After washing, a colorimetric HPA substrate containing 3,3′,5,5′-tetramethylbenzidine (Invitrogen) was added. Adding one volume of 1 M H_2SO_4 stopped the enzymatic reaction, and OD was read at 450 or 450–620 nm. All incubations were performed at room temperature for 1 h, unless otherwise stated.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism V5.04 (GraphPad Software). Data sets were first analyzed with the D'Agostino and Pearson omnibus normality test. Sets conforming to normal distribution were then analyzed further using ANOVA or non-paired two-tailed Student's *t*-test to determine the significance of observed differences. Data sets not exhibiting normal distribution were analyzed using a non-parametric ANOVA, Mann–Whitney *U* test, or the Wilcoxon matched-pairs signed-rank test.

Ethics Statement

All animal experiments were pre-approved and performed in accordance with the Swedish Animal Welfare Act under protocols Dnr 234/12-dnr 11/13 (approved by Stockholms Norra djurförsöksetiska nämnd, Sweden) and Dnr A 59-15 (approved by Umeå försöksdjursetiska nämnd, Sweden).

RESULTS

GC B Cell Responses after Immunization with HIV-1 Env

To determine if potent GC B cell responses occur after a single injection with Env, we devised an injection regimen to characterize the development of GC B cells after immunization with Env in Imject Alum™ adjuvant. By immunofluorescence microscopy, we found that distinct GC formation (PNA^+IgD^-) could be detected on day 6 by histology (**Figure 1A**). The numbers of splenic GCs had significantly increased on day 11, but were reduced in numbers again by day 21 after the immunization. To quantify our findings, we assessed the frequency of splenic GC B cells ($B220^+IgD^-CD95^+GL7^+$) by flow cytometry at the same time points. Consistent with our histological results, we found that the overall frequency of GC B cells had reached detectable levels at day 6, that a major expansion had occurred between days 7 and 11 (**Figure 1B**). We could also quantify the overall reduction of GC B cells between days 11 and 21 after immunization Collectively, these data verify that GC B cell responses develop after a single injection of mice with Env in adjuvant.

Clonal Expansion and Contraction of B Cells in GCs after Immunization with HIV-1 Env in Mice

It has been previously shown that the number of dominant B cell clones in a single GC could vary after immunization with other recombinant proteins (11, 25–27). If a monoclonal B cell population seeds separate GCs after immunization with Env, this could explain the lack of competition between the responses to different epitopes within the antigen. To address this, we isolated single GCs (IgD^-PNA^+) by laser capture microdissection and approximated the relative clonality of these at different time points after immunization. This was done by assessing the number of VDJ regions of heavy chain (Vh) with variable nucleotide lengths

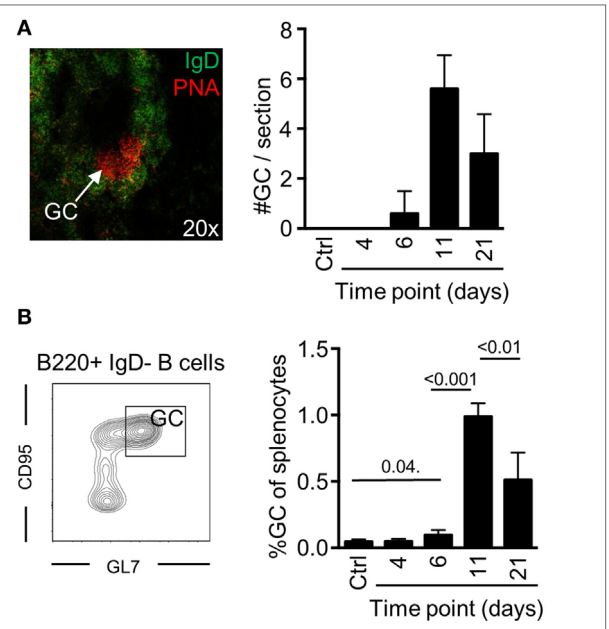

FIGURE 1 | Germinal center (GC) B cell development after a single injection of mice with Env. **(A)** The presence of GC structures was assessed by immunofluorescence microscopy as distinct PNA$^+$/IgD$^-$ areas (red) within follicles (PNA$^-$/IgD$^+$, green) in spleen sections of mice (representative image, left panel). The number of GCs in spleen sections from individual mice was enumerated at the indicated time points (right panel). **(B)** Flow cytometric quantification for the frequency of GC B cells (B220$^+$IgD$^-$GL7$^+$CD95$^+$) of total splenocytes is shown at the indicated time points. $N_{(adjuvant,\ d4)} = 4$ animals; $N_{(d6,\ d11,\ d21)} = 5$ animals.

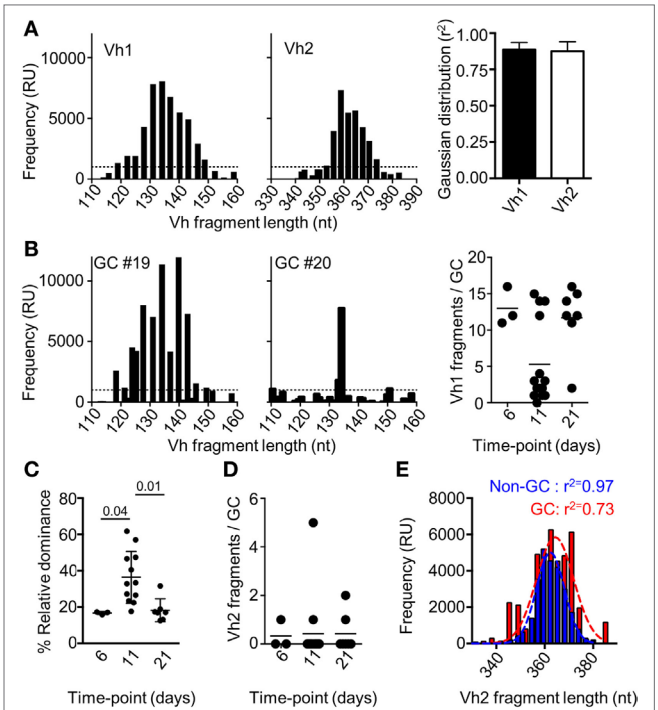

FIGURE 2 | Fragment analysis for the presence of B cell clones of the Vh1 or Vh2 families. **(A)** Splenocytes from uninjected mice ($N = 3$) were assessed for the number of Vh1 (left panel) or Vh2 (mid panel) fragments of different nucleotide length that could be amplified. The distribution of the fragments with respect to their frequency was assessed for adherence to a Gaussian distribution (right panel). **(B)** Fragment analysis of the Vh1 family in single germinal centers (GCs) after isolation by laser capture microdissection. Shown are representative histograms of the Vh1 distribution in polyclonal GCs (left panel) and relatively monoclonal GCs (mid panel), 11 days after injection with Env. Enumeration of how many distinct Vh1 fragments that could be found in each of the isolated single GCs on day 6 ($N = 3$), day 11 ($N = 14$), and day 21 ($N = 7$) was performed (right panel). **(C)** The relative dominance of the VDJ fragment with the highest frequency among all detected Vh1-family VDJ fragments is shown. **(D)** Shown is an enumeration of how many distinct Vh2 fragments that could be found in each of the isolated single GCs at different time-points. **(E)** GC B cells (B220$^+$IgD$^-$GL7$^+$CD95$^+$, red) and non-GC B (B220$^+$IgD$^-$GL7$^-$CD95$^-$, blue) cells were sorted and assessed for the frequency and number of amplified Vh2-family VDJ fragments. The r^2-value indicates the adherence to a Gaussian distribution of different fragments with respect to their relative frequency.

that could be amplified from spleen sections from mice shown in **Figure 1**. We focused on the Vh1 family that cover a large part of the total Vh-repertoire of mice, and on the Vh2-family that cover a limited part of the Vh-repertoire (28). To validate the method, we first amplified Vh1 and Vh2 VDJ regions from a sectioned spleen. As expected, we could amplify a large number of Vh1 and Vh2 VDJ fragments from the polyclonal population of B cells in the spleen section, and the frequency of amplified fragments were closely adhering to a Gaussian distribution with respect to fragment length (**Figure 2A**). Focusing on the Vh1 family, we could amplify a large number of VDJ fragments from single GCs at days 6 and 21 after injection with Env, whereas a significant number of GCs contained a relatively few Vh-1 fragments at day 11 (**Figure 2B**). This suggested that significant clonal selection had occurred between days 6 and 11, but that the GC B cell population had then diversified with respect to fragment lengths between days 11 and 21 after the immunization. To quantify this, we investigated the relative dominance of the most abundant Vh1 VDJ fragment among all amplified Vh1 VDJ fragments from single GCs over time. Consistent with polyclonal GC B cell populations, the dominance of a single VDJ fragment in separate GCs was on average 16.5% (range: 16–17%) or 18% (range: 12–31%) of all VDJ fragments on days 6 or 21 after injection (**Figure 2C**). By contrast, the average dominance of a single VDJ was 36% (range: 18–61%) on day 11 after the injection. This supports that GCs at peak response have reduced B cell clonality, but that fully monoclonal GCs were rare. Instead, GCs at peak response display variable degrees of clonal dominance. A similar variation of clonal dominance in single GCs was previously shown after injection of mice with chicken gamma globulin, *Bacillus anthracis* protective antigen and influenza hemagglutinin (11, 12).

Detection of clones from the Vh2-family in single GCs was rare at all time-points and when detected, comprised up to five fragments (**Figure 2D**). By contrast, a large number of clones of the Vh2-family could be detected after flow cytometric sorting of GC B cells 11 days after immunization of mice with Env (**Figure 2E**). The conflicting data are likely explained by the presence of a cross-section of all responding GC B cell clones

after the flow cytometric sorting, whereas the laser capture microdissection allowed for analysis of GC B cell clones from single GCs. A Gaussian distribution analysis of the sorted cells revealed that non-GC Vh2 B cell clones were normally distributed with respect to their BCR length ($r^2 = 0.97$). By contrast, the distribution of Vh2-related GC B cells was slightly skewed ($r^2 = 0.78$). Even though seeding and recruitment of Vh2-family B cells to single GCs was low in comparison to Vh1 clones, biased selection of GC B cell clones had occurred, if assessed on a global level.

Development of Antigen and Epitope-Specific GC B Cell Responses after Immunization of Mice with HIV-1 Envelope Glycoproteins

A requirement for an investigation to understand if antibodies can mediate a feedback to regulate epitope-specific GC B cells was that we could also measure GC B cell responses to two distinctly different regions of Env. Here, we took advantage of a probe-based system that we had previously used to enumerate subunit-specific plasma cell responses after repeated immunizations with Env (20). To test this system, we first assessed the capacity of splenic GC B cells to bind to Env, the gp120 subunit, or to a gp120 subunit that lack the variable region 3 (gp120ΔV3) 11 days after injection with Env. We found that an average of 45% of GC B cells was specific for Env, and that approximately 50% of those could bind to both gp120 and the gp120ΔV3 probes (**Figure 3A**). Importantly, we had previously shown that repeated injection of Env into mice did produce significant B cell responses to the non-exposed inside of Env trimers (20). Therefore, the Env-specific response was evenly distributed between epitopes that span the gp120 or the gp41 subunits, whereas no significant response had developed against the variable region 3 at this time point.

Next, we assessed changes in frequency of Env-binding GC B cells over time by flow cytometry. We injected mice with Env and found that GC B cells had reached sufficient numbers and affinity for Env to be detectable in our analysis after 11 days (**Figure 3B**). This suggested that significant proliferation, antigen-specific affinity maturation, and selection of GC B cells had occurred during the second week after the injection. The frequency of B cells that could bind to the Env-based probe was not significantly changed between days 11 (median: 41%) and 21 (median: 50%).

Subtracting gp120-specific responses for the total Env-specific response allowed us to determine the specific response to the gp41 subunit of Env. As expected, gp120 and gp41 subunit-specific responses developed with the same kinetics as the total Env-specific response and required between 7 and 11 days to develop sufficient affinity for detection (**Figure 3C**). No further increase in the frequency of gp120- or gp41-binding GC B cells had occurred between days 11 and 21 after a single immunization of mice with Env. Collectively, gp120-specific GC B cells accounted for a median of 51% (day 11) and 58% (day 21) of total Env-specific GC B cells. Consistently, gp41-specific GC B cells accounted for the remaining 49% (day 11) and 42% (day 21) of total Env-specific GC B cells.

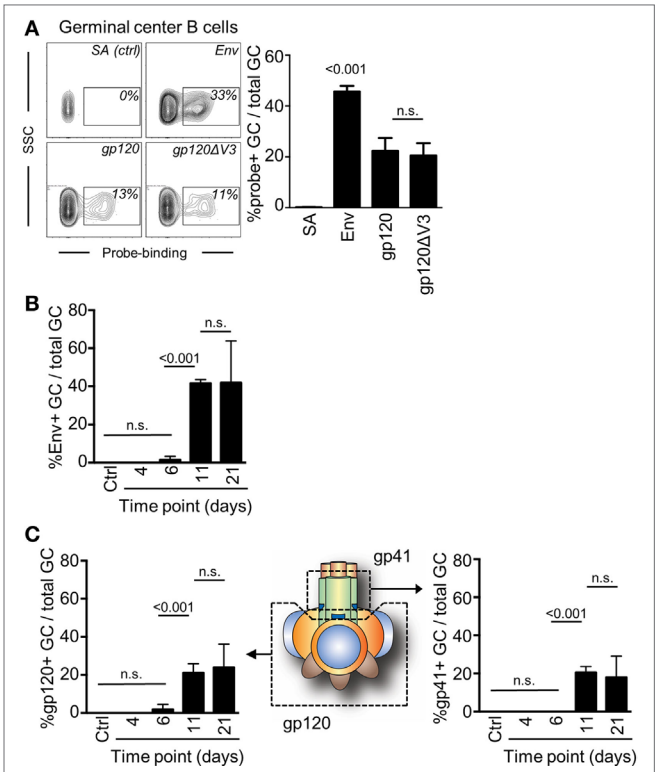

FIGURE 3 | Detection of antigen and subunit-specific germinal center (GC) B cell responses after a single injection of mice with Env. **(A)** The frequency of GC B cells ($B220^+IgD^-GL7^+CD95^+$) 11 days after injection which were able to bind biotinylated Env, gp120, or gp120ΔV3 was assessed by flow cytometry after addition of APC-conjugated streptavidin. Staining of cells with APC-conjugated streptavidin (SA) in the absence of Env was used verify the specificity of the binding. **(B)** The frequency of Env-specific GC B cells of total GC B on days 6, 11, and 21 after injection of mice with Env is shown. **(C)** The frequency of GC B cells ($B220^+IgD^-GL7^+CD95^+$) that could bind to the gp120 subunit of Env was assessed in a similar manner (left panel). By subtraction of the gp120-specific GC B cells from the total Env-specific GC B cells, we could also determine the proportion of gp41-specific GC B cells that had been induced at the same time points (right panel). *N* = 4–5 animals per group.

Regulation of Subunit-Specific GC B Cell Responses to the HIV-1 Envelope Glycoproteins

To study feedback regulation, we generated serum by repeated injections of mice with soluble Env or with gp120, that either contained antibodies to both the gp120 and gp41 subunits (Env injection) or only to the gp120 subunit (gp120 injection). We subsequently normalized the harvested serum so that both had a similar binding capacity to Env with regard to IgG and IgM (**Figure 4A**). Respective serum was then further diluted 2× in PBS and 200 µl was infused into mice that had been immunized with Env 4 days earlier (**Figure 4B**). We chose this time point to allow for similar initiation of the GC response toward Env in all groups prior to the serum infusion (29), and that it was just before GC B cells could be detected by flow cytometry (**Figure 1A**). Moreover, it would allow for similar trafficking and retention of Env to the network of follicular dendritic cells in GC

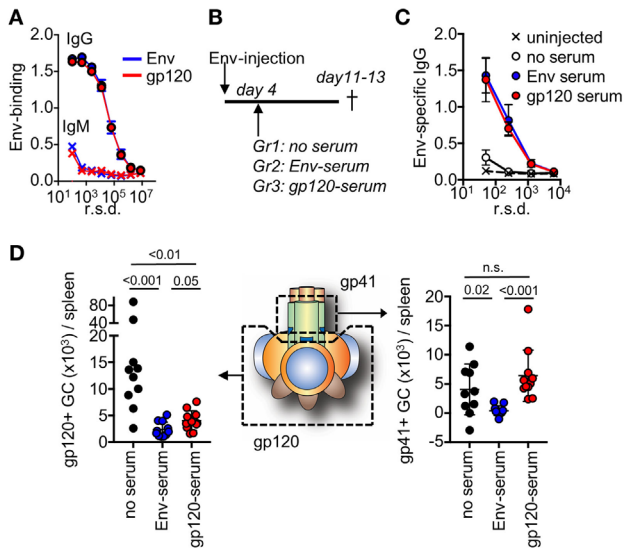

FIGURE 4 | After repeated injections with either Env or the subunit gp120, serum was harvested from mice and assessed for binding to Env. **(A)** Shown is IgG or IgM in serum pre-normalized for Env binding, as assessed by ELISA. **(B)** Mice (N = 5–6/group) were injected with Env and anti-Env or anti-gp120 serum was infused at the indicated time point. The mice were terminated on days 11–13 after start of the experiment. Control mice (N = 4–5/group) did not receive a serum infusion. **(C)** Assessment of Env-specific IgG by ELISA in serum from respective groups 13 days after injection with Env. **(D)** Detection of gp120 and gp41-specific germinal center (GC) B cells in groups of mice that had received Env-specific or gp120-specific serum, or no serum on day 4 after the Env injection. Shown is the accumulated data from two separate experiments.

for the first 4 days after immunization (30, 31). Since the injected serum levels was below those that can be induced by repeated Env injections into BALB/c mice, the potential regulatory function of antibodies on GC B cell responses likely mimic that of the endogenous high-affinity anti-Env antibody response after it has been generated.

Two weeks after immunization of mice with Env, we could detect low-levels of circulating Env-specific IgG after one injection (**Figure 4C**). This represented the background levels of the endogenous response at a time point when Env-specific GC B cells had developed. In both the serum-infused groups, we found distinct levels of circulating Env-binding IgG. Since these levels were significantly higher than that of the endogenous response, this demonstrated that specific IgG from the serum infusion had remained in circulation for more than a week. This is consistent with a half-life of murine IgG of approximately 8 days (32). We did not detect significant antigen-specific IgM in any of immunized animals at this time-point.

To understand if the development of subunit-specific GC B cells had been influenced by the serum injection, we determined the absolute number of splenic gp120- and gp41-specific GC B cells that had been induced in respective groups of mice. Here, we found that a distinct inhibition of gp120-specific GC B cells had occurred in both of the serum-infused groups (**Figure 4D**). This verified that the infused Env-specific and gp120-specific serum had similar capacity to suppress gp120-specific GC B cell responses. By contrast, inhibition of gp41-specific GC B cells had only occurred in the groups of mice that had received Env-specific serum. This suggested that gp41-specific GC B cell responses had been negatively regulated in the presence of high-affinity Env-specific antibodies that target the gp41 subunit, but not by antibodies that targeted the gp120 subunit.

Collectively, these data suggest that high-affinity antibodies at the level of a normal immune response can provide a negative feedback to repress the development of specific GC B cell responses, but that this only occurs if the antibodies and the GC B cells target the same or overlapping epitopes on Env.

DISCUSSION

Here, we performed a characterization of GC B cell responses to Env after a single immunization in mice, and subsequently addressed if antibodies have potential to regulate the development of GC B cells through an epitope-specific feedback mechanism. Our data suggest that single GCs are seeded by a polyclonal B cell population within a week after immunization with Env. To note, only two mice of six had developed distinct GCs at this time point. While we could not definitively rule out contamination from naïve B cells at this early time point, prior to clonal outgrowth, our data are consistent with the diverse early GC response after immunization of mice with chicken gammaglobulin, as has previously been shown (11). During the second week after immunization, varying degrees of clonal dominance is established in single GCs (**Figure 2C**). This coincides with peak frequency of total GC B cells in spleens of injected animals, and the detection of Env-specific GC B cells. To minimize the influence of non-cognate B cells that transport antigen to follicular dendritic cells or residual background from follicular B cells that did not participate in the GC reaction (33–35), we also made a qualitative approximation of clones in single GCs (**Figures 2B,D**). In this setting, we found that 9 of 14 single GCs contained between 1 and 4 distinct Vh1 fragments, where 3 GCs had potential to be fully monoclonal within the Vh1-family VDJ fragment length. During the third and fourth week after immunization, clonal dominance in single GCs had returned to levels that were indistinguishable from day 6. It was previously shown that tens to hundreds of individual B cell clones participate in the initial GC reaction (11). By the spectratyping approach used here, it was not possible to directly enumerate individual B cell clones but it was sufficient to approximate the relative clonality of single GCs at separate time points after injection of mice with Env.

After a single injection of mice with Env, we could demonstrate that up to 50% of the GC B cell response was focused on the gp41 subunit of Env. Consistent with these findings, gp41-specific plasma cells represent up of 50% of all Env-specific B cells after a booster injection (20). This suggests that GC B cells that develop in mice after a single injection of Env may differentiate into plasma cells after a subsequent booster injection. In line with this, the absence of V3-specific GC B cell development after a

single injection with Env could therefore explain the absence of V3-specific plasma cells after a booster injection, as previously shown (13, 20).

Importantly, we proceeded to generate evidence that antibodies can feedback regulate the development of epitope-specific B cells. By injection of high-affinity polyclonal serum in mice at a time point where the endogenous GC response had been initiated, but prior to detection of Env-specific GC B cells, we found that preexisting antibodies to the gp120 subunit could repress gp120-specific but not gp41-specific GC B cells (**Figure 4D**). By contrast, infusion of Env-specific serum could repress both gp120 and gp41-specific GC B cell responses. Since V3-specific GC B cells had not developed after a single injection of mice with Env, we could not assess if also V3-specific GC B cell responses could be suppressed by a similar mechanism. Interestingly, infusion of Env in complex with a V3-specific Fab was recently shown to specifically suppress endogenous V3-directed antibody responses in Guinea pigs (36). This suggests that the development of V3-specific GC B cells may also be regulated by a similar antibody feedback-mediated mechanism as we here describe for gp41-specific GC B cells.

Since T cells are rapidly primed within the first days after antigenic challenge (37, 38), it is unlikely that priming of Tfh cells was affected by the day 4 serum injection. Moreover, presentation of antigenic peptides on MHC class II cannot directly explain a regulatory feedback mechanism that is dependent on the binding specificity of GC B cells. Similarly, a regulatory feedback mechanism that is dependent on the binding specificity of soluble antibodies is difficult to explain by engagement of the constant Fc-region of the infused IgG to the inhibitory Fc-gamma receptor IIb (39). In fact, a recent study demonstrated that antibody feedback of epitope-specific GCs during experimental antigen challenge act independently of Fc-gamma receptor engagement (40).

We therefore propose Env-specific B cell responses to HIV-1 Env are feedback regulated by epitope masking of antigen by high-affinity antibodies, and that this leads to a subsequent inability of low-affinity B cell clones with similar specificity to acquire stimulation *via* their BCR. In GCs, the antibody-mediated occlusion may occur on antigen that has been deposited on the FDC network, as was previously proposed by infusion of IgM (17). In our study, we investigated how early low-affinity GC B cells were affected by infusion of high-affinity IgG.

Clearly, additional research is required to fully understand how the epitope-specific GC B cell response is regulated during the gradual affinity increase and subsequent termination or differentiation of GC B cells during an endogenous immune response after vaccination with HIV-1 Env, but also if and how an antibody-based feedback can regulate the fate of memory B cells after re-challenge, as recently discussed (40–43).

Collectively, we provide data that strongly suggest that the development of GC B cells to a biologically relevant antigen is directly regulated by the presence of physiological levels of circulating antibodies. An affinity-dependent and antibody-mediated feedback to regulate affinity maturation of GC B cells has been suggested (17). We propose that this feedback acts on GC B cells only if they share the same or overlapping specificity as the circulating antibodies. Undoubtedly, the future development of well-defined mouse-derived monoclonal will allow for a more detailed investigation with regards to the biochemical and molecular properties of the inhibitory function of antibodies that target overlapping, partially overlapping, and non-overlapping epitopes of Env. Such data would be invaluable for the future designs of novel antigens for vaccination against HIV-1.

Importantly, the data presented here suggest that non-neutralizing or strain-specific neutralizing determinants on vaccine antigens have potential to suppress the development of bNab only if they share an overlapping binding site with these on Env. Our study therefore validates previous and on-going efforts to develop Env-based vaccine antigens with reduced exposure of non-neutralizing epitopes to the immune system (44–46), and we propose that it is crucial to focus these efforts on areas of Env where non-neutralizing epitopes overlap with broadly neutralizing epitopes.

ETHICS STATEMENT

All animal experiments were pre-approved and performed in accordance with the Swedish Animal Welfare Act under protocols Dnr 234/12-dnr 11/13 (approved by Stockholms Norra djurförsöksetiska nämnd, Sweden) and Dnr A 59-15 (approved by Umeå försöksdjursetiska nämnd, Sweden).

AUTHOR CONTRIBUTIONS

MF: experimental design, performed experiments, analyzed data, and wrote the manuscript. MK: experimental design, analyzed data, and wrote the manuscript. LK, SS, JA: performed experiments.

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