LYMPH NODE T CELL DYNAMICS AND NOVEL STRATEGIES FOR HIV CURE

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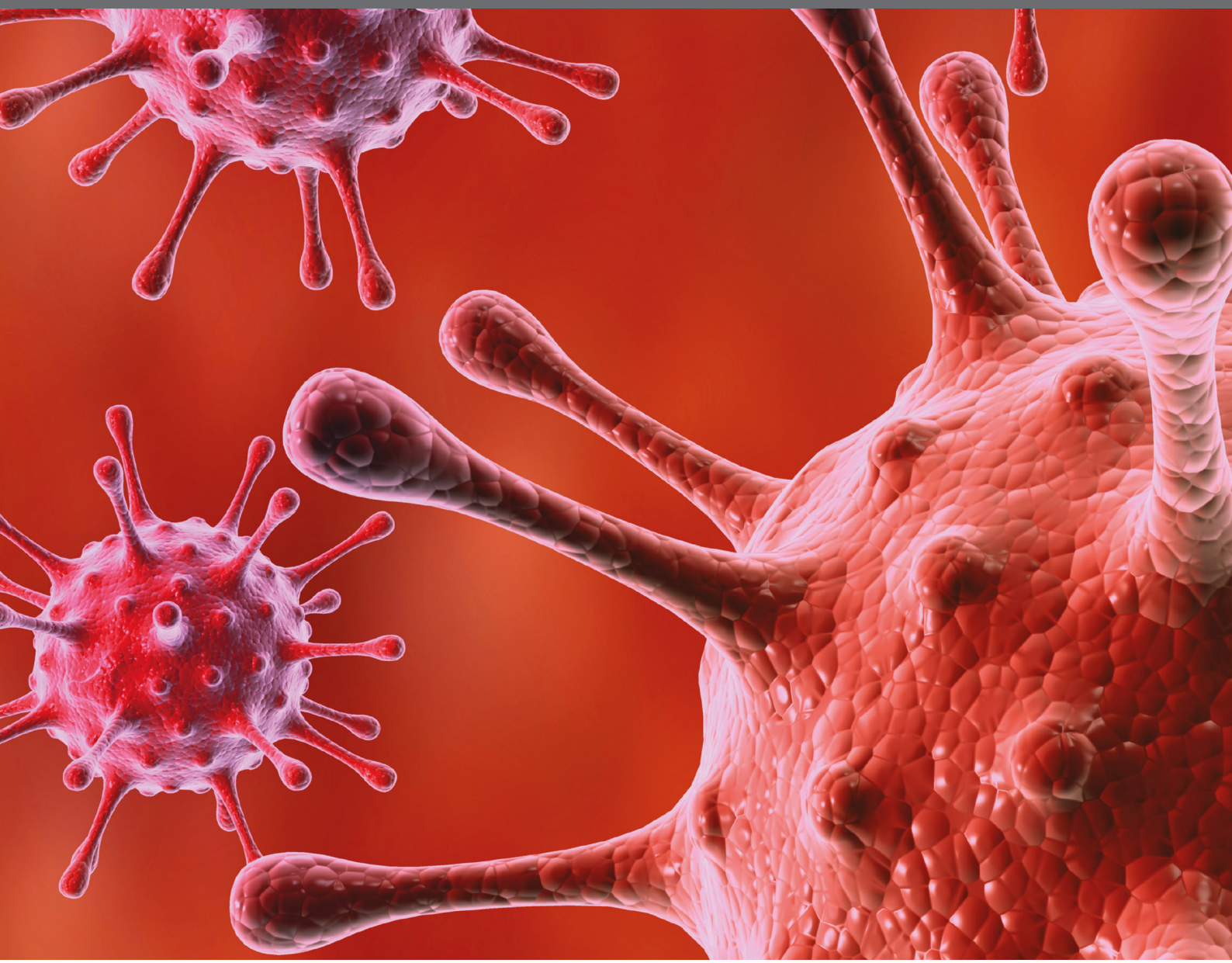

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LYMPH NODE T CELL DYNAMICS AND NOVEL STRATEGIES FOR HIV CURE

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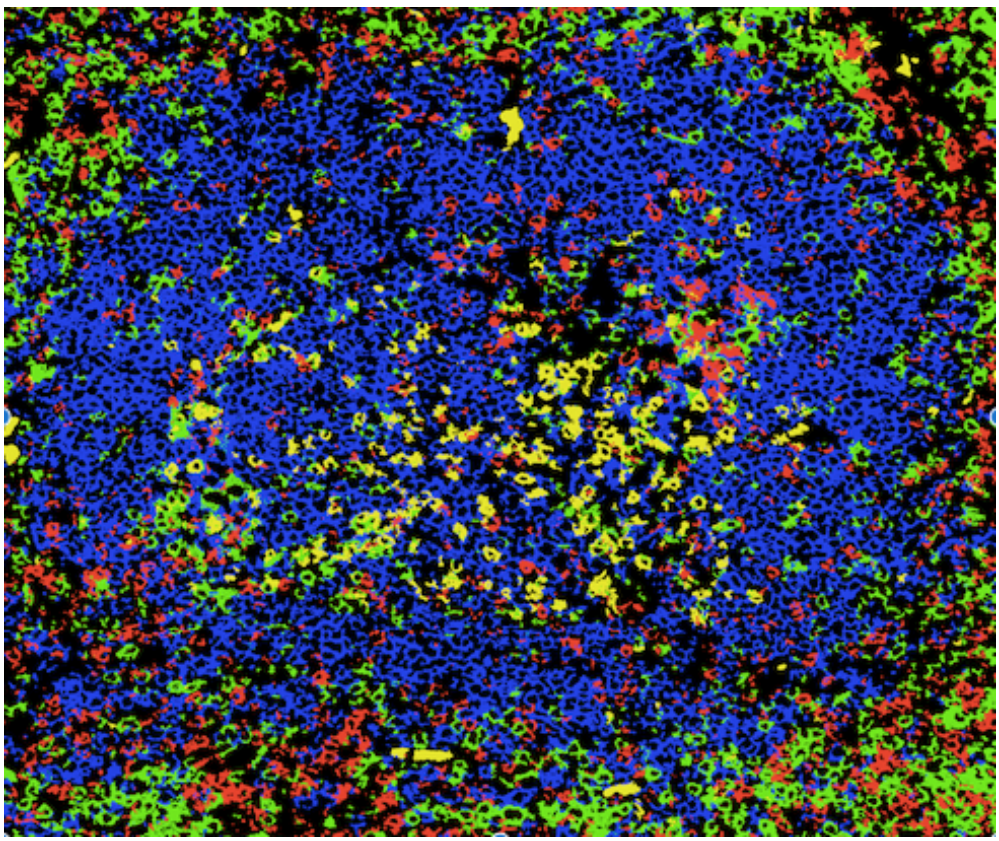

Represents a HIV+ human follicle (CD8-green, CD4-red, CD20-blue, PD-1-yellow). This image was generated by the laboratory: Tissue Analysis Core, Vaccine Research Center, NIAID, NIH, Bethesda, MD, United States.

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Currently, more than 36 million people are infected with HIV. Although the introduction of highly active anti-retroviral therapy (HAART) has led to substantial advances in the clinical management of HIV infected individuals, HAART cannot completely eliminate the virus. This is because CD4 T helper cells, harboring the virus, remain dormant reservoirs. These reservoirs are difficult to measure and are present even in HAART-treated HIV infected individuals with undetectable levels of HIV in the blood. A growing body of studies has revealed follicular helper (Tfh) CD4 T cells, a highly differentiated CD4 T cell population localized in immunologically sanctuary

sites (follicle/germinal center), as a major reservoir of HIV. The present Frontiers in Immunology eBook compiles 16 timely review articles focusing on the dynamics of major follicular immune cell types in HIV/SIV infection and their potential role for disease pathogenesis and the viral persistence in the lymph node.

This eBook provides a comprehensive presentation of recent published work on lymph node and especially Tfh cell dynamics in HIV infection and we hope that it will be useful for our further understanding of how such dynamics affect the interplay between virus and host as well as for the discovery of novel therapeutic targets in the fight against HIV.

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EDITORIAL
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Editorial: Lymph Node T Cell Dynamics and Novel Strategies for HIV Cure

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Keywords: HIV, lymph node, cure, T cells, follicular T helper cells

Editorial on the Research Topic

Lymph Node T Cell Dynamics and Novel Strategies for HIV Cure

Currently, more than 36 million people are infected with HIV. Although the introduction of highly active anti-retroviral therapy (HAART) (1, 2) has led to substantial advances in the clinical management of HIV infected individuals, HAART cannot completely eliminate the virus (3). This is because CD4 T helper cells, harboring the virus, remain dormant reservoirs. These reservoirs are difficult to measure and are present even in HAART-treated HIV infected individuals with undetectable levels of HIV in the blood. A growing body of studies has revealed follicular helper (Tfh) CD4 T cells, a highly differentiated CD4 T cell population localized in immunologically sanctuary sites (follicle/germinal center) (4), as a major reservoir of HIV (5). The present Frontiers in Immunology eBook compiles 16 timely review articles focusing on the dynamics of major follicular immune cell types in HIV/SIV infection and their potential role for disease pathogenesis and the viral persistence in the lymph node (**Figure 1**).

Vaccari and Franchini provide an overview of follicular T cell populations in the SIV NHP model. The phenotype and function of NHP Tfh cells as well as their role in viral reservoir formation and the impact of HIV/SIV infection on Tfh dynamics is discussed. Besides their role as immune suppressors, the possible role of Tfr cells in the development of high avidity antigen-specific antibodies is discussed. The recent literature for follicular CD8 T cell dynamics and *in vivo* manipulation to study their role is reviewed. The authors, convincingly reveal the importance of NHP studies to understand the follicular dynamics in HIV/SIV pathogenesis and explore *in vivo* manipulations targeting these cell populations. Investigation of follicular CD4 T cell heterogeneity is an important parameter for the understanding of generation of neutralizing antibodies as well as the formation of viral reservoir. Velu et al. discuss several follicular CD4 T cell subsets including CXCR3$^+$ Th1-like follicular helper CD4 T cells. High levels of IFNg and IP-10 observed in HIV/SIV could represent a mechanism for the differentiation of Tfh toward a Tfh1 phenotype. Given the differential levels of CCR5 expression by Tfh subsets, future studies should take under consideration this heterogeneity when the virus reservoir is under investigation. Tfh1 cells were found in vaccinated non-human primates the authors suggest that local inflammatory signals could represent critical regulators of Tfh1 cell dynamics. The authors show the need for further mechanistic studies aiming to understand the dynamics of follicular CD4 and CD8 T cells. Tracking the movement of cells between different anatomical compartments in human subjects is highly challenging. Analysis of phenotypically alike cells could represent a starting point for the investigation of cell subsets from compartments with possible dynamic interchange. Banga et al. provide data showing that circulating CXCR3$^+$CXCR5$^-$ CD4 T cells is the major blood compartment containing replication competent virus in cART, aviremic individuals. Among the parameters analyzed, the frequency of PD-1$^+$ cells was significantly correlated with the enrichment of replication competent virus in the circulating CXCR3$^+$ CD4 T cell compartment.

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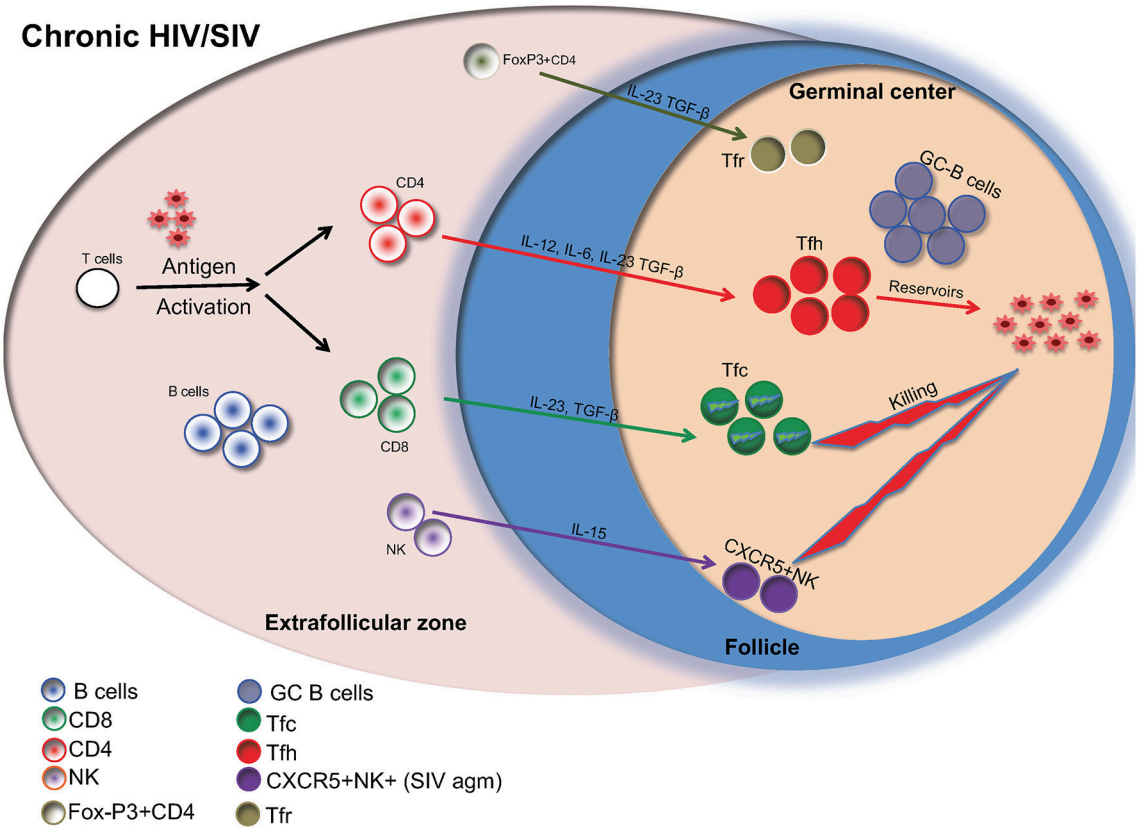

FIGURE 1 | Major follicular cell populations in chronic HIV/SIV infection are shown. Cytokines are important for the differentiation of relevant cells as well as their possible contribution to reservoir formation is also shown.

The data suggest a connection between the presence of infected lymph node and circulating CXCR3+ CD4 T cells. The potential role of CD8 T cells in viral control has been shown by several HIV/SIV studies. Xiao et al. review recent data on CD8 T cells expressing the receptor CXCR5 (fCD8 T cells) and have the ability to migrate into follicular areas. A comparison between fCD8 dynamics in chronic LCMV and HIV infection revealed key transcriptional regulation of these cells in the setting of chronic viral infections. However, what regulates their intra-lymph node trafficking is still unknown. The authors comment on the capacity of the fCD8 T cells for cytokine production and ability for suppressing chronic viral infection. Given this profile, strategies for therapeutic use of fCD8 to purge the virus are also discussed. The dynamics of Tfh CD4 T cells is the outcome of a complex process regulated by multiple factors including tissue inflammation, antigenic stimulation and local immunosuppressor mechanisms. Jaio et al. provide original data regarding the functionality of HIV-specific $CXCR5^{+}CD8^{+}$ T cells in the peripheral blood as well as the role of PD-1 as regulator of this functionality. Furthermore, distribution of CCXR5+CD8 T cells in the lymph node negatively correlated with disease progression. Interestingly, PD-1 expression was constantly retained on $CXCR5^{+}CD8^{+}$ T cells while significantly decreased on $CXCR5^{-}CD8^{+}$ T cells after successful antiretroviral treatment in chronic HIV-infected patients. $PD\text{-}1^{+}CXCR5^{+}CD8^{+}$ T cells may represent a novel therapeutic strategy for the disease control. Kleinman et al. provide an overview of the phenotype, development/homeostasis and function of Treg and follicular regulatory (Tfr) CD4 T cells as well as strategies for their *in vivo* manipulation in an effort to eliminate the virus. The frequency of Treg cells is increased in HIV infection while there are susceptible to viral infection. Therefore, Tregs cells can affect HIV pathogenesis by (i) suppressing antiviral immune responses and (ii) contributing to viral reservoir formation. Huot et al. discuss the differences that exist between non-pathogenic SIV infection in natural hosts and pathogenic HIV/SIV infection in humans and macaques regarding virus target cells and replication dynamics in LNs. They emphasized on the NK cell-mediated control and the impact that these insights on viral dynamics and host responses in LNs of natural hosts have for the development of strategies toward HIV cure. Estes et al. provide insights of imaging techniques which help to characterize the compartmentalization of highly specialized immune and stromal lymph node cell populations, the local interplay between the virus and host cells with respect to viral persistence, immune responses, tissue structure and pathologies, and changes to the surrounding milieu and function of immune cells. Merging imaging platforms with other cutting-edge technologies could lead to novel findings regarding the phenotype, function, and molecular signatures of particular

immune cell targets, further promoting the development of new antiviral treatments and vaccination strategies.

A major obstacle in the fight against HIV is the establishment of a viral reservoir compartment that persists even under long-term successful antiretroviral treatment. Bronnimann et al. provide a comprehensive review of biological factors contributing to the role of B cell follicle as a major site for actively transcribed virus. The role of particular cell types, including Tfh, follicular CD8, Follicular Dendritic Cells (FDCs), γδT, and NK cells in this process is discussed. An overview of "cure strategies" targeting the virus within the follicular area are also presented. Several reports have revealed the importance of Tfh cell compartment for the viral propagation and reservoir establishment. Dave et al. discuss the unique contribution of cervical lymph nodes (CLNs) in establishing/maintaining viral reservoir. CLNs is a draining site for meningeal and nasal lymphatics and virus could access these anatomical sites through CNS and conventional DCs or CD4 T cells infected within the CNS compartment. Similar to peripheral LNs, CLN FDC network can contribute to viral spreading and reservoir establishment. The authors also discuss possible ways to purge the virus from these anatomical sites. Novel, more sophisticated strategies are needed for the elimination of the virus, especially form sites like follicular areas. Chimeric Antigen Receptor genetically engineered T cells (CAR) represents a promising immunotherapy for hematological tumors and actively pursued for solid tumors too. Aid et al. discuss specific cellular signaling molecules and transcriptional factors as regulators of lymph node Tfh cell development. System biology tools can provide valuable information to this regard. The authors describe molecular pathways and particular molecules that could promote the infection of Tfh cells and establishment of the viral reservoir. Further investigation of Tfh cell biology could lead to novel strategies for the efficient depletion of HIV from this T cell compartment. Besides Tfh cells, the role of FDCs in capturing virions and contribute to viral spreading is well-established. Haran et al. provide original data describing the construction of anti-SIV CAR/CXCR5 T cells. The engineered cells have the capacity to populate the follicular area in an *ex vivo* B cell follicle migration assay while retaining their viral suppression activity. The provided data further support the development of CAR technology as an alternative approach for virus elimination in the follicular areas. Wang and Xu review the epigenetic regulation of GC responses, especially for GC B and Tfh cell under normal and during chronic HIV/SIV infection. Ellegård et al. provide original data showing that dendritic cells, natural killer cells, and T cells play critical roles during primary HIV-1 exposure at the mucosa, where the viral particles become coated with complement fragments and mucosa-associated antibodies. The microenvironment together with subsequent interactions between these cells and HIV at the mucosal site of infection will determine the quality of immune response that ensues adaptive activation. George and Mattapallil reviewed the role of IFN-alpha subtypes in HIV infection and discuss the possibility that certain subtypes could be potential adjuncts to a "shock and Kill" or therapeutic vaccination strategy that can lead to better control of the latent reservoir and subsequent functional cure while Wang et al. discuss the potential role of IFN-I as regulator of innate and adaptive immunity, including Tfh cells, in chronic HIV infection and the therapeutic strategies targeting IFN-I in infected individuals.

This eBook provides a comprehensive presentation of recent published work on lymph node and especially Tfh cell dynamics in HIV infection and we hope that it will be useful for our further understanding of how such dynamics affect the interplay between virus and host as well as for the discovery of novel therapeutic targets in the fight against HIV.

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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REVIEW
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T Cell Subsets in the Germinal Center: Lessons from the Macaque Model

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Germinal centers (GCs) are organized lymphoid tissue microstructures where B cells proliferate and differentiate into memory B cells and plasma cells. A few distinctive subsets of highly specialized T cells gain access to the GCs by expressing the B cell zone–homing C-X-C chemokine receptor type 5 (CXCR5) while losing the T cell zone–homing chemokine receptor CCR7. Help from T cells is critical to induce B cell proliferation and somatic hyper mutation and to limit GC reactions. $CD4^+$ T follicular helper (T_{FH}) cells required for the formation of GCs and for the generation of long-lived, high-affinity B cells. Regulatory $CD4^+$ (T_{FR}) and $CD8^+$ T cells co-localize with T_{FH} cells and keep their expansion in check, thus limiting GC reactions. A cytotoxic $CXCR5^{pos}$ $CD8^+$ T cell subset has been described in GCs in humans: although low in number, GC $CD8^+$ T cells can expand rapidly during certain viral infections. Because these subsets find their home in secondary lymphoid tissues (lymph nodes and spleen) that are difficult to obtain in humans, GC–homing T cells have been extensively studied in mice. Nevertheless, significant limitations in using this model, such as evolutionary divergences between mice and humans and the lack of an optimal mouse model for certain human diseases, have prompted investigators to characterize GC–homing T cells in macaques instead. This review will focus on discoveries made in macaques, particularly in the non-human primate models of simian immunodeficiency virus and simian–human immunodeficiency virus infection. Indeed, experimental studies in these models have allowed researchers to gain insight into the relative role of follicular T cell subsets in HIV progression, virus persistence, and specific B cell responses induced by HIV vaccines. These discoveries have prompted the testing of novel approaches aimed to manipulate follicular T cells to increase the efficacy of HIV vaccines and to eliminate HIV reservoirs.

Keywords: T follicular helper cell, T follicular regulatory cells, non-human primate, HIV infections, simian immunodeficiency virus, germinal center

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INTRODUCTION

Effective antibody responses are crucial for preventing viral infections and are the basis for the majority of successful vaccination strategies (1). The quality of such antibodies is largely dependent on T cell–B cell interactions. In physiological conditions, T and B cells are subcompartmentalized within lymphoid lobules of lymph nodes (LNs). B cells reside within the outer cortex areas of the

lobules enriched for the B cell-attracting CXCL13, while T cells express the (C-C motif) receptor 7 (CCR7) and recirculate through the paracortex and interfollicular cortex enriched in CCR7 ligands (CCL21 and CCL19). Following antigenic stimulation, a small number of activated T cells lose CCR7 expression and upregulate CXCR5, the receptor for CXCL13 (2–4). $CXCR5^{pos}$ T cells travel toward the B cell-rich follicles in the outer cortex areas, where they interact with B cells (5–7). Two highly specialized $CXCR5^{pos}$ $CD4^+$ T cell subsets, T follicular helper (T_{FH}) and T regulatory (T_{FR}) cells, have been identified in B cell follicles. Activated T_{FH} cells migrate to the T–B borders and B cell follicles where they are required for the formation and maintenance of germinal centers (GCs) [reviewed in Ref. (8, 9)]. GCs are organized lymphoid tissue microstructures where B cells expand and differentiate during immune responses to appropriate pathogens or antigens (10, 11). In the GCs, T_{FH} cells support B cells class switching recombination and somatic hypermutation (SHM) (10, 12). GC reactions ultimately result in the selection of resting B cell memory cells and long-lived plasma cells producing antibodies with high affinity for the encountered antigen (13). The strong reaction occurring in the GCs needs to be tightly regulated to avoid the generation of autoantibodies and excessive inflammation (14–17). T follicular regulatory cells have recently been described as a subset of $CXCR5^{pos}$ T regulatory cells that co-localize with T_{FH} cells, control their expansion, and modulate T_{FH} cell-driven B cell maturation, antibody class switching, and affinity maturation (14, 16, 17). $CD8^+$ T cells are also part of the follicular T cell population (18–25). Recent studies have started to shed light on the role of these cells in regulating GC reactions and their interaction with T_{FH} and B cells in certain infections (19, 20, 23–25).

Because of their critical role in every step of B cell differentiation, T_{FH} cells have been the focus of intense interest in HIV infection. Indeed, aberrant B cell responses and B cell dysfunction are characteristics of chronic HIV infection (26). T_{FH} cells are infected with HIV (27–29), they accumulate in lymphoid tissues of some individuals during chronic infection (27, 28), and their ability to provide B cell help is impaired (30). Hence, HIV-associated changes in T_{FH} cells most likely affect the generation of effective B cell responses against the virus. Moreover, by homing to the GCs, T_{FH} cells escape immunological control and establish a persistent reservoir (21, 22, 31). The quest for an effective vaccine against HIV has also fueled intense interest in the biology of $CXCR5^{pos}$ T cells and their role in GC reactions. Ideally, an HIV vaccine would induce high affinity broadly neutralizing HIV-1 antibodies capable of neutralizing multiple HIV-1 viral strains. These antibodies show remarkable levels of somatic mutation (32); henceforth, their generation is most likely highly dependent on effective T_{FH}–B cell interactions in GCs.

Much of the current knowledge on the role of GC-resident T cells during HIV infection has been attained by studies performed in non-human primate (NHP) models. Macaques can be infected with the simian immunodeficiency virus (SIV) that closely mimics many aspects of HIV infection (33), giving the NHP model advantages by comparison to both rodents and humans. This review will focus on discoveries made in macaques, on how GC–homing T cells are affected during HIV/SIV infection, and on how HIV-associated changes in these cells may alter antibody responses. Strategies tested in NHP models aimed to target T_{FH} cells to eliminate HIV reservoir from GCs and to increase the effectiveness of HIV vaccine responses will also be discussed.

Characterization of GCs in NHPs

Studies in mice have been fundamental in revealing the phenotype and function of GC-resident T cells and studying their key lineage-specific transcription factors (14, 16, 34). However, the similarity between humans and NHPs makes NHPs optimal for research on complex immunological interactions. Macaques have several advantages over rodents, and the first is that their genetic evolution more closely resembles those of humans (35, 36). For example, evolutionary divergence between the signaling pathways that shape T_{FH} cell differentiation in humans and mice has recently been discovered (37). Second, their immune system also resembles those of humans. Indeed, NHPs have been used to study fundamental aspects of immunology, including the development and maintenance of T cell memory (38), immunodominance (39), and the aging immune system (40). Third, macaques LN's structure is more similar to humans than rodents LN' structure (41). In macaque lobes, T cell zones and B cell follicles can be identified with equal function and cell distribution as in humans. Finally, lymphoid cells and a number of their different subtypes are also identifiable with equivalent markers and methodologies used in humans.

Germinal centers are typically few in LNs of naive animals, with very little T_{FH} cell number (31). Upon vaccination or infection, selected follicles are activated and develop into GCs. The interactions between cognate B and T cells have been reported to occur 1–2 days after antigen exposure (42–44). Studies in macaques have shown that GCs are formed in draining LNs a few days after intramuscular immunization at the same site of the delivery, while they are absent in contralateral LNs (45, 46). GCs in macaques can be readily identified as positive for the B cell marker CD20 and express high levels of the proliferation marker Ki67 ($CD20^{pos}$ $Ki67^{hi}$). Alternatively, Hoechst staining of nuclei is used to discern GCs from the adjacent marginal zone by less intense staining in immunohistochemistry analyses (47). Marginal zone B cells, responsible for an early antibody response to blood-borne pathogens, have been identified in cynomolgus and rhesus monkeys as B cells ($CD19^+$, $CD20^+$) expressing high levels of complement receptor 2 (CD21) and low levels of CD27 (47, 48). CD4, CD20, PD-1, and Ki67 markers were simultaneously used to study B follicles and T_{FH} cells in rhesus macaques (48).

Compared to human subjects, the use of NHPs allows researchers to conduct controlled challenge experiments, multiple live surgeries, and invasive and terminal experiments, ultimately granting access to different tissues to an extent that is not feasible in humans (49). NHP models have also been used to test sampling techniques aimed to study the cellular composition of GCs. While in macaques it is possible to surgically remove the draining LNs at different time points after an immunization or an experimental infection, this procedure is invasive and may disrupt ongoing immune responses. Two studies have used fine-needle aspirations (FNAs) technique to collect cells from

LNs of pigtail and rhesus macaques (50, 51). In both models, T_{FH} cell where readably measurable, suggesting that FNA may be an interesting alternative to collect small numbers of GC cells for further analyses, while maintaining ongoing immune responses.

Characterization of T_{FH} Cells in Macaques

T_{FH} cells in macaques have a phenotype comparable to that of humans. T_{FH} cells are considered a distinct cell subset with a specialized function and a specific transcription factor that differs from other T helper cell subsets. The master regulator of T_{FH} cell differentiation is the transcription factor Bcl-6 (52, 53). While Bcl-6 is the unique marker of T_{FH} cells, other canonical markers used to identify them are CXCR5, PD-1, and the inducible T cell costimulator, ICOS. High expression of PD-1 has been considered an effective way to identify GC–T_{FH} cells in intact tissues (31) when GCs are co-stained. In healthy macaques, PD-1^{hi} cells within the GCs are almost exclusively $CD4^+$ T cells (31). Different combinations of markers have been used to define T_{FH} cell in cell suspensions by flow cytometry, in human and macaques (**Table 1**). The percentage of T_{FH} cells frequency in LNs depends on the choice of the markers used to define and the gating strategy. Because of the unavailability of a cross-reactive antibody for CXCR5, T_{FH} cells were originally identified in secondary lymphoid tissues of pigtail macaques as $CD4^+$ T cells-expressing programmed cell death 1 of PD-1^{hi} and low levels of interleukin-7 receptor alpha (IL-7Rα) chain (CD127) (50, 54). This cell population was only present in spleen and LNs, but not in blood, and expressed high levels of ICOS and Bcl-6. In rhesus macaques, T_{FH} cells were first identified in cell suspension from LNs as central memory ($CD28^{hi}$ $CD95^{hi}$) $CD4^+$ T cells expressing low levels of $CCR7^{lo}$ and high levels of PD-1 and ICOS (55). When the cross-reactive anti-CXCR5 antibody clone MU5UBEE became available, co-expression of CXCR5, coupled with high levels of PD-1 expression, has been widely used to identify and sort T_{FH} cells (56–59). However, others have reported changes in both markers, and particularly in PD-1 following *ex vivo* HIV infection, warning against using only these two markers to define T_{FH} cells (60).

TABLE 1 | Markers to define TFH cells in cell suspension in humans and macaques.

Species	Tissue	Infection/ treatment	TFH definition	Reference
Indian rhesus macaques	LN	SIVmac251	$CD28^{hi}$ $CD95^{hi}$ $CCR7^{low}$ PD-1^{hi}	(55)
Pigtail macaques	LN, spleen	SIVmac239/ SIVmac251	$CD8^-$ $CD45RA^-$ PD-1^{hi} $CD127^{low}$	(50, 54)
Indian rhesus macaques	LN	SIVmac239	$CXCR5^+$ PD-1^+	(31)
Indian and Chinese rhesus macaques	LN, spleen	SIVmac251	$CXCR5^+$ PD-1^{hi}	(61)
Indian rhesus macaques	LN	SIVmac251	$CD95^+$ $FOXP3^-$ $CXCR5^+$ PD-1^{hi}	(57)
Indian rhesus macaques	LN	SIVsmE660	$CXCR5^+$ PD-1^{hi} $FOXP3^-$	(58)
Indian rhesus macaques	LN	SIVmac251, SHIV	$CXCR5^+$ PD-1^{hi}	(62)
Indian rhesus macaques	LN	–	PD-1^{hi} (enriched TFH)	(56)
Humans	LN	HIV+	$CXCR5^+$ PD-1^{hi}	(28)
Humans	LN	HIV+, HIV + ART, and LTNP	$CD45RA^-$ $CXCR5^+$ PD-1^+ BCl-6^+	(27)
Humans	Spleen	HIV+	$CD45RA^-$ $CCR7^-$ $CXCR5^+$ PD-1^+	(29)
Humans	LN	HIV+	$CD45RA^-$ $CXCR5^{hi}$	(30)
Humans	Blood	–	$CD45RA^-$ $CXCR5^{hi}$	(63)
Humans	Blood	HIV+	$CXCR5^+$ $CXCR3^-$ PD-1^+	(64)

ART, antiretroviral treatment; LN, lymph node; SHIV, simian–human immunodeficiency virus; SIV, simian immunodeficiency virus; TFH, T follicular helper cell.

Macaques have been a useful model for validating circulating biomarkers of GC responses that can be easily translated to humans. One example is the measurement of the level of plasma CXCL13. In macaques, CXCL13 is detectable in plasma, it increases following immunization, and its levels are associated with the frequency of T_{FH} cells in LNs (65). Importantly, a substantial frequency of $CD4^+$ T cells expressing CXCR5 is also present in the blood of rhesus macaques, as is the case in humans (63). Phenotypically, circulating T_{FH} (cT_{FH}) cells share common markers with GC-resident T_{FH} cells and can be identified as $CXCR5^{pos}$ PD-1^{pos} $CD4^+$ T cells. However, cT_{FH} cells express lower levels of ICOS and of the activation marker CD69 than GC T_{FH} cells, suggesting that they are present in a resting phase (66). While the origin of cT_{FH} cells is still unclear, the marker expression and ability to interact with B cells and promote B cell responses *in vitro* suggest that they may be circulating counterparts of T_{FH} cells in LNs. In mice, humans, and macaques, circulating $CXCR5^{pos}$ PD-1^{hi} $CD4^+$ T cells are heterogenic and can be divided into subsets based on their expression on (C-X-C motif) chemokine receptor 3 (CXCR3), a marker for $CD4^+$ T helper type 1 (Th1) cells, alone or together with CCR6. $CXCR5^{pos}$ $CXCR3^{neg}$ PD-1^{pos} T_{FH} cells present the most genetic and functional similarities to T_{FH} cells in LNs (64). When the expression of CCR6 is considered, cT_{FH} cells can be further divided into three subpopulations that mirror the unique phenotype and cytokine signature of lineages of non-T_{FH} $CD4^+$ T cells in blood: T_{FH} type 1 ($CXCR3^{pos}$ $CCR6^{neg}$), type 2 ($CXCR3^{neg}$ $CCR6^{neg}$), and type 17 ($CXCR3^{neg}$ $CCR6^{pos}$). More studies are needed to identify the role of these cell subsets in generating or maintaining antibody responses to pathogens.

Functionally, T_{FH} cells help B cells by secreting cytokines and expressing surface molecules and providing survival, proliferation, and differentiation signals [reviewed in Ref. (9, 67).]. In macaques, as in humans, GC-resident T_{FH} cells express the costimulatory receptor ICOS, the costimulatory protein CD40L required for B cell survival, and they produce the B cell helper cytokines IL-21 and IL-4 although T_{FH} cells can also produce other cytokines depending on the stimulus they receive (9). IL-21 signaling is pivotal for B cell differentiation and for the development of B cell memory. *In vitro* IL-21 production is often used as a

means to measure antigen-specific responses, particularly following immunization in humans (68) and macaques (69). However, T_{FH} and cT_{FH} cells produce limited quantities of IL-21. As a result, the tracking of antigen-specific responses by intracellular staining is challenging. A recent study has used the macaque model to develop a cytokine-independent technique aimed improve the quantification of antigen-specific T_{FH} cells. Havenar-Daughton et al. have shown that the co-expression of OX40 and CD25 surface markers is sufficient to identify antigen-specific GC T_{FH} and pT_{FH} cells in the LNs and blood of immunized animals (70). Importantly, this technique offers the possibility to isolate antigen-specific T_{FH} cells by cell sorting, which is not possible with intracellular cytokine detection.

HIV-/SIV-Associated Changes in T_{FH} Cells

HIV infection is associated with numerous B cell anomalies (26). Untreated HIV and AIDS patients develop profound B cell dysfunction, characterized by hypergammaglobulinemia, and polyclonal B cell activation (26, 71–73). The majority of HIV-infected individuals and SIV-infected macaques fail to produce protective antibodies against HIV/SIV and low-affinity B cells mature inappropriately into plasma cells (74). Because T_{FH} cells are required for the induction of high-affinity antibody responses and the generation of long-lived B cell memory (75), several groups have investigated HIV/SIV-associated changes in T_{FH} cells and their possible effect on B cell abnormalities.

Recent data suggest that GC–CXCR5$^+$ PD-1^{hi} T_{FH} cells are susceptible to HIV-1/SIV infection (27, 28, 54, 55, 60). Interestingly, unlike non-T_{FH} CD4$^+$ T cells, T_{FH} cell frequency and number increase in chronic HIV/SIV infection in the LNs of some humans (27, 28) and macaques (31, 54, 55, 57, 58, 60). In both macaques and humans, the increase in T_{FH} cell frequency in chronic infection is approximately 10 times compared to non-infected levels (28, 55). In humans, a median of 60% of HIV-1 RNA-producing cells was found within lymphoid follicles by *in situ* hybridization in chronically infected untreated patients with a median of 17% of follicles tissues per inguinal LN (22).

Remarkably, in all the studies reported, T_{FH} cell expansion is observed only during chronic infection, but not in acute infection. Although the reason for the increase in T_{FH} cell levels during chronic HIV/SIV infection is not clear, different hypotheses have been proposed. The accumulation of T_{FH} cells during chronic SIV and HIV has been associated with immune activation (55) and plasma viremia (57, 58) in some studies. Other studies suggest that this expansion may be driven by prolonged T cell receptor stimulation (62). In mice, LCMV infection redirects CD4$^+$ T cell development away from the Th1 cell responses induced during an acute infection toward T_{FH} cells (76). Others have shown that HIV-specific GC–T_{FH} cells, particularly against the gag, also expanded in chronic infection in humans (27, 28). Finally, effective antiretroviral treatment (ART) decreases the number of T_{FH} cells in humans and macaques, suggesting that active HIV replication is necessary for T_{FH} cell expansion (27, 28).

The increase in T_{FH} cells levels is also associated with increased frequency of activated GC B cells and SIV-specific antibodies (55) in macaques, and plasma cells and immunoglobulin levels in HIV infection (28). Moreover, broadly neutralizing antibodies (bNabs) are present in HIV patients with high levels of circulating CXCR5^{pos} CXCR3^{neg} PD-1^{hi} CD4$^+$ T cells (64). These results suggest that T_{FH} cells may be highly functional during HIV/SIV infection; however, other studies have revealed that they provide inadequate help to B cells (30, 77). GC-resident T_{FH} cells isolated from HIV-infected patients produce less IL-21, a cytokine pivotal for GC formation, GC B cell proliferation, and B cell maturation (9). The replenishment of exogenous IL-21 *in vitro* to T_{FH}/B cell co-cultures or the *in vivo* administration to SIV-infected macaques significantly improves memory B cell levels (30, 78), suggesting that lost IL-21 production may be a contributing factor to the generation of defective memory B cell responses. T_{FH} cells express a number of molecules that restrain them from excessive proliferation such as PD-1 (53). The PD-1 expression is highly increased in HIV-infected CD4$^+$ T cells (31), and the level of its ligand PDL-1 on B cells increases in HIV patients. Interestingly, by blocking the PD-1–PD-L1 interaction, IL-21 production by T_{FH} cells is recovered and B cell functions are restored. Therefore, it is possible that T_{FH} cell impairments may be, at least in part, mediated by HIV-associated changes affecting B cells (30).

Studies in monkeys have reported that during SIV infection, T_{FH} cells express non-characteristic transcriptional factors together with canonical ones and that gene and cytokine expressions are skewed toward CD4$^+$ Th1 cells and interferon (IFN)-γ (78). During chronic SIV infection, IFN-γ-induced genes are upregulated while the expression of the IL-4 gene is downmodulated (55). Accordingly, the majority of GC–T_{FH} cells in chronically infected macaques are positive for CXCR3$^+$ and produce IFN-γ (Th1-type cytokine) alongside IL-21. While these cells are capable of helping B cells *in vitro*, they express higher levels of CCR5 and harbor more SIV-DNA than CXCR3^{neg} GC–T_{FH} cells (79). T-bet, the transcriptional regulator of Th1, is also increased in T_{FH} cells isolated from SIV-infected macaques' spleens (61). Importantly, an association between IFN-γ^{low} IL-21^{hi} GC resident T_{FH} cells and the broad neutralization activity against the envelope was found in simian–human immunodeficiency virus (SHIV)-infected macaques (80). Taken together, these studies suggest that, while functional, T_{FH} cells may undergo changes in levels and function that may affect their ability to help B cells induce high-quality antibodies. These conclusions are corroborated by the lack of responsiveness to other infections or vaccines observed during late HIV/SIV infection.

T_{FR} Cells and SIV-Associated Changes in Macaques

T follicular regulatory cells control the magnitude of GC reactions and avoid the onset of some autoimmune diseases (14–17). The frequency of T_{FR} cells is low in mice, humans, and monkeys compared to other CD4$^+$ T cells subsets, and as for the T_{FH} cells, the percentage varies depending on the markers used to identify this subset. Phenotypically, they share the canonical markers of T_{FH} cells (CXCR5, ICOS, PD-1) and T_{REGS} [FOXP3, CD25, cytotoxic T-lymphocyte-associated protein 4 (CTLA4) positive and CD127 negative] (**Table 2**). Functionally, T_{FR} cells produce IL-10

TABLE 2 | Markers to define TFR cells in cell suspension in humans and macaques.

Species	Tissue	Infection/ treatment	TFH definition	Reference
Indian rhesus macaques	LN	SIVmac251	CD95$^+$ FOXP3$^+$ CD25$^+$ CXCR5$^+$ CCR7$^-$	(57)
Indian rhesus macaques	LN	SIVsmE660	CXCR5$^+$ PD-1^{hi} FOXP3$^+$ CD25$^+$	(58)
Indian rhesus macaques	LN	SIVmac239	CD3$^+$ CD8$^-$ CD25^{hi} CD127$^-$ CXCR5^{hi} (GC:PD-1^{hi})	(82)
Humans	Spleen	HIV+	CD45RA$^-$ CCR7$^-$ CXCR5$^+$ PD-1$^+$ FOXP3$^+$ CD25$^+$	(29)
Humans	LN	HIV+	CD3$^+$ CD8$^-$ CD25^{hi} CD127$^-$ CXCR5^{hi}	(81)

and TGF-β and express the inhibitory molecule CTLA4 (16). T_{FR} cells have been characterized in the LNs of rhesus macaques as FOXP3^{pos} CD25^{pos} CXCR5^{pos} CCR7^{neg} as FOXP3^{pos} CD25^{pos} PD-1^{hi} CD127^{neg} CXCR5^{pos} CD4$^+$ T cells or, alternatively, as CD25^{pos} CD127^{neg} CD3$^+$ CD8$^-$ T cells (57, 58, 81, 82). Depending on the markers used, their frequency ranges between 2 and 5% of CD4$^+$ T cells or CD8$^-$ CD3$^+$ T cells. We showed that an enriched population of T_{FR} cells, obtained from the LNs of macaques by isolating sorted CD25^{pos} CD4$^+$ T cells migrating toward CXCL13, was capable of suppressing autologous GC–T_{FH} cell proliferation *in vitro* (58).

T_{FR} cells are essential to the control of T_{FH} cell numbers in mice (14, 16). T_{FR} cell decrease or stagnation during chronic SIV infection may contribute to the T_{FH} cell dynamic seen in HIV infection. Two macaque studies have shown that T_{FR} cells are susceptible to infection by different SIV strains: SIV_{mac251} and SIV_{smE660} (57, 58). A recent study expanded this knowledge to humans by showing that T_{FR} cells are highly permissive to infection both *ex vivo* and *in vivo* in chronic HIV-untreated patients (81). In a longitudinal study in SIV_{mac251}-infected macaques, we showed that the frequency and number of T_{FR} cells significantly decreased in LNs during the chronic phase and that the reduction was associated with an increase in T_{FH} cell levels (58). These findings were corroborated by the parallel independent study by Chowdhury et al., showing changes in the ratio of T_{FH} to T_{FR} cells in favor of T_{FH} during chronic infection with SIV_{smE660} (59). Interestingly, in a cross-sectional study, Miles et al. showed an increase in the number of GC-resident T_{FR} cells in HIV-infected humans and SIV_{mac239}-infected macaques. In humans, an increase in the CD4$^+$ Foxp3$^+$ cell count was observed when the LN area was considered to account for LN enlargement that occurs during chronic HIV infection (81). Differences in the study design (longitudinal versus cross-sectional), T_{FR} cell definition, and analyses may have contributed to the inconsistent findings in these studies.

The increase of T_{FH} cells in chronic SIV infection has been previously associated with an increase in the titers of gp120-specific antibodies with high avidity (55). Interestingly, we observed an antithetical role of T_{FR} and T_{FH} cells in the avidity of antibodies to the SIV-gp120 protein throughout the infection. T_{FR} cell levels were associated with a reduction of binding high-avidity antibodies to SIV-gp120 in all the infected animals (58). The role of T_{FR} cells in the impairment of humoral immunity during HIV infection remains to be determined. Finally, T_{FR} cells are a relatively newly discovered population, and many of the studies performed on T_{FH} cells in humans and macaques did not include markers for T_{FR} cells exclusion (**Table 1**). Given their changes in frequency, susceptibility to infection and function, discriminating markers for T_{FR} should be included when studying T_{FH} cells, particularly in HIV vaccine studies.

GC-Resident CD8$^+$ T Cells in Macaques

GC-resident or CXCR5^{pos} CD8$^+$ T cells are present in lymphoid tissues of humans (20, 83–85) and macaques (19, 31). In fact, three decades ago, high frequencies of CD8$^+$ T cells were found in inflamed lymphoid follicles in heroin addicts and HIV-related lymphadenopathy (83, 84). However, compared to T_{FH} cells, current research on the CXCR5$^+$ CD8$^+$ T cells is relatively scarce. Several studies suggest that CXCR5$^+$ CD8$^+$ T cells represent a subset of follicular cytotoxic CD8$^+$ T cells and may contribute to virus control in B cell follicles (23). Indeed, follicular cytotoxic CD8$^+$ T cells express granzyme A and B and perforin at higher levels than their CXCR5^{neg} counterpart (85). Interestingly, a study identified a subset of CD8$^+$ T cells with the suppressive activity on T_{FH} cells in rhesus macaques' LNs and humans' tonsils (19). CD8$^+$ with a regulatory function produce IL-10 and express high levels of CXCR5 and the homing cell adhesion molecule CD44. Together these studies suggest that GC-resident CD8$^+$ T cells may be a heterogeneous cell population.

The accumulation of infected T_{FH} cells in LNs during chronic infection is a major obstacle toward eradication. Cytotoxic CD8$^+$ T cells are critical for the clearance of virus-infected CD4$^+$ T cells; thus, studies focused on understanding the phenotype and function of HIV/SIV-specific CD8$^+$ T cells have been performed in humans and macaques. To date, there are conflicting data on the quantity of specific CXCR5^{pos} CD8$^+$ T cells and their ability to clear virus-infected T_{FH} cells. Virus-specific CD8$^+$ T cells are present in GCs of humans and macaques, but they may not be enough to clear the increasing population of infected T_{FH} cells (21, 22), may be functionally impaired/exhausted (25), may exert regulatory instead of cytotoxic function, or be predisposed to provide B cell help once they enter the B cell follicles (18, 19, 25). Two *in vivo* CD8 cell depletion studies have been performed in SIV-infected macaques (24, 25). Fukazawa et al. showed that SIV is restricted to CD4$^+$ T cells in the B cell follicles (with a median of 95% of productively SIV-infected cells) in macaques that are naturally controlling infection (elite controller or EC), but not in animals with normal disease progression. *In vivo* depletion of CD8$^+$ cells in EC macaques resulted in a temporal redistribution of productive CD4$^+$ T cells in the extrafollicular area, until CD8$^+$ T cells absolute count returned to normal levels (24). Li et al. showed higher levels of both follicular and extrafollicular SIV-producing cells after CD8$^+$ cell depletion in normal disease progression macaques, with the greatest increase in the extrafollicular areas (8.9 versus 3.8 cells/mm^2 average change in the follicles) (25). Although these two studies differed in the CD8 depletion protocol (repeated low dose administrations, one single high-dose bolus), both showed

profound depletion in the LNs. It should be noted that *in vivo* CD8 cell depletion may have eliminated other CD8-expressing cell populations (for example, NKs).

Some CXCR5^{pos} CD8$^+$ T cells with the ability to contain LCMV have been found in GCs in mice and in blood of HIV-infected patients (86), where their levels correlated with viral load. In patients with HIV, the number of virus-specific CXCR5^{pos} CD8$^+$ T cell subset is inversely correlated with viral load in LNs (86). Peripheral and GC CXCR5^{pos} CD8$^+$ T cells are also present in SIV-infected macaques, where their levels increase after immunization, and it is higher in macaques controlling infection than ones who do not (87). CD8$^+$ T cells can still contain viral replication in chronic infections although the mechanism of this containment is largely unknown (86). Recent work by Petrovas et al. show that CD8$^+$ T cell in the GC had better killing activity than non-follicular CD8$^+$ T cells, despite being less polyfunctional (20). Taken together, these results suggest that CD8$^+$ T cells could be an effective component of an HIV cure strategy.

T_{FH} Cells as Privileged Latent Reservoir

Lymphoid organs constitute the first established reservoir of HIV infection. In untreated HIV patients, viral replication is found in GCs soon after and all through the duration of infection (88–90), and the free virus can be detected even during clinical latency asymptomatic phase (91, 92). Viral replication is never completely curtailed from the LNs, and it is detected in the GCs till they involute with advancing disease (93). The macaque model of HIV-1 infection has been fundamental to study B cell follicles as immune privileged sites and for extending these observations to gut-associated lymphoid tissue (21, 94, 95). Antiretroviral therapy contains viral replication; however, it fails to eliminate the virus from lymphoid tissues. A steady-state level of very low viremia has also been described among those on ART, but the exact mechanism for persistent viremia during ART is not completely understood (96). Upon ART discontinuation, viral replication rebounds, resulting in titers similar to those observed prior to treatment. Thus, it is possible that some cellular sanctuary may exist, which allow the virus to persist.

Recent data suggest that GC–CXCR5$^+$PD-1^{hi} T_{FH} cells are highly susceptible to HIV-1 infection (27, 28, 60). Some studies report that T_{FH} cells are a preferentially infected by HIV/SIV (27, 57), while others report that the permissiveness of T_{FH} cells is comparable to other subsets of memory CD4$^+$ T cells (54, 55). The levels of the virus entry co-receptor CCR5 expressed by T_{FH} cells varies in different studies (54, 60, 62, 97), possibly depending on the definition used to identify T_{FH} cells. The role of levels of CCR5 expression of T_{FH} cells and susceptibility to HIV/SIV infection is also not clear. While a study reports no association between the levels of co-receptor and susceptibility (60), Xu et al. used NHP to explain the apparent discrepancy between low levels of the HIV co-receptor and the heightened permissiveness to infection (97). The group identified a subset of LN-resident T_{FH} cell precursors expressing intermediate levels of PD-1 and higher levels of CCR5 than fully differentiated PD-1^{hi} T_{FH} cells and showed that in their precursor state T_{FH} cells are highly susceptible to *in vitro* SIV infection. Other parameters may also contribute to the high susceptibility to HIV/SIV infection of T_{FH} cells described in certain studies. Recent work by Ruffin et al. showed that GC–T_{FH} cells from LNs and tonsil obtained from chronically infected patients express low levels of the HIV-1 restriction factor SAMHD1 (98, 99).

Localization of T_{FH} cells within the GCs most likely contributes to their high susceptibility to HIV/SIV infection and their expansion (22, 31). The increase in T_{FH} cell permissiveness, when compared to other memory CD4$^+$ T cell subsets, does not associate with their activation status or levels of HIV co-receptor expression (60). It is possible that their unique localization in the GC may play a role in the heightened susceptibility. Indeed, it has been shown that CD4$^+$ T cells located in the follicles are 40 times more likely to be infected by HIV than those located outside the follicles (22). In the GCs, the virus can be transmitted by follicular dendritic cells (FDCs) that are capable of long-term antigen retention (100). FDCs trap multiple intact viral particles on the surface and efficient transmission to GC-resident CD4$^+$ T cells (101–104). Indeed, FDCs act as HIV "archives" by retaining ART-resistant virus variants that are not present elsewhere (104). While this is an interesting theory, it is also expected that other molecules may be bound together with the virus, such as antibody and complement, and it is not clear how this trapped virus would serve as a source for CCR5-expressing T_{FH} cell precursors (97). Other HIV-/SIV-associated changes in cellular composition within the GCs, such as changes in T_{FH}/T_{FR} cell ratio, may contribute to the accumulation of T_{FH} cells, at least in macaques (57, 58). Moreover, the paucity of virus-specific CD8$^+$ T cells in the B cell follicles, when compared to extrafollicular areas in both HIV (22) and SIV infection (21), as well as cell exhaustion or malfunction, may account for the lack of clearance of infected T_{FH} cells as previously described.

Improving the ability of potent antiviral CD8$^+$ T cells to traffic into B cell follicles may result in the elimination of virus reservoirs. To determine whether the expression of CXCR5 may be sufficient for CD8$^+$ T cells to enter the follicles, Ayala et al. infused six SIV-infected macaques with autologous CD8$^+$ T cells genetically modified to express CXCR5 (105). The engineered T cells were found in abundance within the B follicles, with some cells localized in the proximity of infected T_{FH} cells. While CD8$^+$ T cells used in this study were circulating T cells that were not selected by their specificity to HIV, this study is an important step forward in the further development of strategies aimed to eliminate virus persistence in treated patients (105). Finally, a better understanding of immune cell type localization in the GCs, particularly those with the ability to eliminate infected T_{FH} cells, will be a key for designing new eradication strategies.

Vaccine-Induced T_{FH} Cells

The goal of a vaccine is to induce long-lasting memory responses to the pathogen. HIV presents a greater challenge than other viruses, in part because it replicates in CD4$^+$ T cells and induces profound deregulation of the overall immune system. Neutralizing antibodies against the autologous virus are detectable only after years from seroconversion and only 20% of infected patients develop cross-react antibodies against different gp120 regions (106–108). Although these antibodies have shown protection in non-human macaques' models using SHIV (109, 110), the most

desirable response for an HIV vaccine would be the induction of bNabs. bNabs can act against a wide spectrum of viruses by targeting relatively conserved regions on the surface HIV envelope trimer spike (111). Because of the striking amount of SHM in HIV bNabs (112–114), it is conceivable that T follicular helper cells and GCs play a critical role in generating such antibodies. However, the elicitation of bNabs trough vaccination is challenging. These antibodies are uncommon (produced by 10% of HIV-infected individuals) (115), and conventional HIV vaccines are unable to induce the number of mutations observed in bNabs. Some studies have been performed in macaques to test the ability of different adjuvants to stimulate T_{FH} differentiation, SHMs, and affinity maturation to neutralizing HIV epitopes. Importantly, in this model, the levels of GC-resident T_{FH} cells are associated with the generation of neutralizing antibody breadth during SIV/SHIV infection (80). A study in macaques revealed scarce differences in the mean SHM levels or CDR H3 lengths using eight different adjuvants in combination with a gp140 protein vaccine to immunize macaques (116). PLGA, a toll-like receptor ligand containing nanoparticles adjuvant, induces strong GCs reactions in monkeys (117, 118). A native-like Env trimer, given twice intramuscularly together with a strong adjuvant ISCOMATRIX induced neutralizing antibodies against Tier 2 (difficult to neutralize) viruses (118). Potent T_{FH} cell responses were found in LNs of rhesus macaques after immunization, and no changes were observed in the levels of T_{FR} cells. The effectiveness of this immunization was not tested in challenge experiments.

While the search for a strategy capable of bestowing protection *via* the induction of neutralizing antibodies or even bNAbs continues, non-neutralizing antibodies may also be increased during vaccination by increasing T_{FH} cell differentiation. Of note, the only vaccine to provide low, but significant protection from HIV acquisition in humans induced binding non-neutralizing antibodies to the variable region of the gp120 V2 loop (119, 120). In a retrospective study, it was shown that volunteers vaccinated with an ALVAC-SIV + gp120 alum vaccine had higher levels of IL-21-producing cT_{FH} cells than individuals immunized with strategies that failed to protect (68). Therefore, it is possible that an increase in binding antibodies to gp120, and particularly to the V2 loop, *via* T_{FH} cells may increase the efficacy of an HIV vaccine, despite the absence of neutralization. Studies in macaques have revealed that conventional vectored vaccines indeed stimulate T_{FH} cells in combination with gp120 or gp140 protein boosts. Codelivery of MVA-SIV and gp120 protein in alum increased the levels of $CXCR3^{pos}$ $CXCR5^{pos}$ $CD4^+$ T cells in the blood and LNs of rhesus macaques measured at the peak of immune responses after vaccination. Interestingly, while $CXCR3^{POS}$ cT_{FH} cells favored antibody responses, they were also associated with increased peak viremia upon infection with SIV_{mac251} (121). An Adenovirus 5- based vector vaccine, encoding for Env, Gag, and Nef, followed by a gp120 or gp140 protein boost induced IL-21-producing T_{FH} cells in rhesus macaques' LNs. T_{FH} cell levels measured after 2 weeks from the last immunization were associated with the titers of binding antibodies to the gp120 (69). While this vaccine only protected female macaques from SIV_{mac251} infection, it is noteworthy that only small differences in the levels of IL-21-producing T_{FH} cells were found when animals were stratified by sex. Taken together, these studies suggest that T_{FH} cells are induced by different vaccination strategies, and their induction results in potentially protective antibody responses that are measurable in LNs and blood. Studies comparing different strategies side by side should be performed to shed light on the association between the levels and function of T_{FH} cell induction and vaccine efficacy.

CONCLUSION

The NHP model has played a fundamental role in understanding the dynamics of T_{FH} cells during HIV infection and their role as major sites for viral replication and the establishment of viral reservoirs. This model has been a key to the development of new techniques to study T_{FH} cells and GC responses that can be translated to humans, and it makes it possible to conduct preclinical studies aimed at eradicating HIV. Undoubtedly a fuller appreciation for the range of cells participating in meaningful cellular reservoirs could result in a rational attack on latent HIV-1 and may provide inroads into creating an effective vaccine designed to generate HIV neutralizing antibodies. However, the obvious limitation is that NHPs are not humans. Much of what is learned from non-human primates, especially at the preclinical level, must be validate in humans.

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REVIEW
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Tfh1 Cells in Germinal Centers During Chronic HIV/SIV Infection

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T follicular helper CD4 cells (Tfh) are essential for the development and maintenance of germinal center (GC) reactions, a critical process that promotes the generation of long-lived high affinity humoral immunity. It is becoming increasingly evident that GC-Tfh cells are heterogeneous in nature with some cellular characteristics associated with a Th1, Th2, and Th17 phenotype. Emerging studies suggest that GC-Tfh cells are directed to differentiate into distinct phenotypes during chronic HIV/SIV infection and these changes in GC-Tfh cells can greatly impact the B cell response and subclass of antibodies generated. Studies in HIV-infected humans have shown that certain Tfh phenotypes are associated with the generation of broadly neutralizing antibody responses. Moreover, the susceptibility of particular GC-Tfh subsets to HIV infection within the secondary lymphoid sites can also impact GC-Tfh/B cell interactions. In this review, we discuss the recent advances that show Tfh heterogeneity during chronic HIV/SIV infection. In particular, we will discuss the dynamics of GC-Tfh cells, their altered differentiation state and function, and their impact on B cell responses during HIV/SIV infection. In addition, we will also discuss the potential role of a recently described novel subset of follicular homing CXCR5$^+$ CD8 T cells (Tfc) and their importance in contributing to control of chronic HIV/SIV infection. A better understanding of the mechanistic role of follicular homing CD4 and CD8 T cells during HIV/SIV infection will aid in the design of vaccines and therapeutic strategies to prevent and treat HIV/AIDS.

Keywords: Tfh1 cells, germinal centers, HIV/SIV reservoirs, follicular CD8 T cells, Tfh cells, HIV/SIV infection

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INTRODUCTION

Lymphoid organs are the primary anatomical compartments for the generation of an effective adaptive immune response. CD4 T cells play a central role in the generation of adaptive immunity by providing help to both B cells and CD8 T cells (1, 2). CD4 T helper cells comprise of multiple subsets, including Th1, Th2, Th17, Tfh, Th9, Th22, Th-CTL, and T-regulatory cells (1, 3–5), and the generation of each subset is regulated by specific transcription factors and cognate cytokines (3). Among the various subsets of CD4 T cells, the follicular CD4 T cells (Tfh) reside in the B cell follicles and germinal centers (GC) of lymphoid tissue and play a major role in providing B cell help for the generation of high affinity antibody and long-lived memory B cell response (6, 7). Tfh cells are characterized by the expression of the chemokine receptor CXCR5 (required for homing to B cell follicles), PD-1, CD40L, and ICOS, and the transcription factor Bcl-6 (**Figure 1**) (8). These cells secrete the cytokines IL-21, IL-4, and IL-10 (6). A subset of Tfh cells reside within the GCs

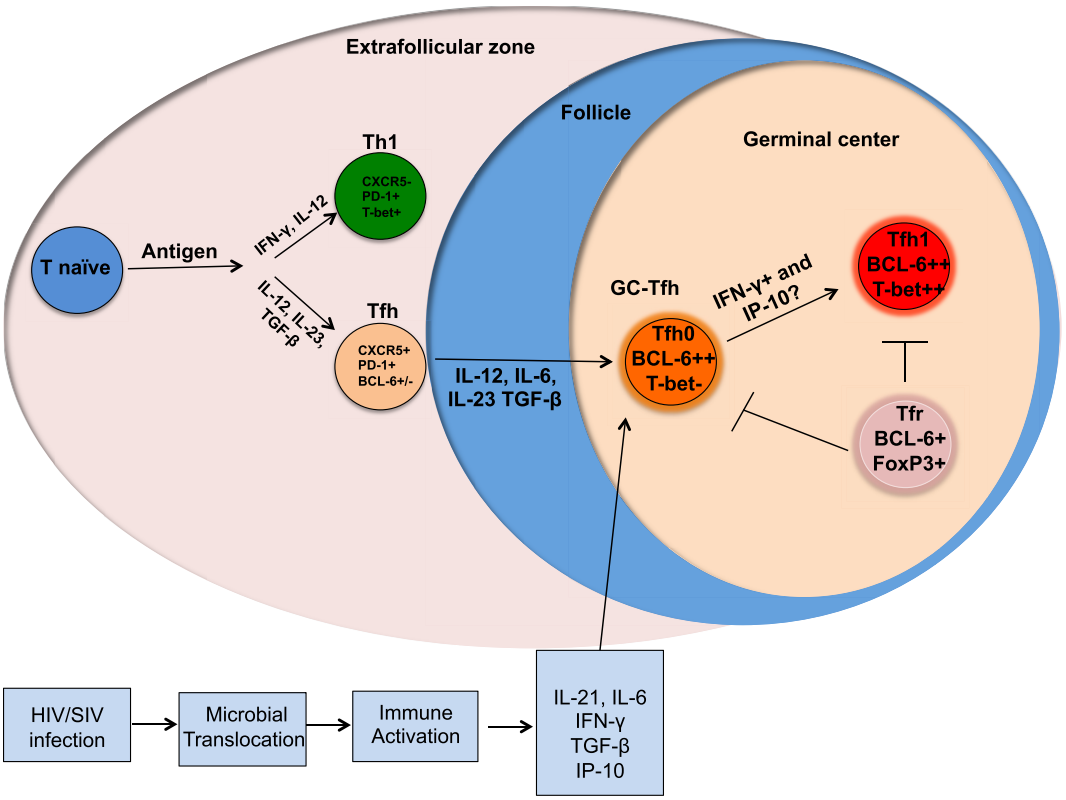

FIGURE 1 | Altered differentiation of Tfh cells during chronic HIV/SIV infection. Following antigenic stimulation naïve CD4 T cells differentiate into different helper T cells and the presence of cytokines, such as IL-12, IL-23, and TGFβ promote differentiation into Tfh cells. Upon further interaction with B cells, these Tfh differentiate into germinal center (GC)-Tfh and migrate to GC. GC-Tfh can further differentiate into Tfh1 cells that can be mediated by the high levels of IFNγ and IP-10 produced during chronic HIV/SIV infection. The GC-resident Tfr cells can regulate the magnitude and function of GC-Tfh.

(GC-Tfh) (**Figure 1**), interact with GC-B cells, and facilitate affinity maturation and Ig class switching (9–12). The GC-Tfh cells express higher levels of PD-1 and Bcl-6 compared to Tfh cells that reside outside the GC. The interaction between Tfh and B cells is mediated by cell associated and soluble factors, including CD40L and ICOS, and IL-21, IL-10, and IL-4 (1, 6). GCs also consist of a subset of regulatory CD4 T cells called follicular regulatory cells (Tfr), which aid in regulating Tfh responses during GC reactions (**Figure 1**) (13, 14). Blood counterparts of lymph node (LN) resident Tfh have also been identified (15) and similar to LN-Tfh cells, these peripheral Tfh cells (pTfh) have been shown to provide help to B cells *in vitro* (15–17).

The linear multistage Tfh differentiation pathway implicates cooperation between multiple antigen-specific interactions and signaling pathways to imprint Tfh differentiation program in the secondary lymphoid organs (7). These include TCR activation, costimulation, cytokines and chemokine receptors. Now it is well established that the co-stimulatory receptors, such as ICOS, CD40L, and cytokines, such as IL-12, IL-23, TGF-β, IL-6, and SLAM family receptors regulate the Tfh differentiation program. Although IL-12 has been shown to be essential for Th1 differentiation, it has also been shown to be important for Tfh cell differentiation in humans (6, 17–20). An early step in the differentiation of human Tfh cells is the upregulation of CXCR5 that is strongly induced by the combination of cytokines IL-12, IL-23, and TGF-β (**Figure 1**) (18). The expression of cell surface CXCR5 allows for trafficking of Tfh cells along a CXCL13 chemokine gradient into lymphoid B cell follicles (21, 22). Recently, Activin A has been identified as a novel regulator that enhances the expression of multiple genes associated with the Tfh program (23), however, this program was conserved in humans and macaques but not in mice.

Tfh cells have been extensively studied in the LN of chronic HIV-infected humans and SIV-infected rhesus macaques (RM) (24–26). HIV infection is associated with altered T and B cell differentiation and enhanced frequencies of Tfh and B cell follicles within secondary lymphoid sites. Characterization of LN Tfh cells during chronic HIV infection has demonstrated impaired B cell help *in vitro* (27, 28). Furthermore, LN-resident Tfh cells are targeted early after SIV infection and constitute a major fraction of latent reservoirs during highly active anti-retroviral therapy (ART) (29–31). Despite their high susceptibility to HIV/SIV infection, many studies including our own reported an accumulation of both tissue resident (32, 33) and circulating Tfh cells during the early chronic phase of HIV/SIV infection (34, 35). In this review, we focus on the recent reports that studied the Tfh cell accumulation, differentiation and heterogeneity during chronic HIV/SIV infection, and discuss the influence of these changes in Tfh cells on the GC response.

DYNAMICS OF Tfh CELLS DURING CHRONIC HIV AND SIV INFECTIONS

Multiple studies including our own have characterized the Tfh cells in the LNs during chronic HIV infection in humans (27, 29, 36, 37) and SIV infection in RMs (33, 35, 38–40). These studies demonstrated a marked increase in Tfh cells during chronic SIV infection and this increase in Tfh cells has been shown to be associated with higher HIV/SIV replication (27, 29, 33, 35, 38). Importantly, this increase in Tfh cells occurs despite their high frequency of infection *in vivo* and *in vitro*. Additionally, Tfh cells constitute a significant portion of the HIV/SIV reservoir (31, 32, 35, 36, 40–42). It has been shown that infection of Tfh cells occurs early in the course of SIV infection and persists throughout the course of disease progression (42). Although longitudinal studies in HIV-infected humans are yet to be done, cross-sectional studies suggest a similar profile of infection (27, 29, 31). However, Tfh in lymphoid tissues are eventually depleted in macaques with end-stage AIDS (40). It is also important to note that rapid progressing SIV infection results in severe follicular involution in lymphoid tissues, while on the contrary, animals that are typical progressors show lymphadenopathy with confluent GCs and follicular hyperplasia (43) (**Figure 2**). In addition, there is increasing evidence to suggest that follicular hyperplasia does not completely resolve following ART (44) and that the preferential carriage of HIV in Tfh cells during ART therapy (31, 45) contributes to the persistent and intractable viral reservoir in ART-treated patients. The mechanisms that contribute to increased Tfh cells during acute HIV/SIV infection are not completely clear. However, the increased levels of IL-6, TGF-β, and IL-21 during chronic HIV/SIV infection could contribute significantly to their expansion (**Figure 1**). In addition, factors such as relative exclusion of follicular CD8 T cells in B cell follicles, lack of regulation by Tfr (T follicular regulatory cells), lack of follicular NK cells, persistence antigen stimulation, and immune inflammation all these above factors contribute to the rapid accumulation of Tfh cells in lymphoid follicles during chronic HIV/SIV infection. Although there is an aberrant expansion of Tfh cells during chronic HIV infection, these cells are providing inadequate help to B cells. One important issue with GC-Tfh cell is the identification of antigen-specific GC-Tfh cells in lymphoid tissues. It is problematic to identify antigen-specific GC-Tfh cells by cytokine production, as GC-Tfh cells have been shown to be a poor cytokine producers compared to other memory CD4 T cell subsets, as biological role of GC-Tfh cell is to provide B cell help. In order to overcome this problem, currently investigators have started focusing on the cytokine-independent activation induced marker methodology assay (AIM assay using OX40 and CD25) to identify

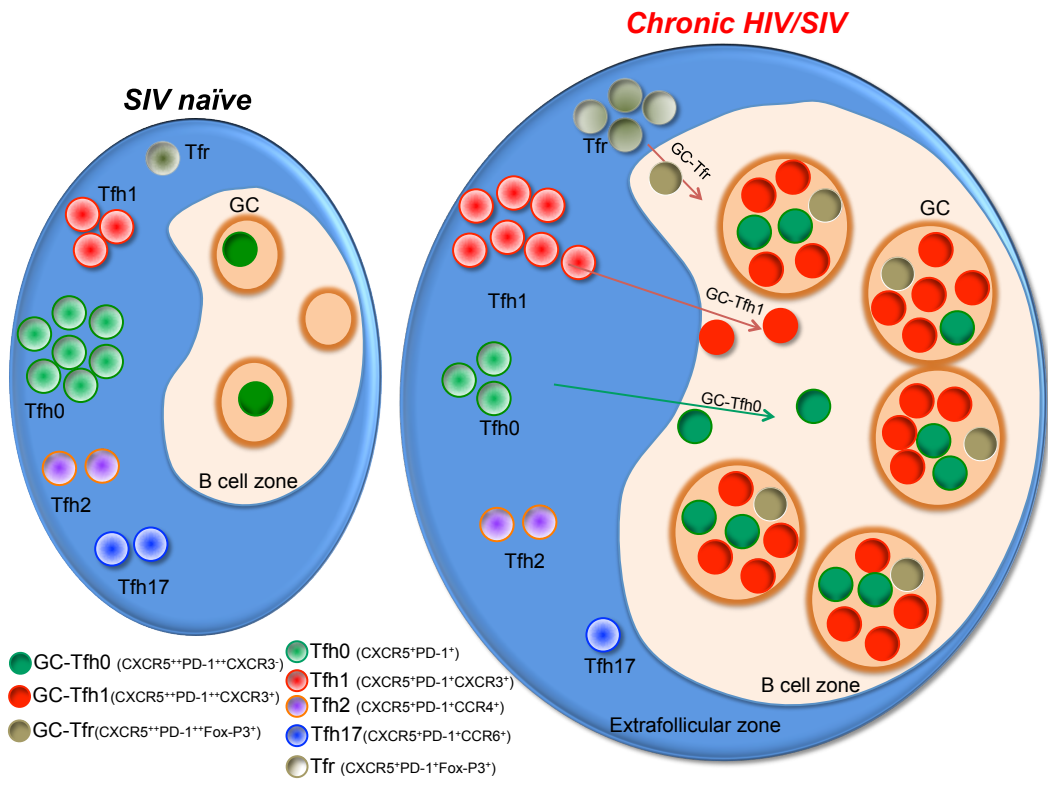

FIGURE 2 | A model showing the status of different germinal center (GC)-Tfh cell subsets during chronic HIV/SIV infection. Tfh cells present as phenotypically distinct subsets with expression of different chemokine receptors pertaining to different lineages of helper CD4 T cells. Upon activation during chronic SIV/HIV infection, there is a massive enrichment of $CXCR3^+$ Tfh cells within the Tfh population in the GC of the lymph node, which in turn drives rapid accumulation of Tfh1 cells. Typical progressors will present with hyperplastic follicles containing a high density of GC-Tfh cells with high levels of CXCR3 expression.

antigen-specific GC-Tfh cells in lymphoid tissues in human and macaques (46, 47). This AIM assay could identify (>10-fold) more antigen-specific GC-Tfh cells compared to cytokine assay. In addition, it has been shown that GC-B cells express higher levels of programmed cell death 1 ligand (PD-L1) in LN during chronic HIV infection, which may suggest a potential role for PD-1/PD-L1 interaction in regulating Tfh cell function (27). Moreover, engagement of PD-1 on Tfh cells leads to a reduction of Tfh cell proliferation, activation, cytokine production, and importantly ICOS expression (48), a key molecule in maintaining a Tfh phenotype. All together these data suggest that impaired Tfh-mediated B cell help diminishes B cell responses during HIV infection and may be regulated by the PD-1 axis on Tfh cells.

PERIPHERAL Tfh VERSUS GC Tfh CELLS

A subset of CD4 T cells in the blood expresses CXCR5 and is referred to as peripheral (pTfh) or circulating Tfh cells (17, 49, 50). These pTfh cells have been identified in mouse, macaques, and humans, and are considered to be the functional equivalent of Tfh cells in the LN. These pTfh cells express CCR7, albeit at low levels indicating that these cells traffic through lymphoid tissue. It has been shown that the pTfh cells express CXCR5 and PD-1 stably (15). However, pTfh express significantly lower levels of PD-1 and do not express Bcl-6 compared to GC-Tfh cells. Similar to LN Tfh, pTfh can also express other chemokine receptors associated with Th1 (CXCR3), Th2 (CCR4), and Th17 (CCR6) cells (17). pTfh can be distinguished into four subsets based on the expression of CXCR3 and PD-1. In humans, the PD-1^{lo} CXCR3^{-} pTfh express high levels of IL-4, do not express IFN-γ, and provide superior B cell helper function compared to PD-1^{+} CXCR3^{+} cells (15, 51). In addition, the presence of higher frequency of PD-1^{lo} CXCR3^{-} pTfh was shown to be associated with the development of a broader neutralizing antibody response in HIV-infected individuals with high viremia (15, 51). However, in another study the ratio of PD-1^{lo} to PD-1^{hi} cells within the CXCR3^{+} pTfh was shown to correlate with increased neutralization breadth in HIV controllers with very low viremia (34). These results suggest that both CXCR3^{-} and CXCR3^{+} pTfh expressing lower levels of PD-1 may be important for the generation of a functional antibody response. More studies in different disease contexts are required to correlate the phenotypic and functional differences previously observed between pTfh and GC-Tfh in order to understand the important dynamics of this subset in blood and tissue.

HIV AND SIV INFECTIONS ALTER Tfh DIFFERENTIATION TOWARD Tfh1 PHENOTYPE

The blood memory Tfh cells have been shown to co-express chemokine receptors associated with other T helper cell lineages, such as CXCR3 (Th1), CCR4 (Th2), and CCR6 (Th17) (17, 34, 52). Recent studies characterizing Tfh cells during chronic HIV/SIV infection have delineated phenotypically distinct subsets of Tfh cells in the circulation (17, 34, 53). Similarly, data from our recent study in macaques revealed that a significant proportion of GC-Tfh cells express the surface markers associated with several CD4 lineages during chronic SIV infection (**Figure 2**). We observed a selective enhancement of CXCR3^{+} Tfh (Tfh1) cells in the blood and LN and rapid depletion of CCR6^{+} Tfh cells (Tfh17) (32) demonstrating that SIV infection alters the balance of different subsets of Tfh cells. Others and we have also observed a marked enhancement of T-bet (Th1 marker) expression on Tfh and GC-Tfh cells (32, 33, 40, 54, 55). Unlike conventional GC-Tfh0 cells (CXCR3^{-}), these GC Tfh1 cells exclusively produced IFN-γ (32). Interestingly, these T-bet^{+} Tfh1 cells also expressed Tfh transcription factor Bcl-6 (32, 54). The immune mechanisms that contribute to induction of Tfh1 cells are not completely understood. A similar Tfh1 phenotype was observed in GC-Tfh of humanized mice infected with HIV (56), during chronic LCMV clone-13 infection (57) and malarial infection (49). On the other hand, this phenotype may not be specific to chronic infections as we recently observed this phenotype on GC-Tfh cells after DNA/MVA SIV vaccination in RM (58). This raises the possibility that the local inflammation alone can induce this CXCR3 phenotype on GC-Tfh cells. One possibility is that the induction of high levels of CXCL10 (IP-10) during chronic infection and after DNA/MVA vaccination may promote induction of CXCR3 on Tfh cells (59).

The Tfh1 cells differ significantly compared to Tfh0 cells in terms of the expression of key molecules. Tfh1 cells, compared to Tfh0 cells express relatively lower levels (MFI) of CXCR5, PD-1, and CCR7 and higher levels of ICOS and IL-21 (**Table 1**). In addition, they exclusively produce IFN-γ express high levels of HIV co-receptor CCR5 and HIV-binding protein α4β7 suggesting that these cells are highly susceptible to HIV/SIV infection. Importantly, these cells also provide help to B cells similar to Tfh0 cells. One possible reason for better B cell help from Tfh1 cells could be the expression of high levels of ICOS, IL-21, and CD40L, as these markers direct recruitment of B cells and more efficiently activate B cells (60).

Tfr CELLS DURING CHRONIC HIV/SIV INFECTION

Foxp3^{+} regulatory cells are important in suppressing different types of immune responses help to maintain homeostasis.

TABLE 1 | Divergent marker profile of Tfh1 cells versus Tfh0 cells.

Markers	CXCR3^{+} Tfh	CXCR3^{-} Tfh
Surface markers		
CXCR5	+++	+++
CXCR3	+++	–
PD-1	++	+++
ICOS	+++	+++
CCR7	++	+/–
CCR5	+++	+/–
α4β7	+++	+/–
Transcription factors		
BCL-6	+++	+++
T-bet	+++	–
Cytokines		
IFN-γ	+++	–
IL-21	+++	+++
CD40L	+++	+++

Recent data in mice, macaques, and humans have identified a subset of Foxp3$^+$ cells that express Tfh markers, such as CXCR5, PD-1, ICOS, and BCL-6, and migrate into the B cell follicles to regulate Tfh and B cell differentiation. These cells are called Tfr (follicular regulatory) cells (13, 14, 61). Although these cells show characteristics of Tfh cells they lack expression of functional molecules required for B-cell help, such as CD40L, IL-4, and IL-21. Deletion of Tfr cells or impairing their follicular localization led to increased number of Tfh and GC B cells in murine models (13, 14, 61). These cells inhibit GC reactions by interacting with Tfh cells. The mechanisms by which Tfr cells limit Tfh cell function are not clearly understood. During chronic HIV/SIV infection, Tfr cells are also expanded in parallel with Tfh cells. Although both Tfh and Tfr expand after SIVmac251 infection (62), higher frequency of Tfr cells is associated with lower Tfh frequency, suggesting that the expansion of Tfr cells diminishes Tfh frequencies and eventually B cell responses and antibody production (55, 63, 64). In line with this, a recent study demonstrated that a deficiency in Tfr cells promotes autoimmunity (65). *In vitro*, Tfr inhibit the ability of Tfh to proliferate and produce critical B cell helper cytokines, such as IL-4 and IL-21, although they maintain ICOS expression. A small fraction of HIV-infected individuals develops broadly neutralizing antibodies and it would be interesting to assess whether these individuals have lower frequencies of Tfr that would aid in the number and quality of Tfh and the generation of NAbs. Given their negative influence on Tfh cells, HIV vaccination modalities that induce lower levels of Tfr may generate stronger Tfh responses and higher quality B cell responses. Further studies are required to elucidate the role of Tfr on humoral immunity both post vaccination and during HIV/SIV infection.

Tfh AND HIV/SIV VIRAL RESERVOIRS

SIV infection of Tfh occurs early during primary infection and persists over the course of the disease (42). Although extensive longitudinal studies have not been carried out in humans, cross-sectional studies suggest a similar temporal profile of HIV infection of Tfh (29, 37). Studies have shown that HIV-specific CD4$^+$ T cells are preferentially infected by HIV/SIV (66, 67). Within the CD4 compartment, the Tfh population has been shown to constitute higher numbers of HIV-infected cells that are more efficient in supporting viral replication and correlate directly with plasma viral RNA levels (36). Despite representing a large fraction of the HIV-infected CD4 pool (29), LN-resident Tfh cells appear to express low levels of the HIV co-receptor CCR5 (27, 29, 32, 33) but do, however, express CXCR4 (40). A recent study suggested that approximately 30% of human Tfh cells may be CCR5$^+$ cells (41). Furthermore, studies have also shown that the proviral DNA sequences in Tfh cells from SIV-infected macaques are predominantly CCR5-tropic (42). The mechanism by which CCR5 tropic HIV/SIV is present at high levels in Tfh cells in macaques is not well understood. This leads to several questions; where and when are Tfh cells becoming infected, are they being infected before migrating into the GC at the stage of Tfh precursors which express high levels of CCR5 (32, 33), a potential mechanism recently described (40). In support of this potential mechanism is the knowledge that Th1 like Tfh cells accumulate during SIV infection, constitute a large proportion of the Tfh subset, and express higher levels of CCR5 and the HIV binding gut homing integrin receptor α4β7 (32). The accumulation of Tfh1 cells throughout the course of SIV infection both in periphery and in LN (32) suggest that Tfh1 cells accumulate rapidly post HIV/SIV infection and could potentially be transdifferentiating either from a different CD4 T helper cell subset or from a precursor population into a committed GC-Tfh phenotype. Several studies have reported the presence of CXCR3$^+$CXCR5$^+$ pTfh cells in HIV-infected individuals and in humanized mice preferentially expressing the HIV-co-receptor CCR5 (56, 68). Therefore, we can speculate that Tfh1 cells that are CCR5$^+$α4β7$^+$ may have the capacity to maintain a dynamic viral reservoir in GCs.

FOLLICULAR HOMING CD8 DURING HIV/SIV INFECTION

As described above, HIV and SIV infection of Tfh occurs very early in the course of infection and persists throughout disease even after ART (29, 42). Similarly, a higher fraction of these Tfh cells is shown to contain replication competent HIV genomes during ART (29, 36). An important question that needs to be addressed is the role of HIV-specific CD8 T cells in targeting and clearing the viral reservoirs that reside within Tfh cells. It is very clear that anti-viral CD8 T cells are critical for HIV/SIV control (69–74) even under ART (75). However, B cell follicles/GCs are considered to be immune privileged for anti-viral CD8 T cells (76, 77). Studies in unvaccinated SIVmac251-infected RM and HIV-infected humans showed that anti-viral CD8 T cells have a limited capacity to migrate to B cell follicles and GC of the lymphoid tissue during chronic infection (76–78) and the exclusion of CD8 T cells from GC sites have been posited as an important mechanism of immune evasion by HIV/SIV. However, recent studies reported the emergence of CD8 T cells expressing the chemokine receptor CXCR5 that is required for homing to B cell follicles (2, 10) during chronic LCMV, EBV, and HIV infections (79–82). Similarly, in the setting of DNA/MVA vaccinated and SIVmac251-infected macaques, we showed a rapid expansion of a novel subset of SIV-specific CD8 T cells expressing CXCR5 (Tfc) in vaccinated controllers after SIV infection (83) and the expansion of these cells was strongly associated with improved control of SIV replication. The higher expansion of these cells correlated strongly with the higher presence of anti-viral CD8 T cells in the GCs. Similar to SIV studies, these cells are shown to be present in the B cell follicles of HIV-infected LN (84). A more recent study carried out a comprehensive analysis of the phenotype, frequency, localization, and function of follicular CD8 T cells in SIVmac251-infected macaques (85). This study demonstrated that follicular CD8 T cell accumulation occurs in pathogenic SIV infection but not in natural infection (African green monkeys). Interestingly, this study provides clues to the cause of mobilization and accumulation of follicular CD8 T cells during pathogenic SIV infection and describes

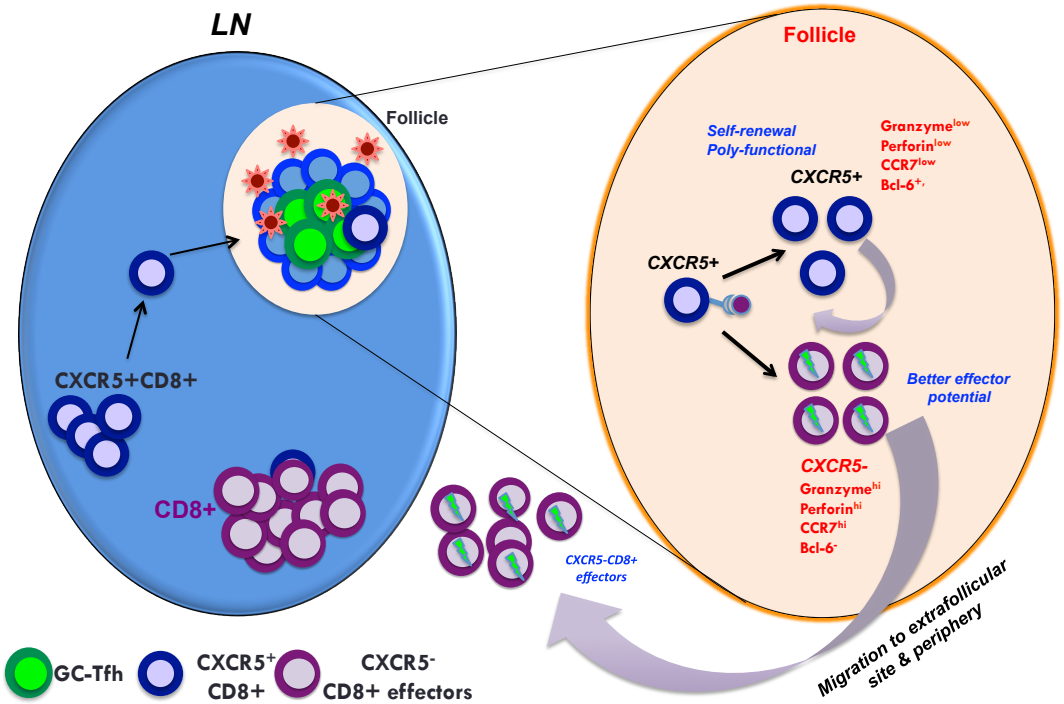

FIGURE 3 | A model showing the predicted role of follicular homing CXCR5⁺ CD8 T cells in controlled SIV infection. In controlled SIV infection, the population of virus-specific CD8⁺ T cells expressing CXCR5 increases in lymphoid follicles. These CXCR5⁺ CD8 T cells can give rise to both CXCR5⁺ and CXCR5⁻ CD8 T cells that may be important for the maintenance of anti-viral CD8 T cells in both follicular and extrafollicular compartments. Thus, generating anti-viral CXCR5⁺ CD8 T cells by vaccination and therapeutic interventions may be critical for the control of chronic HIV/SIV infection.

the process to be largely driven by inflammation and immune activation in and around B cell follicles. Similar to CD8 T cells, a recent study has shown that NK cells also traffic to the GCs, but these cells were seen only in non-pathogenic African green monkeys but these cells are rare SIV mac infection (86). These results clearly demonstrated that anti-viral CD8 T cells can migrate to B cell follicles under conditions of controlled SIV infection.

The mechanisms by which these Tfc contribute to control of HIV/SIV are still under active investigation. Interestingly, Tfc possess a unique gene expression profile that shares both a cytotoxic CD8 T cells and Tfh phenotype (81, 83). These cells display enhanced poly functionality and are capable of restricting the expansion of SIV antigen-pulsed Tfh *in vitro* (83, 84). The Tfc from LN of HIV-infected individuals have been shown to possess higher cytolytic activity than extrafollicular CD8 T cells (79, 80, 82). Similar to HIV/SIV infection, CXCR5⁺ CD8 T cells have been identified during chronic LCMV infection. These cells have been shown to possess stem-cell like properties with self-renewal potential and may prove critical for long-term maintenance of effector CD8 T cells (**Figure 3**) (81). Thus, CXCR5⁺ CD8 T cells may contribute to viral control by replenishing the effector CD8 T cell population required to eliminate persistent virus. It is also important to note that some studies also suggest that the CD8 T cells that localize within B cell follicles may have limited cytolytic capacity and the overall frequencies of virus-specific CD8 T cells are lower in absolute numbers in the intra-follicular compared to extra-follicular compartment (78, 87, 88). However, the potential of Tfc to generate a population of effector CD8 T cells suggests that these cells may also contribute to viral control indirectly through their ability to homeostatically reconstitute the effector CD8 T cell response.

CONCLUSION

The past few years of active research have provided profound insight into the role of follicular CD4 T cells in HIV pathogenesis. It is now well established that the Tfh population represents a major fraction of the viral reservoir and it is essential to develop HIV cure approaches capable of targeting and eliminating these cells. It is also clear that follicular homing CD8 T cells may serve as an important subset in targeting the Tfh reservoir. However, we need to develop a greater understanding of the mechanisms that contribute to the development and maintenance of viral reservoirs in Tfh cells under ART to design strategies to purge virus from this cellular site. The discovery of phenotypically distinct subsets of circulating Tfh cells in HIV infection and the potential for memory recall of Tfh cells in mice warrants further investigation into follicular CD4 T cells in an effort to inform vaccination strategies for HIV. Moreover, the heterogeneous nature of GC-Tfh and circulating Tfh cells can be harnessed for the generation of optimal vaccine-induced HIV-specific B-cell responses. A significant amount of work remains to uncover molecular signals that regulate the generation of follicular CD8 T cells and if this

subset can be achieved by vaccination. In this regard, our ongoing work in nonhuman primates has shown that a CD40L adjuvanted DNA/MVA vaccine can strongly promote the generation of Tfc. Finally, further studies are needed to determine optimal strategies to utilize circulating and GC-Tfh cells during prime and boost immunization strategies to promote robust protective and long-lived antibody responses against HIV infection without promoting increased target cell susceptibility to HIV.

AUTHOR CONTRIBUTIONS

VV, RA wrote the review. VV, GM and RA designed the figures and the table. VV, CI and RR edited the text. VV composed and oversaw the chapters. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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ORIGINAL RESEARCH
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Blood CXCR3$^+$ CD4 T Cells Are Enriched in Inducible Replication Competent HIV in Aviremic Antiretroviral Therapy-Treated Individuals

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We recently demonstrated that lymph nodes (LNs) PD-1$^+$/T follicular helper (Tfh) cells from antiretroviral therapy (ART)-treated HIV-infected individuals were enriched in cells containing replication competent virus. However, the distribution of cells containing inducible replication competent virus has been only partially elucidated in blood memory CD4 T-cell populations including the Tfh cell counterpart circulating in blood (cTfh). In this context, we have investigated the distribution of (1) total HIV-infected cells and (2) cells containing replication competent and infectious virus within various blood and LN memory CD4 T-cell populations of conventional antiretroviral therapy (cART)-treated HIV-infected individuals. In the present study, we show that blood CXCR3-expressing memory CD4 T cells are enriched in cells containing inducible replication competent virus and contributed the most to the total pool of cells containing replication competent and infectious virus in blood. Interestingly, subsequent proviral sequence analysis did not indicate virus compartmentalization between blood and LN CD4 T-cell populations, suggesting dynamic interchanges between the two compartments. We then investigated whether the composition of blood HIV reservoir may reflect the polarization of LN CD4 T cells at the time of reservoir seeding and showed that LN PD-1$^+$ CD4 T cells of viremic untreated HIV-infected individuals expressed significantly higher levels of CXCR3 as compared to CCR4 and/or CCR6, suggesting that blood CXCR3-expressing CD4 T cells may originate from LN PD-1$^+$ CD4 T cells. Taken together, these results indicate that blood CXCR3-expressing CD4 T cells represent the major blood compartment containing inducible replication competent virus in treated aviremic HIV-infected individuals.

Keywords: T follicular helper cells, replication competent virus, circulating T follicular helper cell counterpart, CXCR3, lymph node

Abbreviations: ART, antiretroviral therapy; LNs, lymph nodes; cART, conventional antiretroviral therapy; Tfh, T follicular helper.

INTRODUCTION

Antiretroviral therapy (ART)-treated HIV-infected individuals interrupting treatment experience HIV viremia rebound within 2–3 weeks (1, 2), demonstrating that HIV persists despite therapy and that conventional antiretroviral therapy (cART) does not cure HIV in its actual setting. One of the main mechanisms by which HIV persists is attributed to the ability of the virus to infect activated CD4 T cells entering a quiescent state (3), thereby establishing a latent HIV cell reservoir (4). Latently HIV-infected CD4 T cells are transcriptionally silent and are therefore not targeted by cART or by the immune system (5). As a direct consequence, infected CD4 T cells containing HIV DNA are detectable in virtually all ART-treated HIV-infected individuals (6). Since, ART does not target HIV-infected cells, it has been estimated, based on the half life of memory CD4 T cells, that as long as 70 years of ART might be required for the eradication of the latent reservoir (4). Moreover, mechanisms such as homeostatic proliferation may also contribute to further increase the stability of the latent HIV reservoir (3, 4, 7, 8).

The composition of the latent HIV reservoir is complex and different cell subsets may contribute such as CD4 T cells, monocytes and macrophages (8–10). Among the CD4 T-cell subsets, central memory (CM; defined by $CD45RA^{-}CCR7^{+}CD27^{+}$) and transitional memory (TM; $CD45RA^{-}CCR7^{-}CD27^{+}$) CD4 T cells were identified as major CD4 T-cell populations contributing the most to the latent HIV-1 reservoir in blood (7). Additional CD4 T-cell populations enriched in cells containing replication competent viruses were later described such as memory CD4 T cells with stem-cell like properties (11). On the same line, Descours et al. identified CD32a (low-affinity receptor for the immunoglobulin G Fc fragment) as a specific marker of latently HIV-infected quiescent CD4 T cells (12). However, lymphocytes reside predominantly within lymphoid organs while blood contains only 2% of the total body's lymphocytes. More importantly, lymphocyte populations within the tissues are phenotypically and functionally distinct from those in blood (13). The recently described T follicular helper (Tfh) cells illustrate this difference (14, 15). In this regard, lymph nodes (LN) memory Tfh cells and to a lesser extent memory $CXCR5^{-}PD\text{-}1^{+}$ CD4 T cells were previously shown to serve as the major CD4 T-cell compartments for HIV replication, production and infection in viremic HIV-1-infected individuals (16). In addition, we have recently demonstrated that LN $PD\text{-}1^{+}$/Tfh CD4 T cells isolated from ART-treated aviremic HIV-infected individuals were enriched in cells containing replication competent and infectious HIV as compared to any other PD-1 negative memory CD4 T-cell populations isolated from blood or LN (17). This phenomenon is probably associated with the limited cytotoxic CD8 T-cell access to germinal centers (GCs) (18, 19) where infected Tfh cells locate. However, since HIV-infected effector Tfh cells surviving viral cytopathic effects and escaping extra-follicular CD8 T-cell mediated clearance may convert to memory CD4 T cells and recirculate in blood (20, 21), we hypothesized that blood circulating Tfh cell counterpart (circulating Tfh or cTfh) may therefore be enriched in cells containing replication competent virus.

Circulating Tfh (cTfh) cells were initially defined by the expression of CXCR5 and showed enhanced capacity to provide naïve and memory B-cell help (22). Later, cTfh cells were subdivided into various subsets based on the expression of CXCR3, CCR4, CCR6, PD-1, and ICOS (23–25). Indeed, T-cell migration is orchestrated by the combination of cell-adhesion molecule and chemokine receptor expression (20) Notably, CXCR5 expression allows cell migration to B cell follicles, CXCR3 expression allows cell migration to inflamed tissues and CCR4 and CCR6 expression allows migration to skin and mucosal tissues, respectively (26, 27).

In this context, we have investigated the distribution of replication competent and infectious virus within different blood memory CD4 T-cell populations identified on the basis of their migratory potential determined by the chemokine receptor expression and compared to memory CD4 T-cell populations isolated from LNs of cART-treated HIV-infected individuals. Briefly, blood circulating memory ($CD45RA^{-}$) CD4 T-cell populations were identified on the basis of expression of CXCR3, CXCR5, CCR4, and CCR6, i.e., $CXCR3^{+}CXCR5^{-}$ ($CXCR3^{+}$), $CCR4^{+}CCR6^{-}$ ($CCR4^{+}$), $CCR4^{+}CCR6^{+}$, $CXCR3^{-}CXCR5^{+}$, and $CXCR3^{+}CXCR5^{+}$ CD4 T cells, the latter two populations corresponding to "cTfh" (22, 23) and "Th1-like cTfh" (28). On the other hand, LN memory ($CD45RA^{-}$) CD4 T-cell populations were identified on the basis of the expression of CXCR5 and/or PD-1 expression as previously described (17).

In the present study, we show that blood CXCR3-expressing but not CXCR5-expressing memory CD4 T-cell subset was significantly enriched in cells containing inducible replication competent virus and contributed the maximum to the total pool of cells containing replication competent and infectious virus in blood. However, the enrichment of blood CXCR3-expressing memory CD4 T cells with cells containing replication competent virus was not associated with increased level of activation, HIV coreceptor expression or reduced HIV restriction factor expression, nor with an enrichment in cells harboring central or transitional memory phenotype.

To determine whether blood CXCR3-expressing memory CD4 T cells containing replication competent virus may originate from LN Tfh cells, proviral EnvV1-V4 sequences of blood and LN CD4 T-cell populations of ART-treated aviremic HIV-infected individuals were analyzed but did not indicate virus compartmentalization. We then investigated whether the composition of blood HIV reservoir may reflect the polarization of LN CD4 T cells at the time of reservoir seeding and showed that LN $PD\text{-}1^{+}$/Tfh cells of viremic untreated HIV-infected individuals expressed significantly higher levels of CXCR3 as compared to CCR4 and/or CCR6 which is consistent with previous studies performed in chronically SIV-infected macaques (29).

Taken together, these results indicate that blood CXCR3-expressing CD4 T cells represent the major blood compartment containing inducible replication competent virus in treated aviremic HIV-infected individuals. However, additional studies would be needed to determine their potential origins and the

mechanism by which HIV-infected cells accumulated within this particular subset.

MATERIALS AND METHODS

Ethics Statement

The present study was approved by the Institutional Review Board of the Centre Hospitalier Universitaire Vaudois, and all subjects gave written informed consent.

Study Group and Cell Isolation

Nine viremic untreated HIV-1-infected adult volunteers and 19 aviremic ART-treated HIV-infected individuals were enrolled in the present study (**Table 1**). No predetermined statistical analysis was performed for sample size and was estimated based on a previously published study (17). As inclusion criteria only patients under ART for atleast 12 months with undetectable HIV-1 viremia (<20 HIV-1 RNA copies/mL) were enrolled. As exclusion criteria, individuals experiencing blips of viremia (>50 HIV-1 RNA copies/mL of plasma) within the last 12 months were not enrolled. Leukapheresis and blood samples were obtained at the local blood bank (Centre de transfusion sanguine (CTS), Lausanne, Switzerland). Blood mononuclear cells were isolated as previously described (30).

Reagents and Cell Culture

Cells were cultured in RPMI (Gibco; Life Technologies) containing 10% heat-inactivated fetal bovine serum (FBS; Institut de Biotechnologies Jacques Boy), 100 IU/mL penicillin, and 100 µg/mL streptomycin (Bio Concept).

Antibodies

The following antibodies were used: APC-H7-conjugated anti-CD3 (clone SK7), PB or FITC or PE-CF594-conjugated anti-CD4, PerCP-Cy5.5-conjugated anti-CD8 (clone SK1), (clone RPA-T4), (2G8), PE-conjugated anti-CCR6 (11A9), PE-Cy7-conjugated anti-CCR4 (1G1), V450-conjugated anti-HLA-DR (clone G46-6), PE-Cy7-conjugated anti-CD25 (clone M-A251), PeCy5-conjugated anti-CXCR4 (12G5), AlexaFluor700-conjugated anti-CCR5 (HEK/1/85a), PB or PeCy7 conjugated anti-PD-1 (EH12.1), anti-CCR7 (2H4), AlexaFluor700-conjugated anti-CD27 (M-T271), PE-conjugated anti-Ki67 (B56) purified coating anti-CD3 (clone UCHT1) and anti-CD28 (clone CD28.2) mAbs were purchased from BD (Becton Dickinson, San Diego CA, USA). PB-conjugated anti-CXCR3 (1C6) and PE or APC or PerCP-eFluor710-conjugated anti-CXCR5 mAbs were all purchased from Biolegend (Switzerland). ECD-conjugated anti-CD45RA (clone 2H4) was purchased from Beckman Coulter (Brea CA, USA) and anti-SAMHD1 (611-625) was purchased from Thermo Scientific (Switzerland).

TABLE 1 | Characteristics of study group.

Subject ID	Duration of HIV infection (years)	CD4 count (cells/µl)[a]	Viral load (copies/mL)[a]	Time on HAART (years)	HAART regimen	Assays performed in blood	Assays performed in LN
#10	9.1	543	<20	8.8	EFV, 3TC	INT DNA, VOA, FC	NA
#12	8.4	453	<20	2.9	3TC/r, ABC	INT DNA, VOA, FC	NA
#14	10.1	1,691	<20	3.5	EFV, TDF, FTC	INT DNA, VOA, FC	INT DNA, VOA
#16	25.9	487	<20	2	ETR; DRV/r; RAL	INT DNA, VOA, FC	NA
#18	2.6	376	<20	2.5	FTC, TDF, EFV	INT DNA, VOA, FC	NA
#20	25.3	480	<20	14.1	ETR, DRV/r, RAL	INT DNA, VOA, FC	NA
#26	9.6	666	<20	2	3TC, ABC, DTG	INT DNA, VOA, FC	INT DNA, VOA
#35	2.1	1,219	<20	1.8	FTC, TDF, EVG	INT DNA, SEQ, VOA, FC	INT DNA, SEQ, VOA
#43	2.8	605	<20	2.8	3TC, ABC, DTG	VOA, FC	NA
#45	8	442	<20	7.9	FTC, TDF, ETR	INT DNA, VOA, FC	NA
#75	4.7	417	<20	3.2	FTC, TDF, EFV	INT DNA, VOA	VOA
#94	7	440	<20	6.9	FTC, TDF, EVG	INT DNA, SEQ, VOA	VOA, SEQ
#107	1.9	598	<20	1.8	FTC, TDF, EFV	INT DNA, VOA	VOA
#077	6.6	560	<20	6	3TC, ABC, DTG	SEQ	SEQ
#24	10.7	629	<20	3.9	FTC, TDF, EFV	NA	INT DNA, VOA
#11	16.2	811	<20	11.7	FTC, TDF, ATV/r	NA	INT DNA, VOA
#04	2.9	424	<20	1.8	EFV, TDF, FTC	NA	INT DNA, VOA
#25	28.5	439	<20	4.6	FTC, TDF, RAL	NA	INT DNA, VOA
#42	11.1	949	<20	6.6	FTC, TDF, ATV/r	NA	INT DNA, VOA
#106	0.1	501	160,000	0	NA	NA	MC
#140	0.08	427	360,000	0	NA	NA	MC
#119	1.3	498	13,000	0	NA	NA	MC
#124	0.06	468	510,000	0	NA	NA	MC
#113	31.98	594	640	0	NA	NA	MC
#117	5.42	504	54,000	0	NA	NA	MC
#125	0.16	511	17,000	0	NA	NA	MC
#118	0.06	538	14,000	0	NA	NA	MC
#SA150	10	578	6,900	0	NA	NA	MC

[a]*CD4 cell count and viral load assessments are at the time of enrollment in the study.*

ETR, Etravirine; FTC, Emtricitabine; TDF, Tenofovir disoproxil fumarate; ATV/r, Atazanavir boosted with ritonavir; 3TC, Lamivudine; ABC, Abacavir; DRV/r, Darunavir boosted with ritonavir; EFV, Efavirenz; RAL, Raltegravir; DTG, Dolutegravir; EVG, Elvitegravir; LN, lymph node; INT DNA, integrated DNA; VOA, viral outgrowth assay; FC, flow cytometry; SEQ, sequencing; MC, Mass cytometry; NA, not applicable.

Sorting of Blood and LN Memory CD4 T-Cell Populations

Sorting of chemokine-receptor-expressing memory CD4 T cells was performed using FACS Aria as previously described (17). Briefly, cryopreserved blood mononuclear cells were thawed and CD4 T cells were enriched using EasySep Human CD4 T-cell enrichment kit (StemCell Technologies, Cambridge MA, USA). CD4 T cells were then stained with Aqua LIVE/DEAD stain kit (4°C; 15 min) and then to simultaneously investigate the expression of chemokine receptors on CD4 T cell membrane, with anti-CD3 APCH7, anti-CD4 FITC, anti-CD45RA ECD, anti-CXCR3 PB, anti-CXCR5 APC, anti-CCR4 PE-Cy7 and anti-CCR6 PE (4°C; 25 min). Viable CD4 memory ($CD45RA^-$) $CXCR3^+CXCR5^-$, $CXCR3^-CXCR5^+$, $CXCR3^+CXCR5^+$, $CXCR3^-CXCR5^-CCR4^+CCR6^-$, and $CXCR3^-CXCR5^-CCR4^+CCR6^+$ T-cell populations were then sorted. In parallel, cryopreserved LN mononuclear cells were also thawed and stained with Aqua LIVE/DEAD stain kit (4°C; 15 min) and then with anti-CD3 APCH7, anti-CD4 FITC, anti-CD45RA ECD, anti-PD-1 PE-Cy7, and anti-CXCR5 APC (at 4°C; 25 min) and viable CD4 memory ($CD45RA^-$) $CXCR5^-PD\text{-}1^-$ (DN), $CXCR5^+PD\text{-}1^-$ (single CXCR5), and total $PD\text{-}1^+$ cells were sorted. The grade of purity of the sorted cell populations was >97% in all sorting experiments.

Integrated HIV-1 DNA Quantification

Blood and LN memory CD4 T cells of ART-treated HIV-infected individuals were sorted and CD3 gene copy numbers were determined as previously described (31). The frequency of HIV-1 integrated DNA per million of cells was then calculated as previously described (31).

Viral Outgrowth Assay

Multiple cell concentrations, i.e., fivefold limiting dilutions: 5×10^5, 10^5, 2×10^4, and 4×10^3 for blood CD4 T cell populations and 10^5, 2×10^4, and 4×10^3 for LN CD4 T-cell populations of sorted viable blood and LN memory CD4 T cells isolated from ART-treated HIV-infected individuals were cultured with allogenic fresh CD8-depleted blood mononuclear cells (10^6 cells/mL) from HIV-uninfected individuals in the presence of anti-CD3/anti-CD28 MAb coated plates (10 µg/mL) for 3 days. Cells were then carefully transferred to new uncoated plates post 3 full days of activation. All conditions were cultured in complete RPMI supplemented with IL-2 (50 units/mL) for 14 days. Medium was replenished at day 5, and re-supplemented with cytokines. Supernatants were collected at day 14. The presence of HIV-1 RNA was assessed by COBAS® AmpliPrep/TaqMan® HIV-1 Test (Roche; Switzerland). Wells with detectable HIV-1 RNA (≥20 HIV-1 RNA copies/mL) were referred to as HIV-1 RNA-positive wells. RUPM frequencies (32) were estimated by conventional limiting dilution methods using Extreme Limiting Dilution analysis (http://bioinf.wehi.edu.au/software/elda/) (33). The estimation of each population's contribution to the overall pool of HIV-infected cells within blood compartment or within LN compartment was performed as previously described (17) (Table S1 in Supplementary Material). Briefly, estimated contribution of memory CD4 T cell population A from blood or LN to the pool of cells containing replication competent virus in blood or LN compartments = [(% of memory CD4 T cell population A from compartment X among total memory CD4 T cells from compartment X) × (estimated RUPM freq. of memory CD4 T cell population A from compartment X)] × [% of the estimated number of memory CD4 T cells present in compartment A among the sum of the memory CD4 T cells present in blood or LN]/[(sum of absolute values of cells containing replication competent virus within each memory CD4 T cell population) × 100]. In addition, estimated contribution of memory CD4 T cell population A from compartment X to the pool of cells containing replication competent virus in both blood and LN compartments = [(% of memory CD4 T cell population A from compartment X among total memory CD4 T cells from compartment X) × (estimated RUPM freq. of memory CD4 T cell population A from compartment X)] × [% of the estimated number of memory CD4 T cells present in compartment A among the sum of the memory CD4 T cells present in both blood and LN]/[(sum of absolute values of cells containing replication competent virus within each memory CD4 T cell population from compartment X and Y) × 100]. The estimated number of total blood and LN memory CD4 T cells was obtained from Ganusov et al. (34).

In Vitro HIV-1 Infection Assay

The *in vitro* infection assay was performed as previously described (17). Briefly, preactivated CD8-depleted blood mononuclear cells isolated from HIV-uninfected individuals were washed and exposed for 6 h at 37°C to 100 µl of VOA supernatants (obtained from the highest concentration, i.e., 5×10^5) collected at day 14 from all chemokine receptor expressing blood memory CD4 T-cell populations in VOA. Following 6 h exposure, cells were washed twice with complete medium and cultured for 14 additional days in complete RPMI medium. The presence of infectious HIV-1 particles was determined in culture supernatants at day 0 and 14 post inoculations as assessed by HIV-1 RNA assay (COBAS® AmpliPrep/TaqMan® HIV-1 Test) as previously described (17). For this assessment of HIV RNA, all samples were prediluted 1/10 in basematrix buffer (RUWAG Handels AG).

Proviral Sequencing of Env V1-V4 Region

Proviral sequencing of Env V1-V4 was performed on three aviremic ART-treated HIV-infected individuals (**Table 1**). No predetermined criteria was used to choose patients for phylogenetic sequencing. RNA or DNA was extracted using Qiagen kit QIAAMP DSP VIRUS KIT or AllPrep DNA/RNA, respectively, according to manufacturer's instructions. Fifteen microliters of RNA and DNA (equal to 100,000 cells) were used as input for the first step PCR using SuperScript III RT/Platinum or and Taq High Fidelity Enzyme Mix (Invitrogen). RNA was reverse transcribed using the reverse primer Rev-7659-86 5′ TGGAGAAGTGAATTATATAAATATAAAG [Hxb2 7659←7686]. The 1st round PCR was performed in a 50 µl reaction (0.5 µl (= 5U) High Fidelity Platinum Taq (Life technologies, Darmstadt), 3.5 mM $MgCl_2$, 4 µl of dNTPs and (2.0 mM of each)

forward For6435-675′ ACACATGCCTGTGTACCCACAGACC CCAACCCA) [Hxb2 6435→6467] and reverse Rev7659-86 primers, at 95°C for 10 min followed by 45 cycles (94°C-30 s, 55°C-30 s, 68°C-3 min) and 7 min at 68°C. The 2nd round PCR was performed with 5 µl of first round PCR product in a 50 µl reaction using the same conditions and the primers For6540-62 5′GAGGATATAATCAGTTTATGGGA [Hxb2 6540→6562] and Rev7647-68 5′CACTTCTCCAATTGTCCCTCAT [Hxb2 7647←7668]. To the nested primers were extended with tags that conferred unique identification codes to each plasma or cell fraction. Tagged amplified products were purified using the Ampure beads (Angencourt). Following assessment of DNA concentration (Nanodrop and Qubit) and fragments size (Fragment Analyser, AAT) amplicons were pooled on an equimolar basis. The ends of the DNA were repaired as described by Pacific Bioscience prior to generation of the SMRTbell library (SMRTbell library kit, Pacific Bioscience), which was then purified, quantified and analyzed for fragment size. The library was annealed to sequencing primers at values predetermined by the Binding Calculator (PacBio) and a complex made with the DNA Polymerase (P6/C4 chemistry). The complex was bound to Magbeads and used to set up the required number of SMRT cells for the project. Sequencing was performed using 360-min movie times on the Pacific Biosciences RS11 instrument. Consensus sequences from PacBio reads were generated with accepting only reads where the DNA template had been read at least three times to ensure high quality. All reads were blasted against HIV and converted into fasta files. The resulting sequences were de-multiplexed according to the tags associated with each subcellular fraction and clustered upon 97% identity to exclude differences introduced during PCR amplification. An average of 2,600 reads was obtained ranging between 600 and 5,300 reads. The phylogenetic relationship was inferred by the Maximum Likelihood method based on the General Time Reversible substitution model (GTR + G) and the nucleotide variation within or between plasma and cellular virus pools was estimated using the Gama distributed kimura-two-parameter model and the Mega software package.

Accession Number(s)

Sequences were submitted to GenBank under accession numbers MG755825-MG756598.

Assessment of T-Cell Activation Marker, HIV Coreceptor and Restriction Factor Expression by Flow Cytometry

Cryopreserved blood mononuclear cells from ART-treated HIV-1-infected individuals were thawed and stained with Aqua LIVE/DEAD stain kit (4°C; 15 min) and then with anti-CD3, anti-CD4, anti-CD45RA, anti-CXCR3, anti-CXCR5, anti-CCR4, anti-CCR6 PE, anti-HLA DR, anti-Ki67, anti-CD27, anti-CCR7, anti-CCR5 and anti-CXCR4 antibodies (4°C; 25 min). Regarding SAMHD1 staining, blood mononuclear cells were surface stained with Aqua LIVE/DEAD stain kit (4°C; 15 min) and then with anti-CD3, anti-CD4, anti-CD45RA, anti-CXCR3, anti-CXCR5, anti-CCR4, anti-CCR6 PE. Cells were then permeabilized (Fixation/Permeabilization Kit; BD; 45 min; 4°C) and incubated with anti-SAMHD1 (4°C; 25 min) antibody and then with a PE-conjugated donkey-antirabbit antibody (4°C; 25 min). Cells were then washed and acquired on LSRII SORP.

Flow Cytometry

Data were acquired on a LSR SORP four lasers (405, 488, 532, and 633 nm) and were analyzed as previously described (17, 35).

Mass Cytometry

Mass cytometry experiments were performed as previously described (17). Briefly, cryopreserved LN mononuclear cells isolated from viremic untreated HIV-infected individuals were thawed and resuspended (10^6 cells/mL) in complete RPMI medium. Cell viability was assessed using cisplatin (50 µM; 5 min at RT; Sigma-Aldrich) quenched with fetal bovine serum. Cells were then incubated (30 min; 4°C) with metal conjugated antibodies, i.e., anti-CD3-170, anti-CD4-115, anti-CD8-145, anti-CD45RA-169, anti-CXCR5-153, anti-CXCR3-154, anti-CCR6-141, and anti-CCR4-149 (Fluidigm/DVS Science), washed and fixed (10 min; RT) with 2.4% PFA. Total cells were identified by DNA intercalation (1 µM Cell-ID Intercalator, Fluidigm/DVS Science) in 2% PFA at 4°C overnight. Labeled samples were assessed by the CyTOF1 instrument that was upgraded to CyTOF2 (Fluidigm) using a flow rate of 0.045 mL/min. Data were analyzed as previously described (17).

Statistical Analyses

Statistical significance (*P*-values) was either obtained using two-tailed Chi-square analysis for comparison of positive proportions or using one-way ANOVA (Kruskal–Wallis test) followed by Wilcoxon matched-pairs two-tailed signed rank test as previously described (17, 35). Extreme limiting dilution analysis was applied to obtain statistics for frequencies of cells containing replication competent virus as in previous studies (1, 7, 35). Statistical significance for contribution analysis were either obtained from pairwise comparisons of proportion with FDR correction (multiple tests) or from fisher exact test for pairwise comparisons in case of small sample size. When required, Bonferroni's correction was applied for multiple comparisons. Finally, Spearman rank test was used for correlations.

RESULTS

In the present study, 9 viremic HIV-infected individuals and 19 aviremic ART-treated HIV-infected individuals were enrolled. LN biopsies were collected for all nine viremic HIV-infected individuals. Among the aviremic ART-treated individuals, 12 LN biopsies and 14 leukapheresis samples were collected, amongst which 7 individuals were matched for blood and LNs (**Table 1**). The 19 subjects studied had a documented duration of HIV-1 infection between 1.9 and 28.5 years (mean 10.1 years), a duration of ART between 1.8 and 14 years (mean 4.99 years) and viremia levels <20 HIV-1 RNA copies/mL of plasma for at least 12 months (**Table 1**).

Characterization of CD4 T Cell Populations

The expression of chemokine receptors was first assessed in blood memory CD4 T cells isolated from aviremic ART-treated individuals by multiparametric flow cytometry. To address this issue, blood mononuclear cells were stained with CD3, CD4, CD45RA, CXCR5, CXCR3, CCR4, and CCR6. In parallel, LN mononuclear cells were stained with CD3, CD4, CD45RA, CXCR5, and PD-1 antibodies. Five populations of blood memory ($CD45RA^-$) CD4 T cells were identified on the basis of chemokine receptor expression, i.e., $CXCR3^+CXCR5^-$ ($CXCR3^+$), $CXCR3^-CXCR5^-CCR4^+CCR6^-$ ($CCR4^+$), $CXCR3^-CXCR5^-CCR4^+CCR6^+$, $CXCR3^-CXCR5^+$ (circulating Tfh-like cells; cTfh) (22, 23), and $CXCR3^+CXCR5^+$ (Th1-like cTfh cells) (28) T cells (**Figure 1A**; Figure S1 in Supplementary Material). The expression of CCR4 and coexpression of CCR4 and CCR6 was also assessed on the remaining $CXCR3^+$, cTfh and Th1-like cTfh cells (Figure S2 in Supplementary Material) and revealed no significant differences between the subpopulations ($P > 0.05$).

Lymph node CD4 T-cell populations were identified on the basis of CXCR5 and PD-1 expression. Because of the very low percentage of $PD\text{-}1^+$ CD4 T cells in LNs, it was not possible to sort individual $PD\text{-}1^+$ cell populations and therefore we sorted for the total $PD\text{-}1^+$ CD4 T cell population, $CXCR5^+PD\text{-}1^-$, i.e., single $CXCR5^+$ cells and the $CXCR5^-PD\text{-}1^-$ dual negative cells (DN) cells as previously described (**Figure 1B**). Of note, $PD\text{-}1^+$ cells coexpressing CXCR5 represented 61% of the total LN $PD\text{-}1^+$ CD4 T cell population and were therefore referred to as LN $PD\text{-}1^+$/Tfh cells (**Figure 1C**).

Quantification of Integrated HIV-1 DNA in Sorted Blood and LN CD4 T-Cell Populations

In order to determine the contribution of each CD4 T-cell subset to the pool of HIV-1-infected cells, the percentage of each blood and LN CD4 T cell population on memory ($CD45RA^-$) CD4 T cells and the frequency HIV-1-infected cells containing integrated HIV-1 DNA in blood and LN were determined. In blood, $CCR4^+$ and $CXCR3^+$ CD4 T cell subsets represented the majority, i.e., up to 29 and 22% of the memory CD4 T cells, respectively (**Figure 1D**) followed by cTfh CD4 T cells that represented 13.7% and finally $CCR4^+CCR6^+$ and Th1-like cTfh CD4 T-cell subsets that represented 8.9 and 8.7% of the memory CD4 T cells, respectively. Regarding the LN compartment, LN DN memory CD4 T cell subset represented up to 50% of the LN memory CD4 T cells followed by the single $CXCR5^+$ and $PD\text{-}1^+$/Tfh cells that represented 27 and 20% of the memory CD4 T cells, respectively (**Figure 1E**).

The estimation of frequencies of cells containing integrated HIV-1 DNA among the sorted blood ($N = 12$) and LN memory CD4 T-cell populations ($N = 8$) (**Figure 1F**) revealed no significant differences among memory CD4 T cell populations isolated from blood and/or LN ($P > 0.05$) (**Figure 1F**). In particular, the frequency of blood $CXCR3^+$ CD4 T cells containing integrated HIV-1 DNA reached 5,188 copies per million, 3,882 copies per million in cTfh cells, 4,765 copies per million in Th1-like cTfh cells, 1,732 copies per million $CCR4^+$ CD4 T cells, and 3,965 copies per million in $CCR4^+CCR6^+$ subset of CD4 T cells (**Figure 1F**). Integrated HIV-1 DNA was detected at a mean frequency of 5,281 copies per million cells within LN $PD\text{-}1^+$/Tfh cells CD4 T cells with no significant differences with the LN DN and single CXCR5 CD4 T-cell populations ($P > 0.05$) (**Figure 1F**).

We next evaluated the contribution of blood and LN CD4 T-cell populations to the total pool of cells containing integrated DNA within these compartments. The cumulative data indicated that blood $CXCR3^+$ CD4 T cells contributed the most and represented about 40% of the blood reservoir containing integrated HIV DNA (**Figure 1G**). In the LN compartment, DN CD4 T-cell population contributed the most, reaching up to 48% of the LN reservoir containing integrated HIV DNA ($P < 0.05$) (**Figure 1H**). Finally, we evaluated the overall contribution of each sorted blood and LN CD4 T-cell population to the total pool of cells containing integrated HIV DNA within both blood and LN compartments in matched individuals ($N = 3$). The cumulative data showed that the DN CD4 T-cell population obtained from LNs contributed the most and up to 58% to the total HIV integrated DNA reservoir from blood and LN compartments (**Figure 1I**).

HIV Replication and Production of Blood and LN Memory CD4 T Cell Populations

In order to estimate the frequencies of HIV-1-infected cells containing inducible replication competent virus in both blood and LN compartments, we performed a viral outgrowth assay (VOA) on each isolated blood and LN CD4 T cell population (Figure S3 in Supplementary Material). However, the limited number of cells available prevented us to perform the quantitative virus outgrowth assay (Q-VOA) under conventional experimental conditions, i.e., different cell dilutions and multiple replicates, necessary to generate the frequencies of cells containing replication competent and/or infectious virus within the different memory CD4 T-cell populations for each HIV-1-infected individual. Therefore, a conventional VOA was performed using four single replicate cell dilutions, i.e., 5×10^5, 10^5, 2×10^4, and 4×10^3 cells for all the sorted blood chemokine expressing CD4 T-cell populations ($N = 13$) and three single replicate cell dilutions, i.e., 10^5, 2×10^4, and 4×10^3 cells for all the sorted LN CD4 T-cell populations sorted on the basis of CXCR5 and PD-1 expression ($N = 11$). All cell populations were stimulated with anti-CD3 and anti-CD28 monoclonal antibodies (MAbs) and cultured with allogeneic CD8-depleted blood mononuclear cells isolated from HIV-uninfected individuals for 14 days (Figure S3 in Supplementary Material). The presence of HIV-1 RNA was assessed in the culture supernatants at day 14 as previously described (17). Both the proportion of positive wells and the levels of HIV-1 RNA were generated in the VOA using the highest common concentration of cells (10^5 cells/condition), while the limiting dilution format was used to evaluate the frequencies of cells containing inducible replication competent virus as in previous studies (35). The frequencies of cells containing inducible replication competent virus assessed

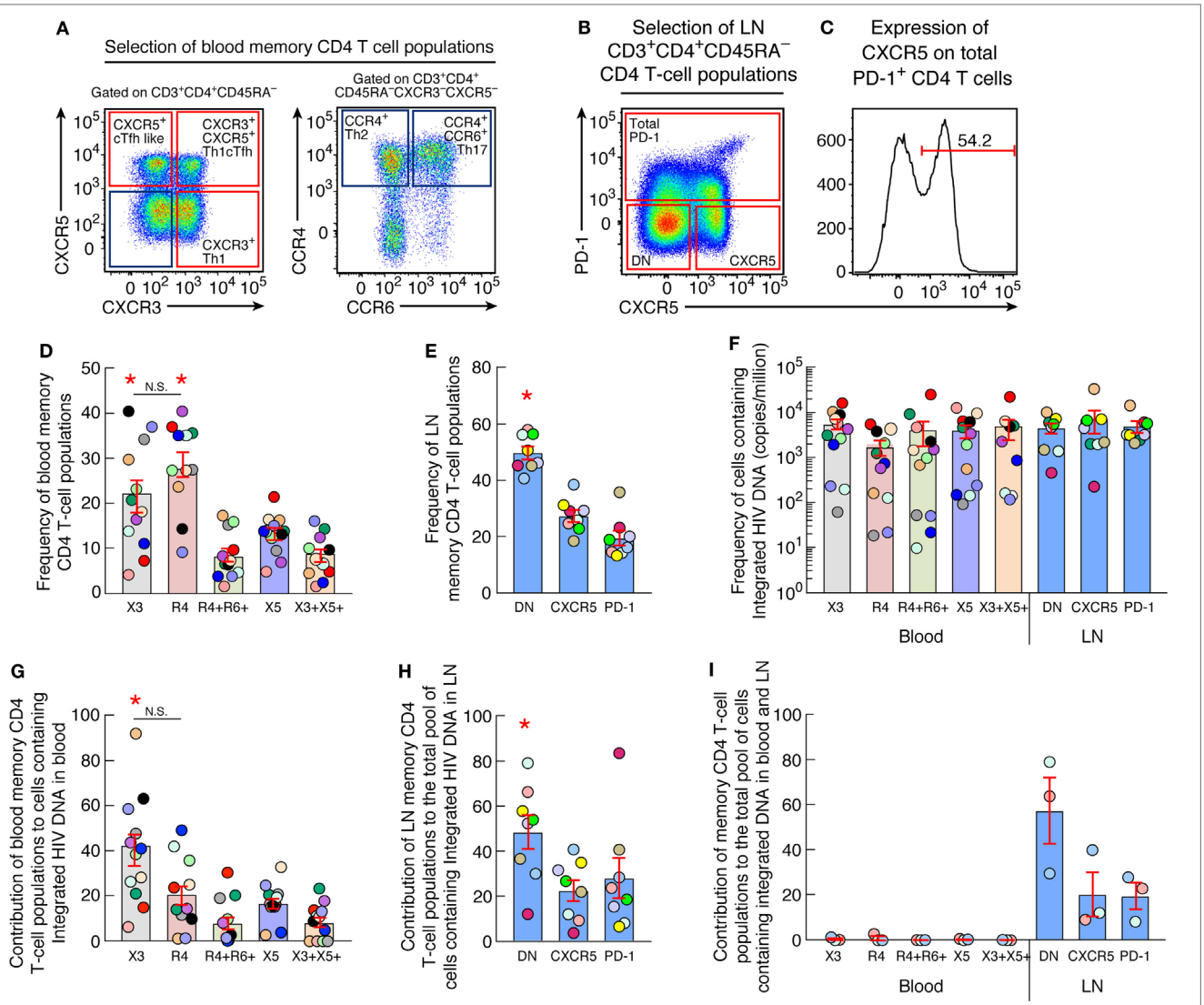

FIGURE 1 | Quantification of HIV Integrated DNA in blood and lymph node memory CD4 T-cell populations **(A)** Representative flow cytometry profile of blood memory ($CD45RA^-$) CD4 T-cell populations expressing chemokine receptors isolated from one representative aviremic long-term-treated HIV-1-infected individual. **(B)** Representative flow cytometry profile of lymph nodes (LN) memory ($CD45RA^-$) CD4 T-cell populations expressing CXCR5 and/or PD-1 isolated from one aviremic HIV-1-infected long term-treated subject. **(C)** Representative flow cytometry profile of surface expression of CXCR5 on LN $PD\text{-}1^+$ memory CD4 T cells isolated from one representative aviremic long-term-treated HIV-1-infected individual. **(D)** Frequency of chemokine receptor expressing blood memory ($CD45RA^-$) CD4 T cells isolated from aviremic long-term-treated HIV-1-infected individuals ($N = 12$). **(E)** Frequency of CXCR5 and/or PD-1-expressing LN CD4 T cells isolated from aviremic long-term-treated HIV-1-infected individual ($N = 8$). **(F)** Frequency of cells containing integrated HIV DNA (copies per million cells) within chemokine receptor expressing blood ($N = 12$) and CXCR5 and/or PD-1 expressing LN ($N = 8$) memory CD4 T-cell populations. **(G)** Contribution of chemokine receptor expressing blood memory CD4 T-cell populations to the total pool of cells containing integrated HIV DNA in blood ($N = 12$). **(H)** Contribution of CXCR5 and/or PD-1 expressing LN memory CD4 T-cell populations to the total pool of cells containing integrated HIV DNA in LN ($N = 8$). **(I)** Contribution of blood and LN memory CD4 T-cell populations of matched individuals to the total body pool of cells containing integrated HIV DNA in blood and LN compartments ($N = 3$). HIV-infected individuals are color coded **(D–I)**. Histograms correspond to mean of blood or lymph node CD4 T-cell population **(D–I)**; red bars correspond to SEM **(D–I)**. "X3" corresponds to blood CXCR3-expressing CD4 T cells; "R4" corresponds to blood CCR4-expressing CD4 T cells; $R4^+R6^+$ corresponds to blood $CCR4^+CCR6^+$ CD4 T cells; "X5" corresponds to blood CXCR5-expressing CD4 T cells; And $X3^+X5^+$ corresponds to blood $CXCR3^+CXCR5^+$ CD4 T cells. "LN" corresponds to lymph node. Red stars indicate statistical significance ($P < 0.05$). Statistical significance (P-values) was obtained using one-way ANOVA (Kruskal–Wallis test) followed by Wilcoxon matched-pairs two-tailed signed rank test **(D–I)**.

by the detection of HIV RNA at day 14 in VOA supernatants was expressed as RNA-unit per million (RUPM) (32) (Figure S3 in Supplementary Material).

We first compared the proportion of HIV RNA positive wells at day 14 in the VOA culture supernatants of all blood ($N = 13$) and LN CD4 T-cell populations ($N = 11$) at the highest common

cell concentration (i.e., 10^5) (**Figure 2A**) (35). Of note, 6 individuals were matched for blood and LN. The data showed that wells containing LN PD-1$^+$/Tfh CD4 T cells were significantly more frequently scored positive (90% of positive wells, corresponding to 9 out of the 11 individuals tested) as compared to LN CXCR5$^+$ and LN DN CD4 T cell populations ($P < 0.05$) (**Figure 2A**), supporting previous observation (17). However, in blood, wells containing CXCR3-expressing CD4 T cells were more frequently scored positive (92% of positive wells, corresponding to 12 out of 13 individuals tested) than any other blood CD4 T-cell populations (**Figure 2A**).

We next assessed the levels of HIV-1 RNA in culture supernatants of all blood ($N = 13$) and LN ($N = 11$) CD4 T-cell populations at the highest common cell concentration (i.e., 10^5) cells at day 0 and 14 (**Figure 2B**) (35). Of note, none of the culture supernatants collected at day 0 were positive for the detection of HIV-1 RNA (data not shown). Consistent with previous study (17), LN PD-1$^+$/Tfh cell VOA culture supernatants contained significantly higher levels of HIV-1 RNA than any other LN memory CD4 T-cell populations ($P < 0.05$) (**Figure 2B**).

However, in blood, CXCR3-expressing CD4 T cells VOA culture supernatants contained higher levels of HIV-1 RNA than any other blood memory CD4 T-cell populations (except from cTfh cells; $P = 0.08$) (**Figure 2B**), suggesting that blood CXCR3-expressing CD4 T cells of ART-treated aviremic HIV-1 infected individuals might be enriched in cells containing replication competent virus.

Blood CXCR3-Expressing CD4 T Cells of Long-term-Treated Aviremic HIV-1-Infected Individuals Are Enriched in Cells Containing Replication Competent Virus

To address the issue of the average frequency of HIV-1-infected cells containing inducible replication competent virus for each memory CD4 T-cell population in blood and LN compartments, the data generated from the single replicate cell dilutions of blood and LN CD4 T-cell populations using the conventional VOA were pooled together such that each patient well at a particular tested concentration in the VOA now represented a replicate; mean frequencies within the cohort were therefore then estimated using extreme limiting dilution assay (33) as previously described (17). The frequencies of HIV-1-infected cells containing inducible replication competent virus are expressed in average RNA-unit per million (RUPM) (32) (**Figures 3A,B**) and statistical significance was obtained following a pair-wise test performed using Extreme Limiting Dilution Assay (33). Consistent with previous study (17), LN PD-1$^+$/Tfh cells were significantly enriched in cells containing replication competent virus reaching 23 cells per million (**Figure 3B**). Interestingly however, the results obtained from blood samples indicated that blood CXCR3-expressing CD4 T cells but not CXCR5-expressing CD4 T cells were significantly enriched in cells containing inducible replication competent virus reaching about 13 cells per million ($P < 0.05$) (**Figure 3A**) while CXCR5-expressing CD4 T cells reached around 4 cells per million.

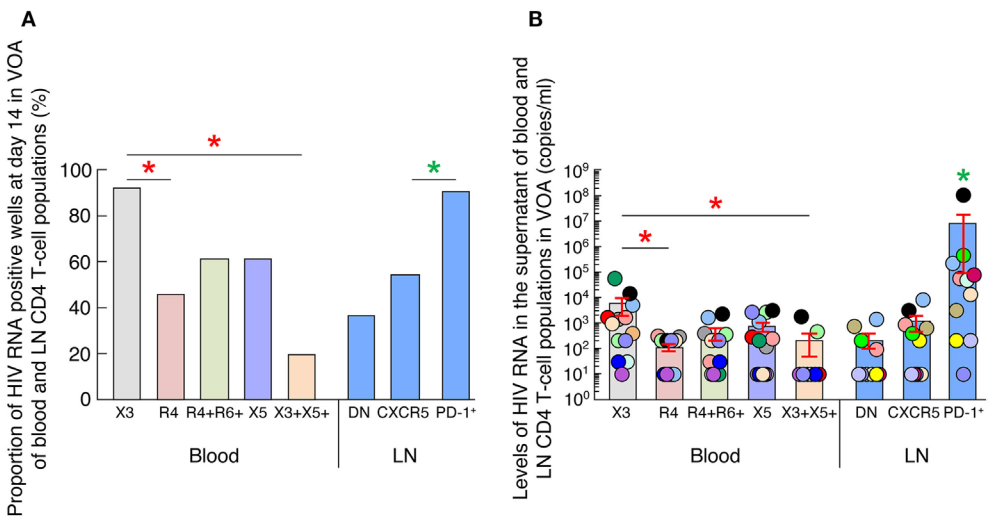

FIGURE 2 | HIV replication and production of blood and lymph node (LN) memory CD4 T cell populations. **(A)** Proportion of HIV RNA positive wells among blood ($N = 13$) and LN ($N = 11$) memory CD4 T-cell populations at day 14 of virus outgrowth assay (VOA). Wells with detectable HIV-1 RNA (≥20 HIV-1 RNA copies/mL) were referred to as HIV-1 RNA-positive wells. **(B)** Levels of HIV-1 RNA in blood ($N = 13$) and LN ($N = 11$) memory CD4 T cell populations at day 14 of VOA. Undetectable values were arbitrarily defined as 10 HIV-1 RNA copies/mL. HIV-infected individuals are color coded (C). "X3" corresponds to blood CXCR3-expressing CD4 T cells; "R4" corresponds to blood CCR4-expressing CD4 T cells; R4$^+$R6$^+$ corresponds to blood CCR4$^+$CCR6$^+$ CD4 T cells; "X5" corresponds to blood CXCR5-expressing CD4 T cells; And X3$^+$X5$^+$ corresponds to blood CXCR3$^+$CXCR5$^+$ CD4 T cells. "LN" corresponds to lymph node. Red stars indicate statistical significance with in blood compartment ($P < 0.05$). Green stars indicate statistical significance within LN compartment ($P < 0.05$). Statistical significance (P-values) was either obtained using two-tailed Chi-square analysis for comparison of positive proportions **(A)** or using one-way ANOVA (Kruskal–Wallis test) followed by Wilcoxon matched-pairs two-tailed signed rank test **(B)**.

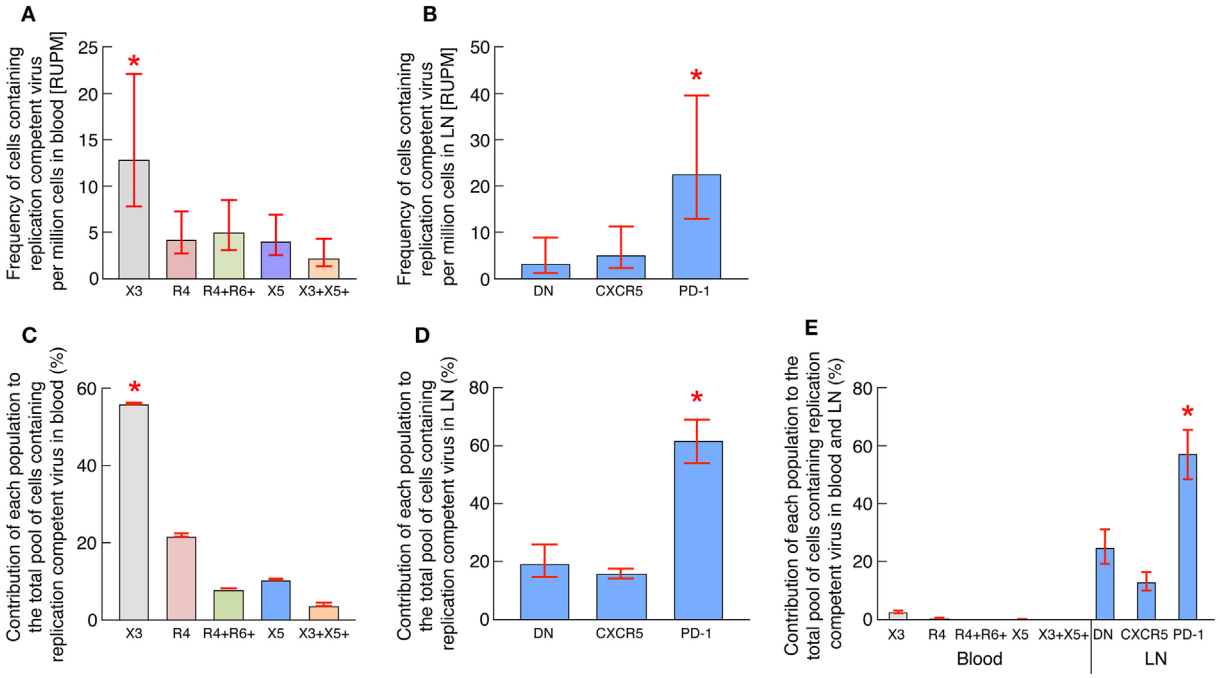

FIGURE 3 | Blood CXCR3-expressing CD4 T cells of long-term-treated aviremic HIV-1-infected individuals are enriched in cells containing replication competent virus. **(A)** Estimated RNA-unit per million (RUPM) frequencies in chemokine receptor expressing blood memory CD4 T-cell populations ($N = 13$). **(B)** Estimated RUPM frequencies in CXCR5 and/or PD-1 expressing lymph node (LN) memory CD4 T-cell populations ($N = 11$). **(C)** Estimated contribution of blood memory CD4 T-cell populations to the pool of cells containing replication competent virus in blood ($N = 13$). **(D)** Estimated contribution of LN memory CD4 T-cell populations to the pool of cells containing replication competent virus in LN compartment ($N = 11$). **(E)** Estimated contribution of blood and LN memory CD4 T-cell populations of matched individuals to the total pool of cells containing replication competent virus in blood and LN compartments ($N = 6$). Contribution of memory CD4 T-cell populations to the pool of cells containing replication competent virus was calculated as previously described (17). Histograms correspond to estimated mean **(A–E)** and red bars correspond to the lower and upper confidence interval at 0.95 **(A–E)**. "X3" corresponds to blood CXCR3-expressing CD4 T cells; "R4" corresponds to blood CCR4-expressing CD4 T cells; $R4^+R6^+$ corresponds to blood $CCR4^+CCR6^+$ CD4 T cells; "X5" corresponds to blood CXCR5-expressing CD4 T cells; And $X3^+X5^+$ corresponds to blood $CXCR3^+CXCR5^+$ CD4 T cells. "LN" corresponds to lymph node. Red stars indicate statistical significance ($P < 0.05$). Statistical significance (P-values) was either obtained using Extreme Limiting Dilution analysis (http://bioinf.wehi.edu.au/software/elda/) **(A,B)**, by pairwise comparisons of proportion with FDR correction (multiple tests) **(C,E)** or by Fisher's exact test for pairwise comparisons **(D)** (36).

On the basis of the above data and since various CD4 T-cell populations may contribute to the total pool of cells containing replication competent virus within blood and LN compartments, the assessment of the relative contribution of each CD4 T cell population was then performed using (1) the frequency of each cell population in each compartment (blood and/or LN), (2) the estimated numbers of CD4 T lymphocytes in different lymphoid organs (34), and (3) the estimated frequencies of cells containing inducible replication competent virus within each blood and LN memory CD4 T cell population as previously described (17). The results indicated that in blood, the CXCR3-expressing CD4 T cells contributed the most to the pool of cells containing inducible replication competent virus in blood and represented about 56% of the blood reservoir containing replication competent virus ($P < 0.05$) (**Figure 3C**). Consistent with previous study (17), LN $PD\text{-}1^+$/Tfh cells contributed the most to the pool of cells containing inducible replication competent virus in LN (represented about 60%) ($P < 0.05$) (**Figure 3D**) and contributed the most to the pool of cells containing inducible replication competent virus in blood and LN (represented about 58%) ($P < 0.05$) (**Figure 3E**).

Taken together, these data indicate that blood CXCR3-expressing CD4 T cells isolated from ART-treated aviremic HIV-1-infected individuals are enriched in cells containing inducible replication competent virus and contributed the most to the HIV reservoir in blood.

Blood CXCR3-Expressing CD4 T Cells of ART-Treated Aviremic HIV-1-Infected Individuals Represent the Major Source of Infectious HIV-1 in Blood

In order to determine whether the virus obtained in the VOA culture supernatants of blood memory CD4 T-cell populations was infectious, we performed an *in vitro* HIV-1 infection assay. For these purposes, day 14 VOA culture supernatants of the highest cell concentration (i.e., 5×10^5) were used to inoculate preactivated CD8-depleted blood mononuclear cells isolated from HIV negative individuals as previously described (17). Culture supernatants were collected at day 0 and 14 and assessed for the presence of HIV-1 RNA. Of note, none of the culture supernatants collected at day 0 had detectable levels of HIV-1

RNA (data not shown). However, after 14 days of culture, HIV-1 RNA was more frequently detected in culture supernatants of CXCR3-expressing CD4 T cells (8 out of the 13 ART-treated aviremic HIV-1-infected individuals tested) as compared to any other blood CD4 T-cell populations (**Figure 4A**). In addition, the levels of HIV-1 RNA detected in culture supernatants of blood CXCR3-expressing CD4 T cells were significantly higher as compared to any other blood CD4 T-cell subsets ($P < 0.05$) (**Figure 4B**). Finally, we determined the relationship between HIV-1 RNA levels detected in the VOA and those detected in the *in vitro* HIV-1 infection assay. The results indicated a strong correlation between HIV-1 RNA levels detected in VOA culture supernatants and HIV-1 RNA levels detected in the *in vitro* HIV-1 infection assay ($r = 0.8175$, $P < 0.0001$) (**Figure 4C**).

Taken together, these data suggest that blood CXCR3-expressing CD4 T cells of ART-treated aviremic HIV-1-infected individuals represent the major source of infectious HIV-1 in blood.

Phylogenetic Sequence Analyses of the Highly Variable EnvV1V4 Region in Sorted Blood and LN Memory CD4 T-Cell Populations

In order to address the phylogenetic relationship between HIV sequences obtained from blood and LN CD4 T-cell populations, we sorted blood and LN CD4 T-cell populations of matched ART-treated HIV-infected individuals as previously mentioned and single genome sequencing of the highly variable region of the gp160 virus envelope, the EnvV1V4 region, was performed using next generation sequencing platform from PacBio Systems as previously described (37).

The phylogenetic analysis revealed that the sequences obtained from the individual #35 and #077 were monophyletic while those obtained from individual #094 were not (**Figure 5A**), indicating varying degree of virus diversity. In all three ART-treated HIV-infected individuals tested, phylogenetic inference revealed that virus pools from any given cell population were closely related to the virus from all other cell fractions demonstrating a lack of virus compartmentalization (**Figure 5B**).

PD-1 Expression on Blood CXCR3-Expressing CD4 T Cells Positively Correlates with the Levels of HIV-1 RNA Produced in the VOA Culture Supernatants

Next, we explored whether specific parameters such as the level of T-cell activation (assessed by HLA-DR and Ki-67 expression), T-cell differentiation (assessed by CD45RA, CCR7 and/or CD27 expression), HIV coreceptor (CCR5 and CXCR4) or restriction factor (SAMHD1) expression may be associated with the increased frequency of cells containing replication competent virus within blood CXCR3-expressing CD4 T cells. To address this issue, the level of HLA-DR, Ki-67, CCR5, CXCR4, SAMHD1, CD45RA, CCR7, and CD27 were determined on the various blood CD4 T-cell populations of HIV-1-infected ART-treated individuals by flow cytometry (**Figures 6A–F**). The cumulative data indicated that blood CXCR3-expressing CD4 T cells were not significantly enriched in activated T cells expressing HLA-DR or Ki-67 ($P > 0.05$) (**Figures 6A,B**), in cells expressing the HIV

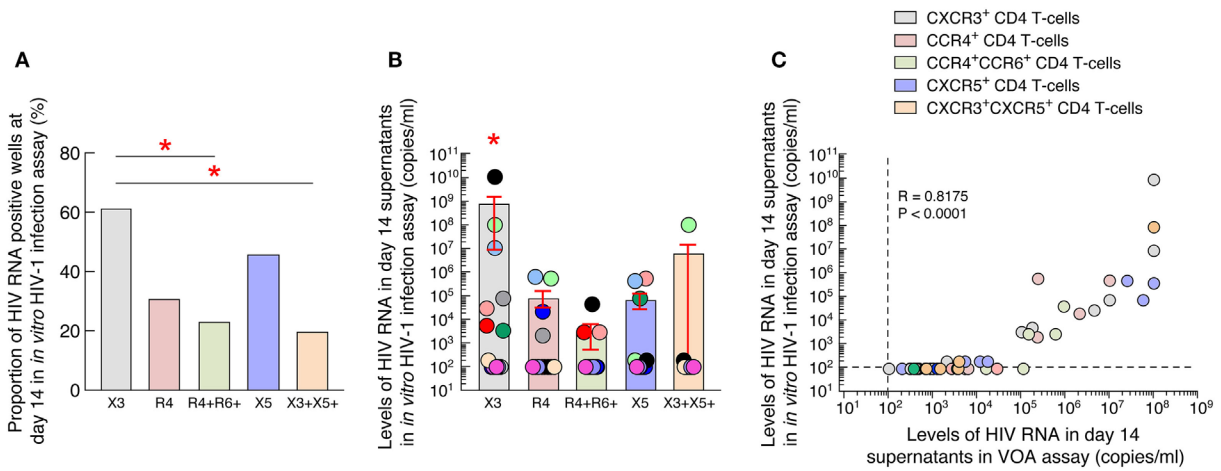

FIGURE 4 | Blood CXCR3⁺ CD4 T cells of antiretroviral therapy (ART)-treated aviremic HIV-1-infected individuals represent the major source of infectious HIV-1 in blood. **(A)** Proportion of HIV-1-infected individuals with detectable HIV-1 RNA (≥200 HIV-1 RNA copies/mL) in the *in vitro* HIV-1 infection assay. **(B)** Levels of HIV-1 RNA in blood memory CD4 T cell populations at day 14 of *in vitro* HIV-1 infection assay ($N = 13$). **(C)** Correlation between HIV-1 RNA levels measured in VOA and in *in vitro* HIV-1 infection assay in supernatants of blood memory CD4 T-cell populations ($N = 13$). Undetectable values were arbitrarily defined as 100 HIV-1 RNA copies/mL **(B,C)**. Each individual is uniquely color coded in **(B)**. Each CD4 T-cell population is uniquely colored in **(C)**. Histograms correspond to mean **(A,B)**; red bars correspond to SEM **(B)**. "X3" corresponds to blood CXCR3-expressing CD4 T cells; "R4" corresponds to blood CCR4-expressing CD4 T cells; R4⁺R6⁺ corresponds to blood CCR4⁺CCR6⁺ CD4 T cells; "X5" corresponds to blood CXCR5-expressing CD4 T cells; And X3⁺X5⁺ corresponds to blood CXCR3⁺CXCR5⁺ CD4 T cells. Red stars indicate statistical significance ($P < 0.05$) **(A,B)**. Statistical significance (*P*-values) was either obtained using two-tailed Chi-square analysis for comparison of positive proportions **(A)** or using one-way ANOVA (Kruskal–Wallis test) followed by Wilcoxon matched-pairs two-tailed signed rank test **(B)** or Spearman rank test for correlation **(C)**.

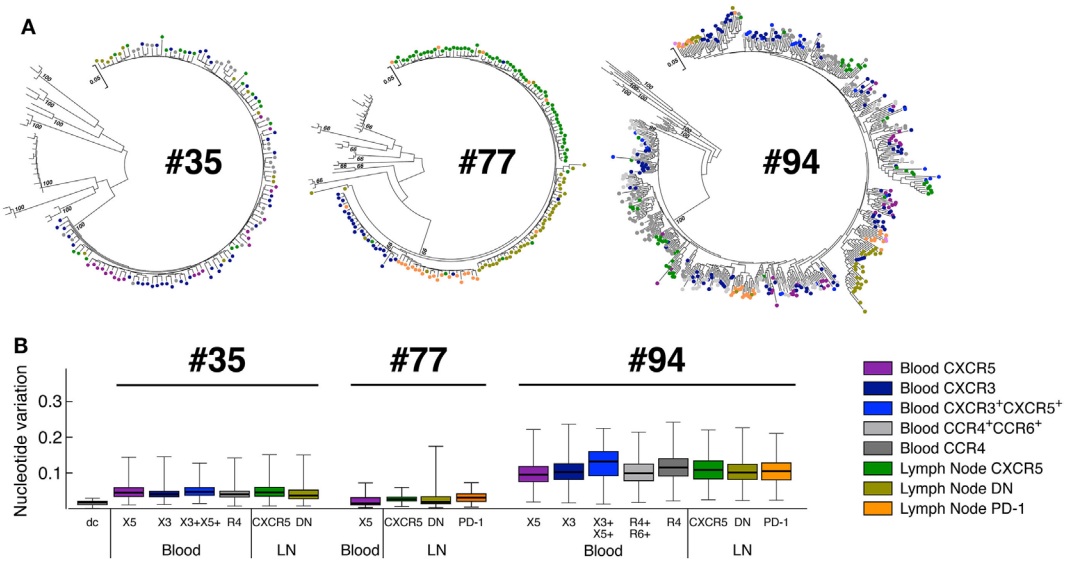

FIGURE 5 | Phylogenetic relationship of HIV-1 envelope sequences derived from CD4 T-cell populations. Sequences of the highly variable EnvV1V4 region (Hxb 6540-7668) were derived from blood and LN memory CD4 T-cell populations isolated from aviremic-treated HIV-1-infected individuals ($N = 3$). Virus quasi-species were amplified and sequenced [single molecule, real-time (SMRT) method/PacBio Systems]. **(A)** Phylogenetic relationship of HIV-1 envelope sequences derived from CD4 T-cell populations ($N = 3$). The phylogenetic relationship was inferred by the Maximum Likelihood method based on the General Time Reversible substitution model (GTR + G). Each tree includes reference sequences from subtype B and non-B HIV-1-infected individuals. **(B)** Nucleotide variations observed within each blood and LN memory CD4 T-cell population estimated using the Gama distributed kimura-two-parameter. CD4 T-cell populations were color coded **(A,B)**. dc corresponds to the laboratory isolate control used to monitor sequence diversity induced by the method. "X3" corresponds to blood CXCR3-expressing CD4 T cells; "R4" corresponds to blood CCR4-expressing CD4 T cells; $R4^+R6^+$ corresponds to blood $CCR4^+CCR6^+$ CD4 T cells; "X5" corresponds to blood CXCR5-expressing CD4 T cells; And $X3^+X5^+$ corresponds to blood $CXCR3^+CXCR5^+$ CD4 T cells. "LN" corresponds to lymph node.

coreceptors CXCR4 or CCR5 ($P > 0.05$) (**Figures 6C,D**). In addition, blood CXCR3-expressing CD4 T cells were not significantly enriched in central memory ($CCR7^+CD27^+$) nor in transitional memory ($CCR7^-CD27^+$) CD4 T cells ($P > 0.05$) (**Figure 6F**). Finally, blood CXCR3-expressing CD4 T cells did not express significantly lower levels of the host restriction factor SAMHD1 ($P > 0.05$) (**Figure 6E**).

Since, a recent study highlighted that blood CD32-expressing CD4 T cells were enriched in HIV-infected cells (12), therefore we assessed the level of CD32 expression on chemokine expressing CD4 T-cell populations (**Figure 6G**). The cumulative data indicated that blood CXCR3-expressing CD4 T cells were not significantly enriched in cells expressing CD32 ($P > 0.05$) (**Figure 6G**).

Finally, since PD-1 expression on CD4 T cells has been associated with increased frequencies of HIV-infected cells (16, 17), we next assessed the level of PD-1 expression on each blood memory CD4 T-cell population of ART-treated HIV-infected individuals. The cumulative data indicated that blood CXCR3-expressing CD4 T cells were not significantly enriched in cells expressing PD-1 ($P > 0.05$) (**Figure 6H**), however, the frequency of PD-1 expressing blood CXCR3-expressing CD4 T cells positively correlated with the levels of HIV-1 RNA produced in the VOA culture supernatants of CXCR3-expressing CD4 T cells ($r = 0.628$, $P = 0.05$) (**Figure 6I**), suggesting that $PD\text{-}1^+CXCR3^+$ CD4 T cells might be enriched in cells containing inducible replication competent virus in blood.

LN PD-1 Expressing CD4 T Cells Express High Levels of CXCR3

In order to determine the potential origin of blood CXCR3-expressing CD4 T cells, the expression of CXCR3, CCR4 and/or CCR6 was assessed on LN memory ($CD45RA^-$) CD4 T-cell populations isolated from viremic untreated HIV-infected individuals (**Figure 7**). The data showed that all PD-1-expressing LN CD4 T-cell populations of viremic HIV-infected individuals were enriched in $CXCR3^+$ CD4 T cells in comparison to $CCR4^+$, single $CCR6^+$, $CCR4^+CCR6^+$, and $CCR4^-CCR6^-$ CD4 T cells ($P < 0.05$) (**Figure 7**). In contrast the PD-1-negative CD4 T-cell populations expressed either comparable levels of CXCR3 and CCR4 (single CXCR5 LN CD4 T cells; $P > 0.05$) or were dominated by CCR4-expressing and $CCR4^-CCR6^-$ CD4 T cells (DN LN CD4 T cells; $P < 0.05$) (**Figure 7**).

Taken together, these data suggest that CXCR3-expressing blood CD4 T cells may originate from LN $PD\text{-}1^+$ CD4 T cells. However, further longitudinal analyses would be needed to support this hypothesis.

DISCUSSION

One of the major barriers to HIV eradication is the presence of cells containing latent, transcriptionally silent, but inducible replication competent virus in ART-treated aviremic HIV-infected individuals (4, 8, 38). Therefore, great effort was placed

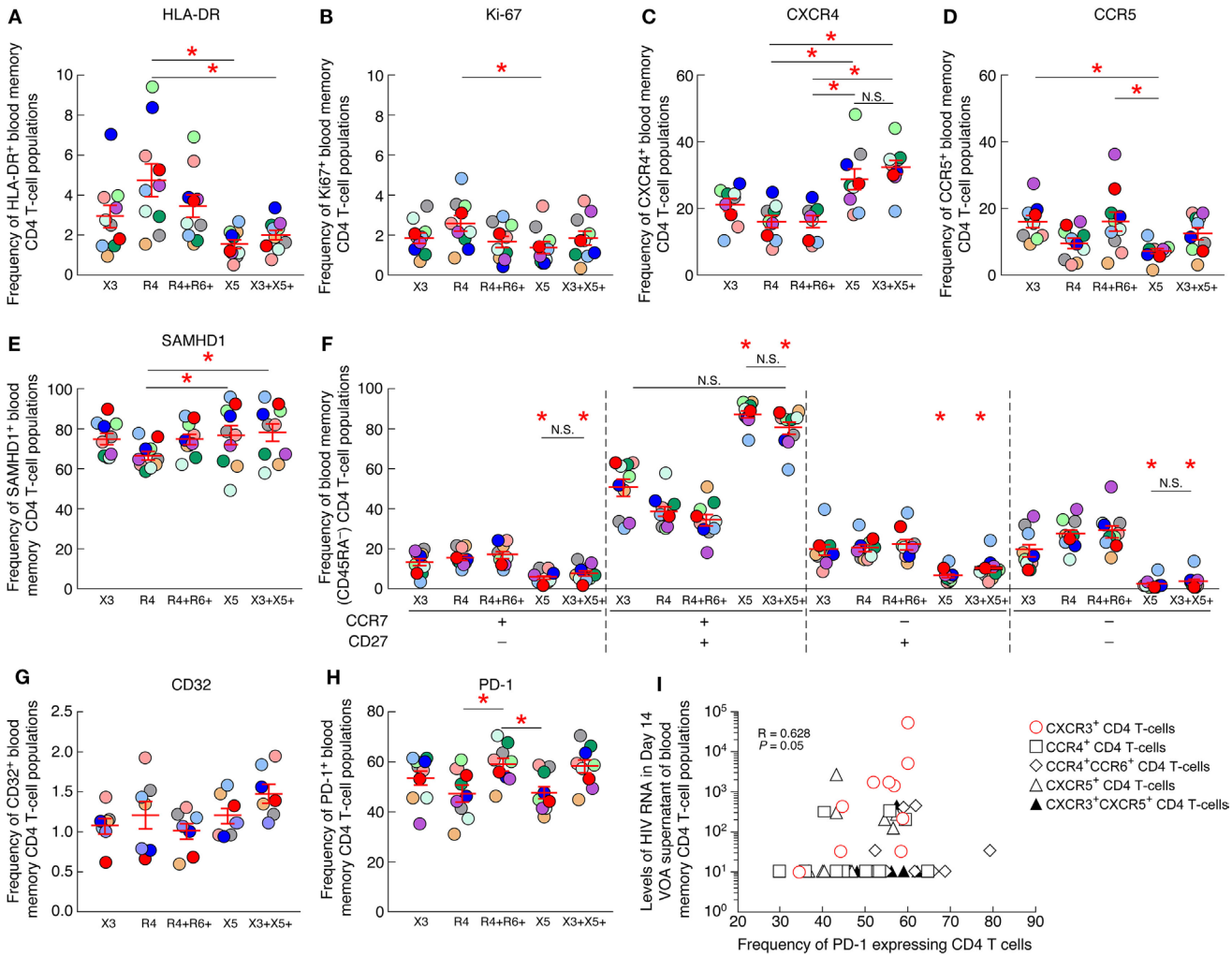

FIGURE 6 | PD-1 expression on blood CXCR3+ CD4 T cells positively correlates with the levels of HIV-1 RNA produced in the virus outgrowth assay (VOA) culture supernatants. Percentage of expression of HLA-DR **(A)**, Ki-67 **(B)**, CXCR4 **(C)**, CCR5 **(D)**, SAMHD1 **(E)** on chemokine receptor expressing blood memory (CD45RA−) CD4 T-cell populations isolated from aviremic long-term-treated HIV-1-infected individuals ($N = 10$). **(F)** Differentiation profile of chemokine receptor expressing blood memory (CD45RA−) CD4 T-cell populations isolated from aviremic long-term-treated HIV-1-infected individuals ($N = 10$). Percentage of CD32+ ($N = 7$) **(G)** or PD-1+ ($N = 10$) **(H)** chemokine receptor expressing blood memory (CD45RA−) CD4 T-cell populations isolated from aviremic long-term-treated HIV-1-infected individuals. **(I)** Correlation between HIV-1 RNA levels detected in day 14 VOA supernatants of chemokine receptor expressing blood memory CD4 T-cell populations and the percentage of PD-1-expressing CD4 T cells within each chemokine receptor expressing blood memory CD4 T-cell population. Undetectable values were arbitrarily defined as 10 HIV-1 RNA copies/mL **(I)**. Red bars correspond to SEM **(A–H)**. "X3" corresponds to blood CXCR3-expressing CD4 T cells; "R4" corresponds to blood CCR4-expressing CD4 T cells; R4+R6+ corresponds to blood CCR4+CCR6+ CD4 T cells; "X5" corresponds to blood CXCR5-expressing CD4 T cells; And X3+X5+ corresponds to blood CXCR3+CXCR5+ CD4 T cells. Each HIV-infected individual was color coded **(A–H)**. Red stars indicate statistical significance ($P < 0.05$) **(A–H)**. Statistical significance (P-values) was either obtained using one-way ANOVA (Kruskal–Wallis test) followed by Wilcoxon matched-pairs two-tailed signed rank test **(A–H)** or Spearman rank test for correlation **(I)**.

to characterize HIV-infected cells in blood and tissues (39). In this regard, we have recently shown that LN PD-1+/Tfh cells were enriched in cells containing inducible replication competent virus in ART-treated aviremic HIV-infected individuals (17), which is probably associated with the reduced capacity of CD8 T cells and cART to penetrate into germinal center (GC) areas (19, 40).

Since memory LN Tfh cells may egress from GCs and recirculate in blood (20), we hypothesized that the LN Tfh cell counterpart recirculating in blood might also be enriched in HIV-infected cells in ART-treated aviremic HIV-1-infected individuals. We therefore determined the frequency of cells containing integrated HIV-1 DNA or replication competent virus and their contribution to the HIV reservoir in blood and LN CD4 T-cell populations defined by chemokine receptor expression. Since the blood circulating Tfh counterpart has usually been identified on the basis of expression of chemokine receptors CXCR5 (22) and/or CXCR3 (20), we have first gated

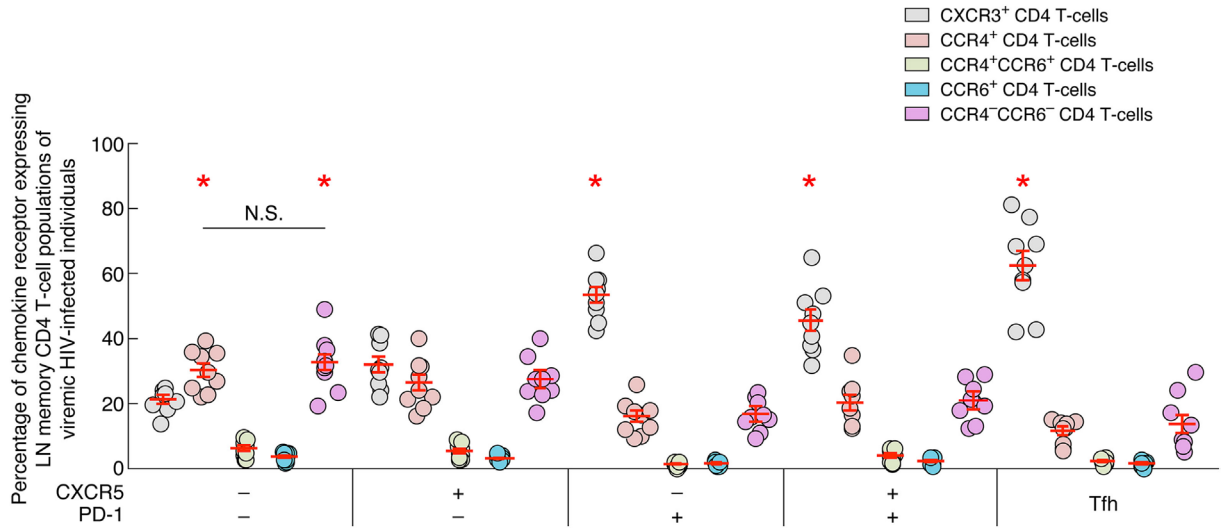

FIGURE 7 | Chemokine receptor expression on lymph node memory CD4 T cell populations of viremic HIV-infected individuals. Percentage of chemokine receptor expression on lymph nodes (LNs) CD4 T-cell populations of viremic HIV-infected individuals ($N = 9$). Red bars correspond mean ± SEM. CD4 T-cell populations were color coded. Red stars indicate statistical significance ($P < 0.05$). Statistical significance (P-values) was either obtained using one-way ANOVA (Kruskal–Wallis test) followed by Wilcoxon matched-pairs two-tailed signed rank test.

on and sorted for CXCR5 and/or CXCR3-expressing memory CD4 T-cell populations. In addition, cells expressing neither CXCR5 nor CXCR3, have previously been shown to be enriched in TH2-like (expressing CCR4$^+$) and TH17-like (expressing CCR4 and CCR6) cells (41). Therefore, in addition to the CXCR5$^+$ and/or CXCR3$^+$ cells, we chose to sort for CCR4$^+$ and CCR4$^+$CCR6$^+$ subsets amongst the CXCR3 and CXCR5 dual negative cells. Of note, the three other remaining populations, i.e., CXCR3$^+$, CXCR5$^+$, and CXCR3$^+$CXCR5$^+$ cell populations also contained cells expressing CCR4 and/or CCR6, but showed no significant differences between them. Therefore, in the present study, we focused on and compared chemokine receptor expressing blood CD4 T-cell populations including CXCR5$^+$ and CXCR5$^+$CXCR3$^+$ blood memory CD4 T cells, corresponding to "cTfh" (22, 23) and "Th1-like cTfh" (28) CD4 T cells, respectively, CXCR5$^-$CXCR3$^-$CCR4$^+$, CXCR5$^-$CXCR3$^-$CCR4$^+$CCR6$^+$ blood memory CD4 T-cell populations and LN CD4 T-cell populations including LN PD-1$^+$/Tfh CD4 T cells.

Interestingly, we did not observe significant differences in terms of frequencies of cells containing HIV-1 integrated DNA within and across blood and LN compartments. However, we showed that the blood CXCR3-expressing CD4 T cells contributed the most (up to 56%) to the HIV reservoir in blood, while DN LN CD4 T cells contributed the most to the HIV reservoir in LN.

We then evaluated and compared the capacity of blood and LN memory CD4 T-cell populations isolated from ART-treated aviremic HIV-1-infected individuals to support active virus replication and produce infectious viruses. In this context, we showed that, consistent with previous studies, LN PD-1$^+$/Tfh cells were the largest source of inducible replication competent virus. Interestingly, we showed that, in blood, CXCR3-expressing CD4 T cells but not CXCR5-expressing CD4 T cells were enriched in inducible replication competent and infectious virus and contributed the most to the replication competent reservoir in blood.

Unfortunately, the results obtained using the VOA assay did not allow to determine whether CXCR3-expressing CD4 T cells contained more cells with intact provirus or whether the intact provirus was more easily inducible in CXCR3-expressing CD4 T cells as compared to other cell populations. One way to address this issue would be to perform full length sequencing of HIV provirus and to determine HIV provirus integration sites within each CD4 T-cell populations isolated from blood and LN compartments. Interestingly, the recent study by Lee et al. highlighted the presence of higher proportion of intact proviruses within blood type 1 helper (Th1) CD4 T cells (42). Since, blood CXCR3-expressing CD4 T cells are usually enriched in cells harboring Th1 functions (41, 43, 44), it is well possible that blood CXCR3-expressing CD4 T cells would also be enriched in cells containing a higher proportion of intact provirus. Full length sequencing of HIV provirus and HIV integration site determination would also help to determine whether the accumulation of replication competent virus within one particular subset is associated with homeostatic T-cell proliferation or with a higher infection rate of blood CXCR3-expressing CD4 T-cell precursor. Indeed, recent studies have highlighted the role of homeostatic clonal expansion of blood HIV-infected memory CD4 T cells in HIV persistence (42, 45). Notably, Lee et al. also suggested that Th1 cells containing intact provirus may have accumulated in blood through clonal expansion (42).

Furthermore, various other parameters including epigenetic modifications such as DNA methylation of HIV gene promoters or HIV provirus integration site may also potentially contribute to explain the relative lack of inducibility of cells containing intact proviruses (46, 47). In this regard, recent integration site analyses performed on blood CD4 T cells of

treated HIV-infected patients demonstrated that intact HIV provirus may be enriched in transcriptionally silent parts of the genome, supporting the relative difficulty to reactivate HIV replication by VOA (46). Therefore, additional studies would be needed to determine the potential mechanism by which HIV-infected cells accumulate within blood CXCR3-expressing CD4 T cells.

To determine the potential origin of blood CXCR3-expressing memory CD4 T cells containing replication competent virus, we performed proviral sequencing of EnvV1V4 region amplified from CD4 T-cell populations isolated from blood and LN compartments. The results obtained indicated that proviral sequences amplified from blood and LN CD4 T-cell populations were intermingled with each other without any indication of compartmentalization. Therefore, these results although limited to certain cell populations indicated that the virus was highly related between the different cell populations, suggesting dynamic interchanges between the two compartments.

We then conducted a series of experiments to determine the potential parameters associated with the enrichment of cells containing replication competent virus within blood CXCR3-expressing CD4 T cells. Notably, blood CXCR3-expressing CD4 T cells did not express significantly higher levels of HIV coreceptors CCR5 and/or CXCR4, were not significantly more activated or did not express significantly lower levels of SAMHD1 restriction factor as compared to the other blood chemokine receptor expressing CD4 T cells. In addition, blood CXCR3-expressing CD4 T cells did not express significantly higher levels of CD32 or PD-1, however, the level of PD-1 expression on blood CXCR3-expressing CD4 T cells directly correlated with the level of HIV RNA produced in the VOA supernatants suggesting that blood CXCR3-expressing CD4 T cells containing replication competent virus might express PD-1.

We then hypothesized that blood CXCR3-expressing CD4 T cells containing replication competent virus might have originated from HIV-infected LN CD4 T cells, that would have been infected during the viremic phase and would recirculate in blood after treatment initiation. Indeed the low state of activation and cell cycle progression of CXCR3-expressing memory CD4 T cells as assessed by the levels of HLA-DR and Ki-67 expression, respectively, also suggests that they may represent those cells that may have exited the tissue sites where prior virus replication may have taken place. Therefore, in order to address the origin of these cells, we assessed the chemokine receptor expression on various LN memory CD4 T cells defined by CXCR5 and PD-1 expression isolated from untreated viremic HIV-infected individuals. Even though the analysis was cross-sectional and not longitudinal, the results obtained indicated that CXCR3 is the most dominant chemokine receptor expressed on PD-1$^+$ CD4 T cells including Tfh cells in viremic HIV-infected individuals whereas PD-1-negative CD4 T-cell populations, i.e., DN and single CXCR5 CD4 T-cell populations expressed either comparable levels of CXCR3 and CCR4 or expressed higher levels of CCR4. It is therefore possible that HIV-infected CXCR3-expressing CD4 T cells may originate from HIV-infected PD-1$^+$CXCR3$^+$ LN CD4 T cells in general and from Tfh cells in particular. Indeed, it has been clearly established that LN GC Tfh cells downregulate CXCR5 expression in order to egress from the B cell follicle post-GC response (48, 49). However, longitudinal assessment of chemokine receptor expression on LN Tfh cells from the viremic phase of infection till the control of infection post-ART treatment may provide further evidence for the chemokine receptors that are retained on these cells to orchestrate further migration from tissues and recirculation in blood.

Taken together, our data highlight the heterogeneity in the CD4 T-cell populations harboring cells containing inducible replication competent virus in blood and LNs of aviremic ART-treated HIV-infected individuals. In particular, we show that blood CXCR3-expressing CD4 T cells but not CXCR5-expressing CD4 T cells were enriched in inducible replication competent virus and contributed the most to the replication competent reservoir in blood. However, additional studies would be needed to determine their potential origins and the mechanism by which HIV-infected cells accumulated within this particular subset.

ETHICS STATEMENT

The present study was approved by the Institutional Review Board of the Centre Hospitalier Universitaire Vaudois, and all subjects gave written informed consent.

AUTHOR CONTRIBUTIONS

RB, FP, AR, AN, GP, WP, and MP designed the experiments. RB, FP, AR, and AN performed the experiments. RB, FP, AR, AN, WP, GP, and MP interpreted the data. MC and J-MC provided the samples. RB, GP, and MP wrote the manuscript. All the authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Differentiation and Function of Follicular CD8 T Cells During Human Immunodeficiency Virus Infection

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The combination antiretroviral therapeutic (cART) regime effectively suppresses human immunodeficiency virus (HIV) replication and prevents progression to acquired immunodeficiency diseases. However, cART is not a cure, and viral rebound will occur immediately after treatment is interrupted largely due to the long-term presence of an HIV reservoir that is composed of latently infected target cells that maintain a quiescent state or persistently produce infectious viruses. CD4 T cells that reside in B-cell follicles within lymphoid tissues, called follicular helper T cells (TFH), have been identified as a major HIV reservoir. Due to their specialized anatomical structure, HIV-specific CD8 T cells are largely insulated from this TFH reservoir. It is increasingly clear that the elimination of TFH reservoirs is a key step toward a functional cure for HIV infection. Recently, several studies have suggested that a fraction of HIV-specific CD8 T cells can differentiate into a CXCR5-expressing subset, which are able to migrate into B-cell follicles and inhibit viral replication. In this review, we discuss the differentiation and functions of this newly identified CD8 T-cell subset and propose potential strategies for purging TFH HIV reservoirs by utilizing this unique population.

Keywords: follicular CD8 T cells, B-cell follicles, human immunodeficiency virus infections, human immunodeficiency virus reservoir, CXCR5$^+$CD8 T cells

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INTRODUCTION

Human immunodeficiency virus (HIV)-specific CD8 T cells play an important role in suppressing HIV replication (1–5). The onset of HIV-specific CD8 T cell responses is concomitant with a reduction in plasma viremia (6–8). The rapidity and magnitude of HIV-specific CD8 T-cell responses correlate inversely with set-point viremia in hyperacutely infected patients (9). However, diminished HIV-specific CD8 T cell responses are accompanied by disease progression (10). Furthermore, elite controllers of HIV infection exhibit specific major histocompatibility complex (MHC) class I alleles and a wide spectrum of HIV-specific CD8 T-cell responses (11–15). Additionally, CD8 T cell-specific epitope mutants emerge to evade CD8 T-cell recognition during HIV infection (16, 17). Most direct evidence comes from rhesus macaques with chronic simian immunodeficiency virus (SIV) infection, in which transient CD8 T cell depletion resulted in a substantial increase in plasma viremia, while the subsequent replenishment of CD8 T cells led to a reduction in viremia (18–20). Despite the importance of HIV-specific CD8 T cells, they are not capable of fully eliminating HIV-infected target cells, mainly CD4 T cells. A wide variety of extrinsic and intrinsic factors are required to cripple HIV-specific CD8 T-cell mediated inhibition of HIV replication. One key factor lies in the functional exhaustion of HIV-specific CD8 T cells due to persistent T-cell receptor (TCR) stimulation and inhibitory microenvironments (21–23). Additionally, recent progress has been made to realize that HIV preferentially targets TFH cells in B-cell follicles for both long-term latent infection and the persistent production of infectious viral particles (24–28), and the majority of HIV-specific CD8

T cells are excluded from B-cell follicles (29–31). Therefore, the exhaustion of HIV-specific CD8 T cells and the anatomical separation of latently infected TFH cells and HIV-specific CD8 T cells might represent two primary barriers for HIV-specific CD8 T cells to eradicate HIV infection. Combination antiretroviral therapeutic (cART) is extremely effective at decreasing viremia to an undetected level (32–34); however, the viremia rebounds soon after the cessation of treatment (35–37). These facts further suggest that exhausted HIV-specific CD8 T cells cannot efficiently inhibit residual viral replication in the presence of effective cART treatment. However, a small fraction of CD8 T cells has been discovered to appear in B cell follicles in HIV infection as early as 1980 and 1990s (38–43). In 2007, Quigley et al. also reported that early effector memory $CXCR5^+CD8$ T cells infiltrated into B cell follicles in human tonsil (44). Until recently, several groups reported a novel subset of exhausted HIV-specific CD8 T cells expressing CXCR5 and capable of migrating to B-cell follicles during HIV/SIV infection that rekindled interest in the filed (45–54). In this review, we focus on understanding the properties of HIV-specific CXCR5-expressing follicular cytotoxic cells and propose strategies for the functional cure of HIV infection by combining cART and $CXCR5^+CD8$ T cells.

IMMUNE EXHAUSTION OF VIRUS-SPECIFIC CD8 T CELLS DURING CHRONIC HIV INFECTION

In response to an acute viral infection, virus-specific CD8 T cells recognize viral peptide–MHC class I complexes presented on the surface of antigen-presenting cells and subsequently become activated by signals transduced from TCR complexes and co-stimulatory receptors (55–57). The activated CD8 T cells in turn undergo dramatic proliferation and differentiate into effector CD8 T cells that are capable of efficiently clearing virally infected target cells by secreting anti-viral cytokines, such as TNF-α and INF-γ, as well as cytotoxic molecules, including perforin and granzymes. In the case of mouse lymphocytic choromeningitis virus (LCMV)-Armstrong and human influenza infection, and in response to smallpox and yellow fever vaccines, a large number of effector CD8 T cells with potent anti-viral functions eventually eradicate infectious viral particles within 8–10 days (58–60). Consistent with the resolution of viral infection and inflammation, the majority (>90%) of virus-specific effector CD8 T cells die of apoptosis, while a small fraction of these effector cells will survive and progressively differentiate into memory CD8 T cells (61–63). Memory CD8 T cells possess a stem cell-like property, being able to maintain themselves long-term through antigen-independent self-renewal driven by the cytokines interleukin-7 and IL-15 (59, 64). Most importantly, quiescent memory CD8 T cells largely preserve the epigenetic modification features of genes associated with effector functions that are developed at the effector stage, allowing these cells to rapidly exert multiple effector functions and efficiently clear invaded viruses soon after re-infections occur (65–67).

In contrast to acute viral infection, the continuous stimulation by persistent viral antigens due to unresolved chronic viral infection leads to a distinct differentiated state of activated virus-specific CD8 T cells termed immune exhaustion (68–71). Distinct from memory CD8 T cells, exhausted CD8 T cells exhibit several unique features, including, but not limited to, reduced cell proliferation potential upon re-stimulation, enhanced turnover rate due to being more prone to apoptosis, programmed and hierarchal loss of the ability to secrete cytokines and release cytotoxic granule components, prolonged and enhanced expression of an array of inhibitory receptors, altered epigenetic and metabolic signatures, and a failure to further convert to traditional memory CD8 T cells (72–76). The exhaustion of CD8 T cells was first discovered in a mouse model of chronic infection with LCMV and later on confirmed in various chronic viral infections in human, such as HIV and the hepatitis C and B viruses (21, 68, 77–80).

Similar to chronic LCMV infection in mice, chronic HIV infection does not clonally delete HIV-specific $CD8^+$ T cells; instead, these cells also undergo a progressive and hierarchical loss of effector functions and display enhanced expression of a set of inhibitory receptors, such as programmed cell death-1 (PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), lymphocyte activation gene 3, and T cell immunoglobulin domain and mucin domain 3 (Tim-3), and a failure to differentiate into classical memory CD8 T cells evidenced by elevated apoptosis, diminished proliferation potential, and a rapid loss of CD127 expression (81–90). Moreover, at the genome-wide transcriptome level, similarities have been observed between exhausted LCMV-specific CD8 T cells and HIV-specific CD8 T cells (88, 91). The antigen load appears to be a critical cause that drives the development of these shared transcriptional signatures associated with CD8 T cell exhaustion in both chronic LCMV and HIV infection (23, 69, 92). The durable exposure to persistent antigen stimulation profoundly impacts the intrinsic epigenetic program and alters the expression mode of key transcriptional factors, such as T-bet, Eomes, TCF-1, Batf, and Id2-E2A, in exhausted LCMV- and HIV-specific CD8 T cells (23, 45, 72, 91, 93). The co-expression of inhibitory molecules, such as PD-1, CTLA4, and Tim-3, further promotes the extent of CD8 T cell exhaustion (22, 94–96). Additionally, the lack of optimal CD4 T cell help, at least partially mediated by IL-21 secreted from this population, represents another important factor for CD8 T cell exhaustion in both chronic LCMV and HIV infection (97–102). Furthermore, regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) may further corroborate the progress of CD8 T cell exhaustion (103–107). The general similar characteristics between exhausted LCMV- and HIV-specific CD8 T cells highlight the great value of murine LCMV chronic infection as an informative experimental system to explore and reveal novel aspects of CD8 T-cell immunity in chronic HIV infection, even though murine LCMV infection is not an ideal model for HIV virology.

Although exhausted CD8 T cells are unable to differentiate into classical memory T cells, they are also not all terminally differentiated cells, which is supported by the consequences of the partial rescue of proliferative potential and effector function of exhausted CD8 T cells by targeting the PD-L1/PD-1 inhibitory pathway with an antibody blockade both *in vivo* (mouse chronic LCMV infection and rhesus macaque chronic SIV infection)

and *in vitro* (co-culturing PD-L1 blockade antibodies with HIV-specific exhausted CD8 T cells) (108–110).

Furthermore, at the population level, exhausted CD8 T cells are not functionally inert and still maintain the critical ability to suppress viral replication during chronic LCMV and HIV infection (16–19, 111). The non-terminal differentiation state and partially preserved effector function of exhausted CD8 T cells provide precious opportunities for therapeutically targeting and reinvigorating exhausted CD8 T cells, which can possibly lead to the efficient control of chronic viral infection.

DIFFERENTIATION OF THE FOLLICULAR CXCR5-EXPRESSING CD8 T-CELL SUBSET DURING HIV INFECTION

Although exhausted, virus-specific CD8 T cells preserve a certain ability to mediate an imperative suppression of viral replication in both chronic LCMV and HIV infection (3, 112–114). Given that the majority of virus-specific CD8 T cells are functionally exhausted, it is of great interest to investigate whether the exhausted $CD8^+$ T cell pool contains a specific subset that are responsible for effectively keeping viral replication in check during chronic viral infection. Our recent study has found that during mouse chronic infection with the LCMV-Cl13 strain, but not acute infection with the LCMV-Armstrong strain, a unique subset of exhausted CD8 T cells expressing the chemokine receptor CXCR5 was differentiated (45). These virus-specific $CXCR5^+CD8$ T cells possess the ability to migrate into B-cell follicles. Furthermore, $CXCR5^+CD8$ T cells express lower levels of inhibitory receptors, such as PD-1, 2B4, and Tim-3, than their $CXCR5^-$ counterparts, and accordingly, these cells demonstrate more potent cytotoxicity than the $CXCR5^-$ subset. The Id2/E2A axis was found to play an important role in the generation of this subset. Specifically, E2A promotes the generation of this population while Id2 antagonizes this effect. In patients with chronic HIV infection, a virus-specific $CXCR5^+CD8$ T cell subset was also identified in blood and lymph nodes, and the number of HIV-specific $CXCR5^+CD8$ T cells inversely correlated with the viral load in blood. Similar to the scenario in chronic LCMV infection, HIV-specific $CXCR5^+CD8$ T cells also show up in the follicular zone (45). Furthermore, HIV-specific $CXCR5^+CD8$ T cells exhibit a reduction in Id2 expression compared to HIV-specific $CXCR5^-CD8$ T cells. These similar characteristics of $CXCR5^+CD8$ T cells during both chronic LCMV and HIV infection indicate that the differentiation of this unique subset might represent a common mechanism for defense against chronic viral infection.

Several other groups have also reported $CXCR5^+CD8$ T cell populations during chronic LCMV infection, SIV and HIV infection. In chronic SIV and HIV infection, these reports uniformly demonstrated the follicular localization of $CXCR5^+CD8$ T cells in lymphoid tissues (46, 47, 49, 53, 115, 116). The follicular location may depend on CXCR5 expression (117). However, in LCMV-Cl13 infection in mice, Im et al. found that the majority of these cells were localized in the T-cell zone (52), while we reported that these cells preferentially localized to the B-cell zone (45). This divergence remains an important issue to be further clarified and a possible explanation may be that Im et al. used antibody recognizing TCF-1 to stain $CXCR5^+CD8$ T cells. As TCF-1 is also highly expressed in T-cell zone residing naïve and memory T cells (118, 119), which may potentially cause false positive. Intra-vital multi-photon confocal microscopy represents a reliable tool to visualize the dynamics of follicular-residing lymphocytes in a real-time pattern, which may provide more solid evidence as to the exact locations of virus-specific $CXCR5^+CD8$ T cells in lymphoid tissues during chronic viral infection. Furthermore, both studies found that $CXCR5^+CD8$ T cells preserved a better proliferative potential than $CXCR5^-CD8$ T cells (45, 52). We also defined the continuous conversion of $CXCR5^+CD8$ T cells into $CXCR5^-CD8$ T cells during LCMV chronic infection in mice, which was likely driven by elevated Id2 expression in $CXCR5^+CD8$ T cells (45). The replenishment of this population critically depends on new emigrants from the thymus (45). It is worthwhile to investigate whether these features also hold true in chronic SIV and HIV infection, which can be determined by using non-human primate models and a bone marrow–liver–thymus humanized mouse model, respectively.

It should be noted that in chronic LCMV-Cl13 infection in mice, viruses seldom infect cells residing in B-cell follicles, while in chronic SIV and HIV infection, viruses predominantly and productively infect follicle-residing TFH cells (25, 120–122). Therefore, in LCMV-Cl13 infection, the antigen loads and inhibitory microenvironment in B-cell follicles are relatively friendly toward virus-specific $CXCR5^+CD8$ T cells, and B-cell follicles may function as a sanctuary for virus-specific $CXCR5^+CD8$ T cells to prevent the rapid loss of their number and effector functions. In contrast, in chronic SIV and HIV infection, viral replication is more concentrated in TFH cells in B-cell follicles (29, 120, 123). Therefore, the high antigen loads in B-cell follicles may drive the more severe exhaustion of follicle-residing HIV-specific $CXCR5^+CD8$ T cells. The enhanced strength and duration of TCR stimulation from high antigen loads cause the rapid loss of these exhausted cells by apoptosis, which may partially explain the scarcity of this subset in B-cell follicles in chronic SIV and HIV infection. PD-1 is a central mediator that negatively regulates the exhaustion of virus-specific CD8 T cells (81, 124). In chronic LCMV infection, virus-specific $CXCR5^+CD8$ T cells were found to express relatively lower PD-1 levels compared to virus-specific $CXCR5^-CD8$ T cells (45). In contrast, during chronic HIV infection, HIV-specific $CXCR5^+CD8$ T cells expressed higher levels of PD-1 than their $CXCR5^-$ counterparts (47). This divergence in PD-1 expression in virus-specific $CXCR5^+CD8$ T cells during chronic LCMV and HIV infection might be largely attributed to the different antigen load levels in B-cell follicles during chronic LCMV and HIV infection (**Figure 1**) (**Table 1**).

THE FUNCTIONALITY OF FOLLICULAR CXCR5-EXPRESSING CD8 T-CELL SUBSET DURING HIV INFECTION

In LCMV-Cl13 infection, compared to $CXCR5^-CD8$ T cells, virus-specific $CXCR5^+CD8$ T cells exhibit elevated effector cytokine

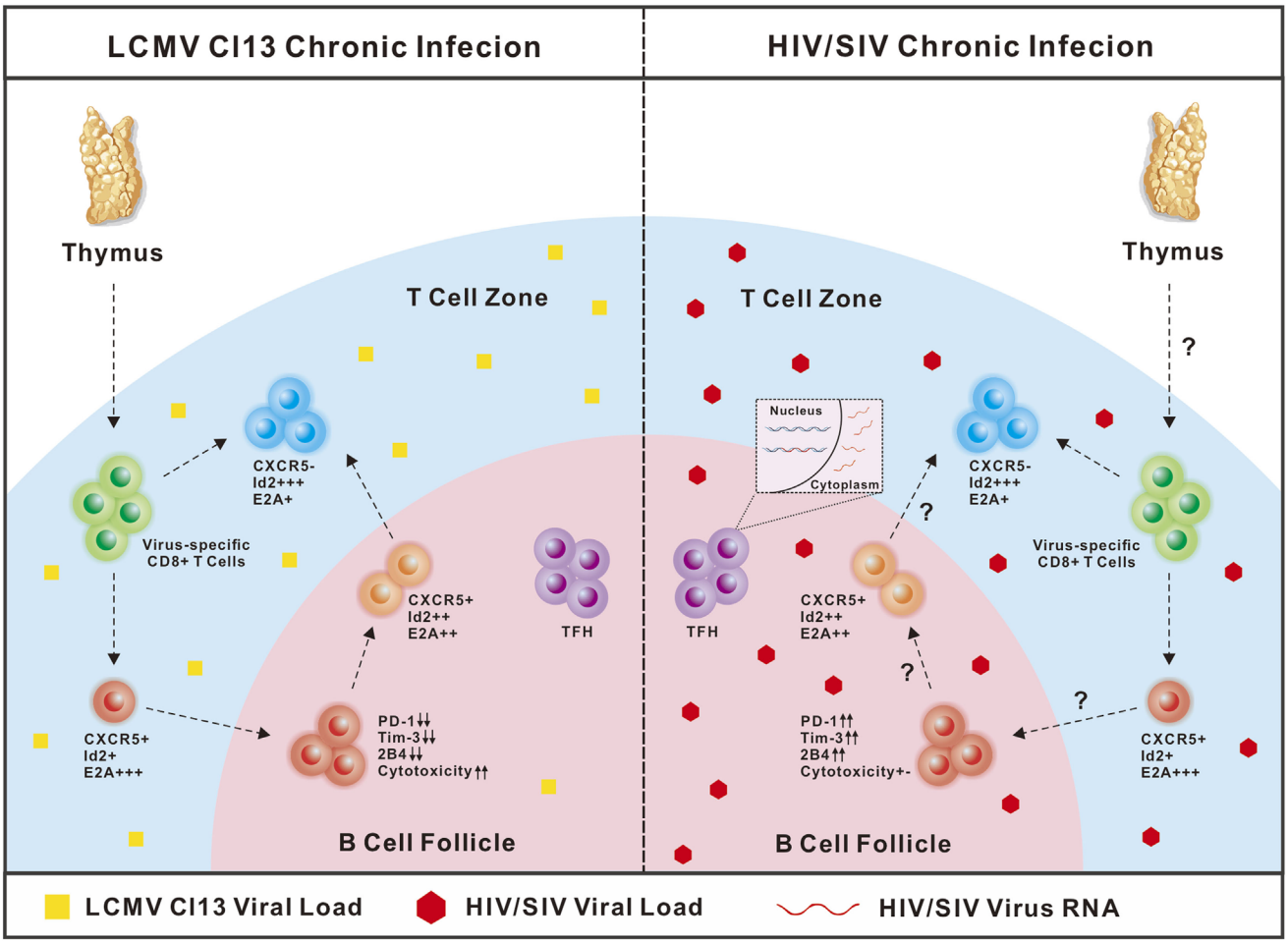

FIGURE 1 | Comparison of CXCR5⁺CD8 T cells in lymphocytic choromeningitis virus (LCMV)-Cl13 and human immunodeficiency virus (HIV) infection. In chronic LCMV-Cl13 infection, viruses seldom infect B-cell follicles, thus B-cell follicles function as a sanctuary for CXCR5⁺CD8 T cells to prevent rapid exhaustion. In contrast, HIV virus preferentially targets TFH cells in B-cell follicles for productive and latent infection, thus accumulating high antigen loads in B-cell follicles may drive more severe exhaustion of CXCR5⁺CD8 T cells.

TABLE 1 | The similarities and differences of CXCR5⁺CD8 T cells in lymphocytic choromeningitis virus (LCMV)-Cl13 and human immunodeficiency virus (HIV)/simian immunodeficiency virus (SIV) infection.

Similarities	• The number is inversely correlated with viral load in blood • The subset was identified in blood and lymphoid organs • Preserve a better proliferative potential than CXCR5⁻CD8 T cells • Transcription factor feature: Id2 ↓, E2A ↑ • Possess the ability to migrate into B cell follicles	
Differences	**Lymphocytic choromeningitis virus**	**SIV/HIV**
	Viruses seldom infect cells residing in B-cell follicles, providing a friendly microenvironment (low antigen load) for CXCR5⁺CD8 T cells	Viruses predominantly infect follicle-residing TFH cells leading to a nasty microenvironment (high antigen load) for CXCR5⁺CD8 T cells
	Lower programmed cell death-1 (PD-1) expression than CXCR5⁻ counterparts	Higher PD-1 expression than CXCR5⁻ counterparts
	More potent cytotoxicity than CXCR5⁻ counterparts (e.g. IFN-γ, TNF-α, and degranulation)	Controversial issue: enhanced or comparable effector functions relative to CXCR5⁻CD8 T cells?

expression, including IFN-γ and TNF-α, in response to antigen stimulation. They also display enhanced degranulation. Consistent with these characteristics, these cells are more efficient at killing target cells *in vivo* than the CXCR5⁻ counterparts. Furthermore, when adoptively transferred to CD8-deficient recipients chronically infected with LCMV-Cl13, virus-specific CXCR5⁺CD8 T cells, but not CXCR5⁻CD8 T cells, can effectively inhibit viral replication in recipients (45). Together, these results demonstrate

that virus-specific CXCR5$^+$CD8 T cells preserve better effector functions than CXCR5$^-$CD8 T cells in suppressing chronic viral infection. However, there are conflicting results regarding the functionality of CXCR5$^+$CD8 T cells in chronic HIV infection. Several reports have shown that compared to CXCR5$^-$CD8 T cells, CXCR5$^+$CD8 T cells show an increase in the production of IFN-γ, TNF-α, and perforin; enhanced degranulation and cytolytic activities (45, 46, 53, 115). In contrast, a recent study demonstrated the comparable production of cytolytic proteins between HIV-specific CXCR5$^+$CD8 T cells and CXCR5$^-$CD8 T cells in lymphoid tissues from patients with chronic infection, but much lower than that of HIV-specific CD8 T cells in blood (50). It should be noted that HIV-specific CXCR5$^+$CD8 T cells are still exhausted cells. Similar to LCMV-specific CXCR5$^+$CD8 T cells, these cells are most likely heterogeneous, consisting of newly recruited cells (less exhausted due to the short exposure time to antigens) from thymic outputs and past generated cells (more exhausted due to the concentrated viral replication in B-cell follicles). Whole body viral loads and disease progression might potentially influence the ratio between newly and past generated HIV-specific CXCR5$^+$CD8 T cells. Interestingly, Miles et al. reported that the majority of follicular CD8 T cells are regulatory CD8 T cells with the expression of CD44 and CXCR5. This regulatory subset expresses less perforin and high level of Tim-3 to inhibit IL-21 production by TFH cells and impairs GC function in SIV and *ex vivo* HIV infection (49). However, whether these regulatory CD8 T cells are SIV- or HIV-specific awaits further investigation. Furthermore, we may not rule out the possibility that certain subset of antigen-specific CD8 T cells become *de novo* CXCR5-expressing cells and be included in the analysis in responding to antigen stimulation. Furthermore, the more exhausted state of CXCR5$^+$CD8 T cells from more concentrated viral antigens in B-cell follicles may also explain their lower cytolytic activities when compared to total HIV-specific CD8 T cells in blood. Next, it is interesting to directly compare the functional capacity of HIV-specific CXCR5$^+$CD8 T cells from lymphoid tissues and blood (50).

Although there are conflicting results regarding the expression of inhibitory molecules and their functional capacities, it is a consensus that the number of HIV- or SIV-specific CXCR5$^+$CD8 T cells inversely correlates with plasma viremia and disease progression (31, 45, 48, 54, 116), highlighting a critical functional role of this subset in viral control during chronic SIV or HIV infection. Given the possible downregulated effector functions and cytolytic activities, this important characteristic of CXCR5$^+$CD8 T cells might be largely attributed to their non-terminal differentiation state and better-retained proliferative potential. Indeed, HIV-specific CXCR5$^+$CD8 T cells express less Id2 (promoting terminal differentiation) and higher TCF-1 (promoting memory differentiation and proliferative potential) than CXCR5$^-$CD8 T cells (45, 47, 51, 125). In a chronic LCMV-Cl13 infection model, overexpressing Id2 or ablating TCF-1 leads to the impaired generation of virus-specific CXCR5$^+$CD8 T cells and accordingly to increased viral loads (45, 47, 52). Moreover, virus-specific CXCR5$^+$CD8 T cells, but not CXCR5$^-$CD8 T cells, respond to the PD-1–PD-L1 pathway blockade and increase clonal expansion (45, 52). In chronic HIV infection, memory-like HIV-specific CXCR5$^+$CD8 T cells may persist longer than their CXCR5$^-$ counterparts at population levels and continuously kill virus-infected cells. By contrast, because B-cell follicle-residing TFH cells are major virus producers compared to other CD4 T cell types in the T cell zone in HIV infection (24, 120), it is reasonable to infer that HIV-specific CXCR5$^+$CD8 T cells, but not CXCR5$^-$CD8 T cells, have chances to come into contact with and kill these target cells. Therefore, HIV-specific CXCR5$^+$CD8 T cells primarily rely on their memory-like properties and unique anatomical location for their critical control of viral replication in the context of chronic HIV infection.

STRATEGIES FOR EMPLOYING CXCR5$^+$CD8 T CELLS TO PURGE HIV RESERVOIRS IN B-CELL FOLLICLES

It has been well-documented that virus-specific CD8 T cells are required for the elimination of HIV reservoirs (3, 112, 126). Accumulating evidence has demonstrated that TFH cells in B-cell follicles of lymphoid tissue serve a major HIV reservoir, as viruses preferentially target TFH populations for productive and latent infection (25, 26). Taking into account that a limited number and exhausted state of HIV- or SIV-specific CXCR5$^+$CD8 T cells were present in B-cell follicles, this unique strategy largely protects these viruses from the attacks mediated by virus-specific CD8 T cells. Indeed, in elite controllers from chronic SIV infection, SIV-specific CD8 T cells can effectively control viral replication at extra-follicular sites; however, the majority of these cells fail to migrate to B-cell follicles to clear SIV-producing TFH cells (19, 30, 31). In ART-treated, aviremic non-human primates and patients, lymph node PD-1$^+$TFH populations also serve as a major reservoir for active and persistent viral transcription (28). Thus, HIV reservoirs harbored in TFH cell populations in lymph node B-cell follicles represent a major obstacle for a functional cure for HIV infection. To this end, the appearance of a large number of HIV-specific CXCR5$^+$CD8 T cells equipped with potent cytotoxic functions is a prerequisite for effectively eliminating TFH reservoirs under cART treatment. Additionally, CXCR5$^+$CD8 T cells are not stable and will eventually convert into CXCR5$^-$CD8 T cells, which will exit B-cell follicles (45). Therefore, we speculate that the rational design of strategies for a functional cure for HIV infection will rely on the following three important aspects: (1) enhanced virus-specific CXCR5$^+$CD8 T cell differentiation, (2) preserved lineage stability, and (3) functional reinvigoration.

In chronic LCMV-Cl13 infection, we have shown a greater therapeutic potential for LCMV-specific CXCR5$^+$CD8 T cells than the CXCR5$^-$ subset upon adoptive transfer to chronically infected mice, as well as synergistic effects that reduce the viral load when combined with anti-PD-L1 treatment (45). In an SIV model or HIV patients, it is also worth testing the efficacy of this combination for suppressing HIV replication and latency in TFH cells in non-human primates or patients under ART treatment. Virus-specific CXCR5$^+$CD8 T cells, but not CXCR5$^-$CD8 T cells, are PD-1 pathway blockade responders (52). In this regard, PD-1 blockade antibodies can effectively expand transferred virus-specific CXCR5$^+$CD8 T cells and boost the effector functions

of these cells. Velu et al. demonstrated that during chronic SIV infection, PD-1 blockade resulted in rapid expansion of virus-specific CD8 T cells with improved functionality (127). However, the reduction of plasma viral load seemed not to be that impressive, which may be due to a very limited number of CXCR5$^+$CD8$^+$ T cells *in situ*. Therefore, the combination of PD-1 blockade with adoptive transfer of large number of virus-specific CXCR5$^+$CD8 T cells may further improve the control of viral replication. Furthermore, the PD-1 pathway blockade may also have partial effects on TFH cells, which express a high abundance of PD-1. The activation of TFH cells latently infected with viruses by the PD-1 antibody blockade may enhance the transcription of viral genes, which may increase antigenic exposure for cytotoxic killing due to the transfer of virus-specific CXCR5$^+$CD8 T cells. In addition to the adoptive transfer of *in vitro* expanded endogenous CXCR5$^+$CD8 T cells from blood, it is also possible to transfer genetically modified virus-specific CD8 T cells over-expressing transcriptional factors that promote the differentiation and lineage stabilization of CXCR5$^+$CD8 T cells, such as E2A and Bcl-6 (**Figure 2**). Besides, adoptive transfer of antiviral chimeric antigen receptor (CAR) T cells co-expressing the follicular homing chemokine receptor CXCR5 could potently suppress SIV replication *in vivo* (128). In addition to adoptive transfer, therapeutic vaccination plus the PD-1 pathway blockade may also boost the differentiation and functional rescue of virus-specific CXCR5$^+$CD8 T cells (129, 130). Furthermore, a recent study has demonstrated that a novel IL-15 agonist ALT-803 could activate and direct SIV-specific CD8 T cells into B cell follicles *via* upregulation of CXCR5 (131). Thus, the combination of IL-15 agonist and strategies mentioned above may offer a new immunotherapeutic agent for purging HIV reservoirs in B-cell follicles.

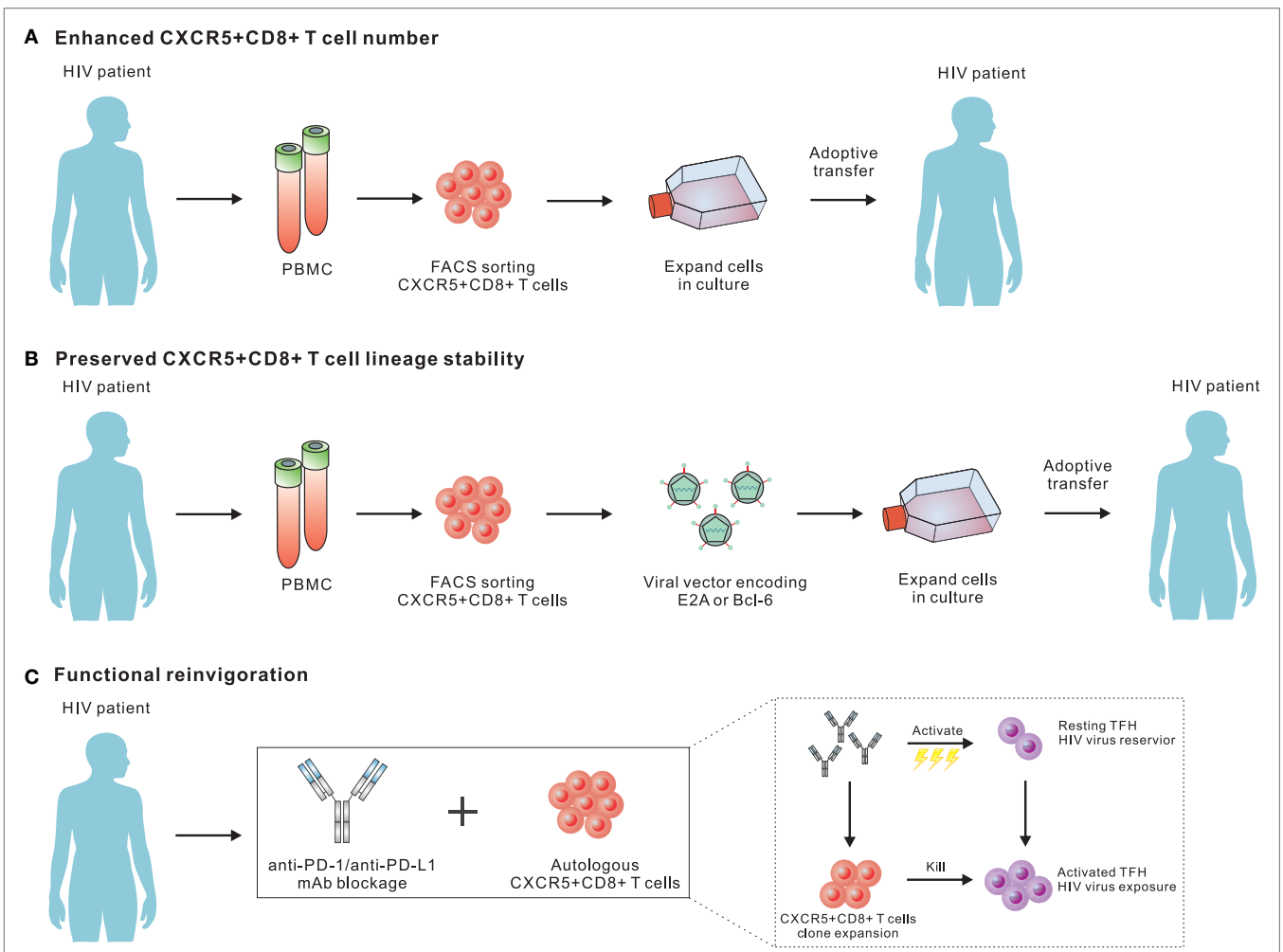

FIGURE 2 | Potential strategies for employing CXCR5$^+$CD8 T cells to purge human immunodeficiency virus (HIV) reservoirs in B cell follicles. **(A)** Adoptive transfer of *in vitro* expanded endogenous CXCR5$^+$CD8 T cells from blood. As CXCR5$^+$CD8 T cells from peripheral blood will further differentiate into CXCR5$^-$CD8 T cells upon antigen re-stimulation, the development of *in vitro* culturing conditions optimal for both expanding and preserving the migratory and functional characteristics of CXCR5$^+$CD8 T cells should be a focus for future investigations. **(B)** Transfer genetically modified virus-specific CD8 T cells over-expressing transcriptional factors that promote the differentiation and lineage stabilization of CXCR5$^+$CD8 T cells. **(C)** Programmed cell death-1 pathway blockade may effectively expand transferred virus-specific CXCR5$^+$CD8 T cells and boost the effector functions.

CONCLUSION

Based on the phenotypic, anatomic, and functional characterization of virus-specific CXCR5$^+$CD8 T cells in chronic viral infection, this subset has drawn immense attention and many new findings have been learned allowing us to better understand its features. Published data have firmly established the notion that this population is localized in B-cell follicles and plays a critical role in repressing viral load during chronic HIV infection. However, many important questions remain unanswered regarding the basic biology of this unique subset. For example, little is known regarding the origin and early fate commitment of this subset. Additionally, we barely know the cytokine milieu that is involved in the differentiation of this subset. Understand the characteristics of these cells will facilitate the optimization of *in vitro* culture conditions for the efficient expansion of these cells for therapeutic purposes.

It is also important to investigate whether and how these cells kill TFH cells that are actively transcribing viral RNAs in B-cell follicles during HIV infection. It is also of great interest to examine whether this population is required for the elimination of latently infected TFH cells after "shock and kill" intervention. Furthermore, we need to develop various immune strategies, such as vaccination, that can be utilized to efficiently induce and stabilize this population. Provided that the major reservoir is harbored in B-cell follicle TFH cells, and CXCR5$^+$CD8 T cells are the sole virus-specific population that has the chance and ability to gain access to these reservoirs, understanding the molecular mechanisms underlying the differentiation, migration, and function of this unique subset will definitely provide important insights that will allow us to harness this population for a functional cure against HIV infection.

AUTHOR CONTRIBUTIONS

MX, RH, and LY wrote and edited the manuscript. XC designed the figures.

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Dichotomous Roles of Programmed Cell Death 1 on HIV-Specific CXCR5$^+$ and CXCR5$^-$ CD8$^+$ T Cells during Chronic HIV Infection

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Background: CXCR5$^+$CD8$^+$ T cells have been demonstrated to play an important role in the control of chronic viral replication; however, the relationship between CXCR5$^+$CD8$^+$ T cells, HIV disease progression, and programmed cell death 1 (PD-1) expression profile on CXCR5$^+$CD8$^+$ T cells during HIV infection remain poorly understood.

Methods: We enrolled a total of 101 HIV patients, including 62 typical progressors, 26 complete responders (CRs), and 13 immune non-responders (INRs). Flow cytometric analysis, immunohistochemical staining, and relative function (i.e., cytokine secretion and PD-1 blockade) assays were performed to analyze the properties of CXCR5$^+$CD8$^+$ T cells.

Results: HIV-specific CXCR5$^+$CD8$^+$ T cells in the peripheral blood and distribution of CXCR5$^+$CD8$^+$ T cells in the lymph node (LN) were negatively correlated with disease progression during chronic HIV infection. PD-1 was highly expressed on CXCR5$^+$CD8$^+$ T cells and positively associated with peripheral CD4$^+$ T cell counts. Functionally, IFN-γ and TNF-α production of CXCR5$^+$CD8$^+$ T cells were reduced by PD-1 pathway blockade, but the production of IFN-γ and TNF-α from CXCR5$^-$CD8$^+$ T cells increased in response to TCR stimulation. Interestingly, PD-1 expression was constantly retained on CXCR5$^+$CD8$^+$ T cells while significantly decreased on CXCR5$^-$CD8$^+$ T cells after successful antiretroviral treatment in chronic HIV-infected patients.

Conclusion: PD-1$^+$CXCR5$^+$CD8$^+$ T cells are functional cytotoxic T cells during chronic HIV infection. PD-1$^+$CXCR5$^+$CD8$^+$ T cells may represent a novel therapeutic strategy for the disease.

Keywords: HIV, CXCR5$^+$CD8$^+$ T cells, programmed cell death 1, cytotoxic T cells, CXCR5$^-$CD8$^-$ T cells

INTRODUCTION

HIV-specific CD8$^+$ T cells play a critical role in controlling HIV replication and there is direct relationship between HIV-specific CD8$^+$ T cells and HIV control (1, 2). Emerging data suggest that if HIV-specific CD8$^+$ T cells can be effectively harnessed, HIV could be eliminated (3–5). Although HIV-specific CD8$^+$ T cells showed an exhausted phenotype to a certain extent during

chronic HIV infection, they can still inhibit viral replication. Some studies have shown that a subset of $CD8^+$ T cells expressing the chemokine receptor CXCR5 share some features of HIV-specific $CD8^+$ T cells and play a pivotal role in the control of viral replication during chronic viral infections (6–8). In addition, it was reported that $CXCR5^+CD8^+$ T cells exhibit a more potent proinflammatory function than $CXCR5^-CD8^+$ T cells during chronic HIV infections (6, 9). Despite these findings, the role of $CXCR5^+CD8^+$ T cells in the context of a chronic HIV infection, as well as the effect of ART on $CXCR5^+CD8^+$ T cells require further clarification.

One of the important characteristics of a chronic HIV infection is $CD8^+$ T cell dysfunction associated with the expression of the programmed cell death 1 (PD-1) inhibitory receptor (10–13). PD-1 is a central regulator of $CD8^+$ T cell exhaustion, and blockade of the PD-1 pathway has a beneficial effect on enhancing T cell immunity in chronic viral infections (10–12, 14–17). It has also been reported that a blockade of the PD-1 pathway did not completely restore T cell function (18–20); this means that other mechanisms impacting $CD8^+$ T cell functionality may exist. There are different opinions regarding the expression of PD-1 on $CXCR5^+CD8^+$ T cells during chronic viral infections. He et al. (6) reported lower levels of PD-1 expression on $CXCR5^+CD8^+$ T cells compared to $CXCR5^-CD8^+$ T cells during chronic viral infection, whereas other researchers (8, 9, 21, 22) reported a higher PD-1 expression on $CXCR5^+CD8^+$ T cells. $CXCR5^+CD8^+$ T cells share multiple characteristics with follicular helper T cells (TFHs; $CXCR5^+CD4^+$ T cells) (6, 8, 9, 21). PD-1 is highly expressed on TFHs and is a critical functional molecule for TFHs (23–25). Whether PD-1 is also a critical functional molecule for $CXCR5^+CD8^+$ T cells during chronic HIV infection remains unknown.

In this study, we enrolled HIV-infected typical progressors (TPs), antiviral therapy complete responders (CRs) and immune non-responders (INRs), and analyzed the $CXCR5^+CD8^+$ T cells from blood and lymphoid tissue specimens. The data showed that $CXCR5^+CD8^+$ T cells were negatively associated with HIV disease progression during chronic HIV infection. $CXCR5^+CD8^+$ T cells exhibited different PD-1 expression profile and response to PD-1 blockade compared to $CXCR5^-CD8^+$ T cells. Thus, $PD\text{-}1^+CXCR5^+CD8^+$ T cells can be classified as functional cytotoxic T cells (CTLs) during chronic HIV infection.

MATERIALS AND METHODS

Subjects

All samples were collected with the approval of the Beijing 302 Hospital Research Ethnics Committee and were written informed consent in accordance with the Declaration of Helsinki. The methods were carried out in accordance with approved guidelines and regulations. Peripheral blood for isolation of peripheral blood mononuclear cells (PBMCs) were obtained from HIV-infected patients. Lymph node (LN) biopsies were collected from nine treatment naïve patients. HIV status was defined according to previous reports (26, 27). Patients included a cohort of 62 TPs (who exhibited a typical progressive disease without receiving antiviral treatment), 26 CRs to antiviral therapy for more than 2 years with peripheral $CD4^+$ T cell counts above 350 cells/μL and plasma HIV-1 RNA <80 copies/mL, and 13 INRs to antiviral therapy for more than 2 years with $CD4^+$ T cell counts below 200 cells/μL and plasma HIV-1 RNA <80 copies/mL. Exclusion criteria included coinfection with HBV, HCV, tuberculosis, pregnancy, and moribund status (28). The detailed information for the donors is listed in **Table 1**.

Plasma HIV-1 RNA Monitoring

The HIV-1 RT-PCR Assay V2 (QIAGEN, Hilden, Germany) and CFX96 Real-Time System (Bio Rad Laboratories, Hercules, CA, USA) were used to quantify the HIV-1 RNA levels in plasma as previously described (11, 29). The cut-off value was 80 copies/mL.

Flow Cytometry

For phenotypic staining, PBMCs and milled LN cells were extracellularly stained using antibodies specific to respective markers, including anti-CD3-PerCP (BD Biosciences, Franklin Lakes, NJ, USA), anti-CD8-FITC (eBioscience, Waltham, MA, USA), anti-CXCR5-eFlour450 (eBioscience, Waltham, MA, USA), anti-PD-1-BV500 (BD Biosciences, Franklin Lakes, NJ, USA), and HIV Pentamer-PE (HIV-1 gag p17 76-84R, HIV-1 gag gp41 67-75R, and HIV-1 nef 72-82R) (Proimmune, Oxford, UK) for 30 min at room temperature. The cells were washed with FACS buffer, and assessed by flow cytometry (6). After extracellular staining, the cells were permeabilized, fixed and stained using the Permeabilization/Fixation Kit according to manufacturer's instructions (eBioscience, Waltham, MA, USA). The cells were then incubated for 30 min at 4°C with antibodies specific to

TABLE 1 | Subject characteristics.

	Typical progressors (TPs) (cells/μL)			CRs	INRs
	CD4 ≤ 200	200 < CD4 ≤ 350	CD4 > 350		
Cases (*n*)	17	25	20	26	13
Age (years)	30 (19–62)	32 (22–62)	26 (20–36)	34 (21–55)	35 (22–51)
Gender (M/F)	17/0	25/0	20/0	26/0	13/0
$CD4^+$ T (cells/μL)	152 (25–190)	270 (205–341)	441 (361–813)	456 (352–727)	140 (35–197)
Viral load (copies/mL)	77,371 (16,514–1,210,000)	44,373 (5,032–333,000)	20,990 (2,541–111,615)	<80	<80

Data are expressed as the median (range), unless otherwise stated.
CRs, complete responders; INRs, immune non-responders; M, male; F, female.

granzyme B-FITC (BD Biosciences, Franklin Lakes, NJ, USA) and perforin-Alexa647 (BD Biosciences, Franklin Lakes, NJ, USA).

After stimulated by overlapping peptides covering the HIV-1 pol, gag, and env antigens (JPT, Berlin, Germany) for 8 hrs with or without PD-L1(10 μg/mL) in the presence of brefeldin A and CD107a-eFluor660 (eBioscience, Waltham, MA, USA), extracellular stained, and permeabilized, the PBMCs or sorted cells were then incubated for 30 min at 4°C with antibodies specific to IFN-γ-eFlour506 (eBioscience, Waltham, MA, USA) and TNF-α-PE-Cy7 (eBioscience, Waltham, MA, USA). Flow cytometric acquisition was performed on a FACSVerse or Caliber.

Immunohistochemistry and Confocal Microscopy

Paraffin-embedded sections of acetone-fixed LN biopsies were incubated with anti-CD8, anti-CXCR5, and anti-CD20 antibodies overnight at 4°C after the endogenous peroxidase activity was blocked with 0.3% H_2O_2. 3-amino-9-ethyl-carbazole (red color) was used as the substrate followed by counterstaining with hematoxylin for single staining according to previously described protocols (30–32). Images (100×, 400×) were acquired with an Olympus CX31 microscope and Olympus FV1000 confocal microscope.

Cell Sorting

Positive selection was adopted for obtaining $CD8^+$ T cells from PBMCs using the MiniMACS system (Miltenyi Biotech, Bergisch-Gladbach, Germany) according to the manufacturer's instructions. $CD8^+$ T cells were then stained with anti-CD8-PE-Cy7 (BD Biosciences, Franklin Lakes, NJ, USA) and anti-CXCR5-Alexa Flour 488 (BD Biosciences, Franklin Lakes, NJ, USA) for 30 min at room temperature and sorted using a FACSAriaII. The purity of the sorted cells was >95% for all sorting experiments.

Killing Assay

To investigate the killing capacity of $CXCR5^+CD8^+$ T and $CXCR5^-CD8^+$ T cells, we co-cultured PBMCs, purified $CXCR5^+CD8^+$ T cells or $CXCR5^-CD8^+$ T cells with Jurkat cells [HIV-infected $CD4^+$ T cells (33)] integration of the HIV cDNA stimulated by overlapping peptides covering the HIV-1 pol, gag, and env antigens (JPT, Berlin, Germany). The total cells were stimulated by the peptide pools (1 μg/mL, 100 μL per sample) and brefeldin A for 8 h at 37°C in the presence of 5% CO_2 before conducting surface and intracellular staining (6). To evaluate the level of apoptosis, the cells were washed with FACS buffer and stained with CD4-APC (eBioscience, Waltham, MA, USA), 7-AAD-PerCP (BD Biosciences, Franklin Lakes, NJ, USA) and Annexin V-PE (Southern Biotech, Birmingham, AL, USA). Flow cytometric acquisition was performed on a FACSVerse or Caliber.

Statistical Analysis

All data were analyzed using SPSS version 22. A non-parametric Kruskal–Wallis test was used for multiple comparisons among different groups, and a Mann–Whitney *U* test was used for the comparison between two groups. A paired Student's *t*-test was adopted for the analysis with or without PD-L1. The correlations between variables were evaluated by a Spearman rank correlation test. For all tests, P values <0.05 indicated a significant difference (28).

RESULTS

HIV-Specific $CXCR5^+CD8^+$ T Cells Were Negatively Correlated with Disease Progression during Chronic HIV Infection

To investigate circulating $CXCR5^+CD8^+$ T cells, we first detected the frequency of total and HIV-specific $CXCR5^+CD8^+$ T cells. There was a small population of $CXCR5^+CD8^+$ T cells (**Figures 1A,B**) in healthy controls. The frequency of total $CXCR5^+CD8^+$ T cells was obviously increased in the HIV-infected patients compared with the healthy controls (**Figures 1A,B**). Among the $Pentamer^+$ CTLs, we clearly identified one population of $CXCR5^+CD8^+$ T cells, indicating that chronic HIV infection can induce HIV-specific $CXCR5^+CD8^+$ T cells. A correlation analysis demonstrated that there was a positive correlation between $CXCR5^+CD8^+$ T cells and peripheral $CD4^+$ T cell counts (**Figure 1C**; $r = 0.367$; $p = 0.003$). Furthermore, the increased amount of HIV-specific $CXCR5^+CD8^+$ T cells was associated with higher $CD4^+$ T cell counts (**Figures 1D,E** left), with better correlation coefficient and significance probability than that of $CXCR5^-CD8^+$ T cells (**Figure 1E** right). In addition, the absolute counts (**Figure 1F**) of peripheral HIV-specific $CXCR5^+CD8^+$ T cells were inversely correlated with the viral load. Thus, these data suggest that chronic HIV infection induced $CXCR5^+CD8^+$ T cells, which are associated with disease progression during chronic HIV infection.

$CXCR5^+CD8^+$ T Cells in LN Correlated with $CD4^+$ T Cell Counts

To visualize $CXCR5^+CD8^+$ T cells in the LN, immunohistochemical staining was performed using antibodies against CXCR5, CD8, and CD20. Double-positive staining of CXCR5 (dark blue) and CD8 (red) was defined as $CXCR5^+CD8^+$ T cells, CD20 was used for the identification of germinal center (GC). As shown in **Figure 2A**, the LNs from HIV-infected patients with low $CD4^+$ T cell counts (<200 cells/μL) exhibited an impaired lymphoid structure, including broken lymphoid follicles, few $CD8^+$ T cells, and enhanced tissue fibrosis. Moreover, few $CXCR5^+CD8^+$ T cells were found (**Figure 2A** left). By contrast, in the LNs from HIV-infected patients with $CD4^+$ T cell counts above 200 cells/μL, the lymphoid structure remained relatively intact, accompanied by normal lymphoid follicles and lymphocyte distribution (**Figure 2A** middle and right). There were more $CXCR5^+CD8^+$ T cells distributed in the LNs with higher $CD4^+$ T cell counts by quantitative analysis (**Figure 2B**). In addition, confocal images confirmed CXCR5 and CD8 double staining of T cells and the enhanced distribution of $CXCR5^+CD8^+$ T cells in the LNs from patients with higher $CD4^+$ T cell counts (**Figure 2C**). Both CD8 (**Figure 2D** left) and CXCR5 (**Figure 2D** middle) can be found in GCs, and also $CXCR5^+CD8^+$ T cells were localized in and out of GCs (**Figure 2D** right). Thus, consistent with the peripheral lymphocytes, patients of higher $CD4^+$ T cell counts exhibited more $CXCR5^+CD8^+$ T cells residing in the LN, where $CXCR5^+CD8^+$

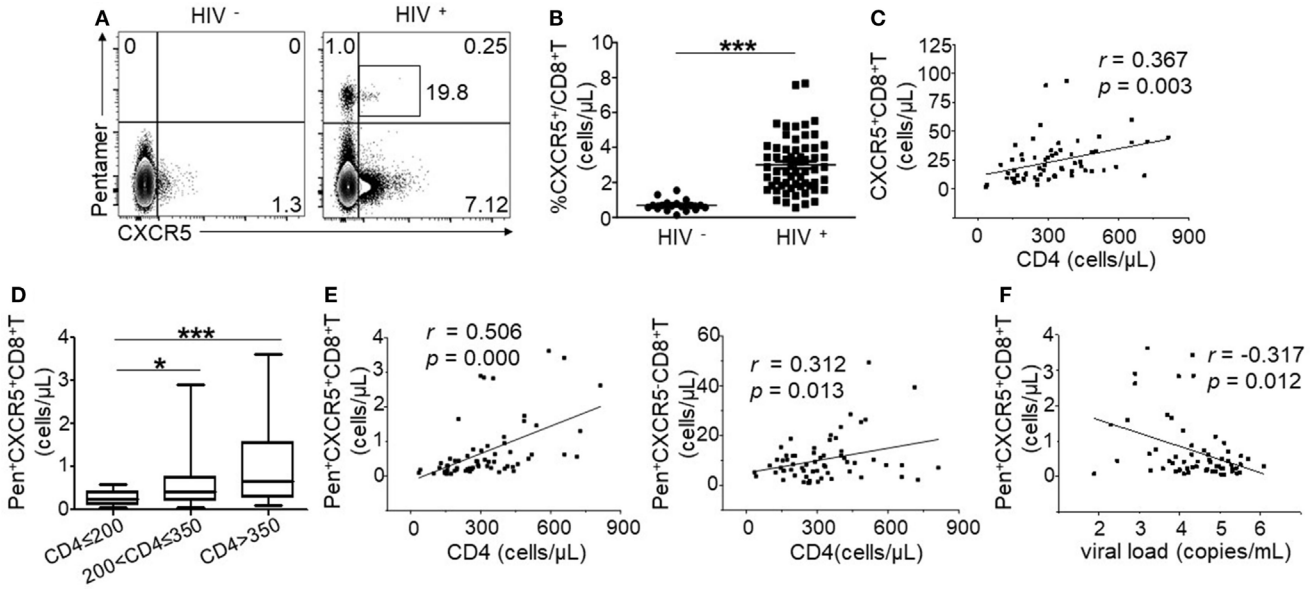

FIGURE 1 | HIV-specific CXCR5+CD8+ T cells are negatively correlated with disease progression during chronic HIV infection. **(A)** Representative flow cytometric data of CXCR5 and Pentamer staining from the peripheral blood of healthy controls and HIV-infected patients (gated on CD3+CD8+ lymphocytes). The values in the quadrant represent the frequency of individual populations. The number in the box represents the frequency of CXCR5+ expression among the Pentamer+ population. **(B)** Comparison of the percentage of CXCR5+CD8+ T cells between the healthy controls and typical progressers. **(C)** Positive correlation between peripheral CXCR5+CD8+ T cells and CD4+ T cell counts ($p = 0.003$). **(D)** Statistical analysis of the number of CD3+CD8+Pentamer+CXCR5+ T cells in individual groups with different CD4+ T cell numbers. Multiple comparisons between the different groups were made using a Kruskal–Wallis H non-parametric test (*$p < 0.05$, ***$p < 0.001$). **(E)** Positive correlation between the number of CD3+CD8+Pentamer+CXCR5+ T cells (left, $p = 0.0001$), CD3+CD8+Pentamer+CXCR5− T cells (right, $p = 0.0013$) and CD4+ T cell counts. **(F)** Negative correlation between the absolute cell number of CD3+CD8+Pentamer+CXCR5+ T cells and plasma viral load. Each dot represents one individual. The correlation analysis was evaluated *via* a Spearman rank correlation test. Solid line, linear growth trend; *r*, correlative coefficient. *p* values are shown.

T cells can be found in and out of GCs. One integrated LN and the relevant mononuclear cell was gotten, and the results of flow analysis showed that there were higher PD-1 expression on CXCR5+ T cells and HIV-specific CXCR5+ T cells than that of CXCR5− T cells (**Figure 2E**).

PD-1 Was Highly Expressed on CXCR5+CD8+ T Cells and Negatively Associated with HIV Disease Progression

Programmed cell death 1 is an important functional marker highly expressed on CD4+ follicular T cells; however, the function of PD-1 on CXCR5+CD8+ T cells remains controversial (7, 34). Thus, we analyzed the expression of PD-1 on the total and HIV-specific CXCR5+CD8+ T cells. Compared with CXCR5−CD8+ T cells, CXCR5+CD8+ T cells exhibited enhanced PD-1 expression (**Figure 3A** left), which was more evident in HIV-specific CTLs (**Figure 3A** right). Statistically analysis demonstrated that PD-1 exhibited a higher expression on CXCR5+CD8+ T cells compared to CXCR5−CD8+ T cells at various stages of disease progression (**Figure 3B**) for both the total and HIV-specific CTLs. Interestingly, PD-1 expression showed an exact opposite pattern on CXCR5− and CXCR5+CD8+ T cells during the disease progression. CXCR5−CD8+ T cells showed significantly lower PD-1 expression in patients with CD4+ T cell counts more than 350 cells/μL for both the total and Pentamer+CD8+ T cells (**Figure 3B**; $p < 0.05$), whereas CXCR5+CD8+ T cells exhibited elevated PD-1 expression in patients with higher CD4+ T cell counts (**Figure 3B**; $p < 0.05$). The higher frequency of PD-1+CXCR5+CD8+ T cells was associated with lower viral load ($r = -0.27$, $p = 0.033$; **Figure 3C** right), while there were tendency positively correlation between the frequency of PD-1+CXCR5+CD8+ T cells and CD4+ T cell counts ($r = 0.21$, $p = 0.100$; **Figure 3C** left). In addition, the mean fluorescence intensity (MFI) of PD-1 expression on HIV-specific CXCR5+CD8+ T cells increased with the elevation of peripheral CD4+ T cell counts (**Figure 3D**).

PD-1 Pathway Blockade Reduced IFN-γ and TNF-α Production of CXCR5+CD8+ T Cells in Response to TCR Stimulation

Considering a PD-1 pathway blockade as an immunological strategy to restore CTL function during chronic HIV infection (14, 17, 21), we investigated the effect of a PD-1 pathway blockade on both CXCR5− and CXCR5+CD8+ T cells in response to short-term TCR stimulation *in vitro*. After an 8-h stimulation using HIV-1 derived overlapping peptides with or without anti-PD-L1, the levels of IFN-γ, TNF-α, and CD107a were detected as functional markers of HIV-specific CTL responses. In agreement with previous reports (11), the PD-1 blockade significantly restored the production of IFN-γ and TNF-α (**Figures 4A,B**) in CXCR5−CD8+ T cells. Unexpectedly, unlike CXCR5−CD8+

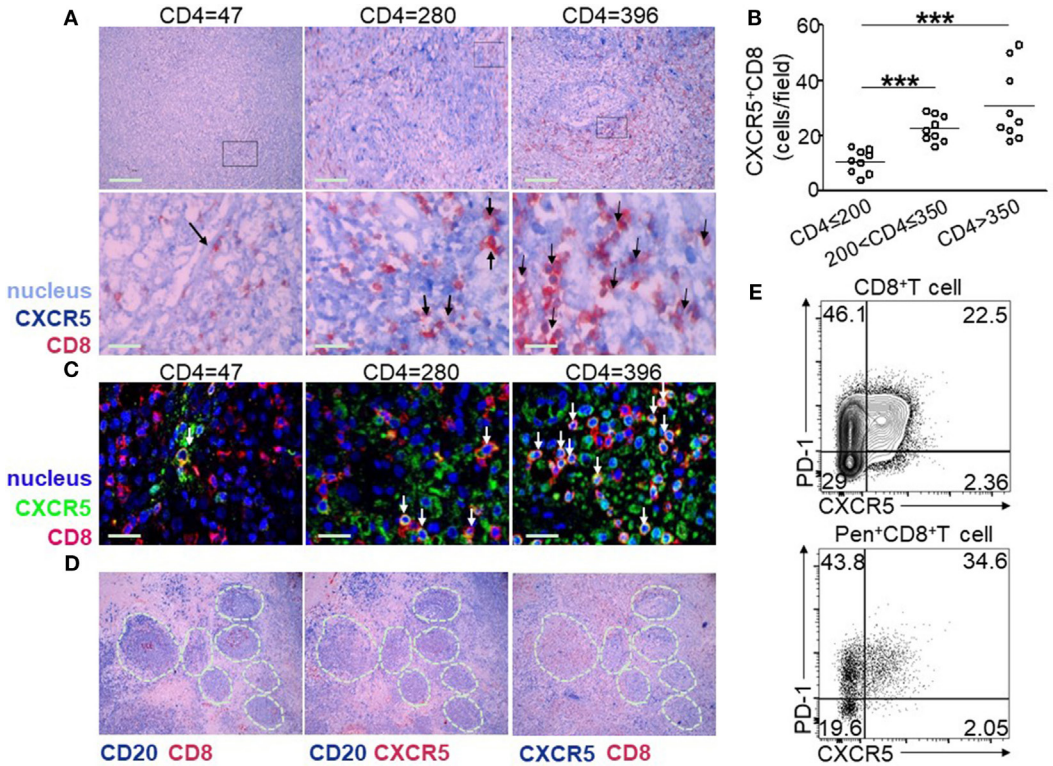

FIGURE 2 | Lymph node (LN) CXCR5+CD8+ T cells are associated with peripheral CD4+ T cell counts. **(A)** Representative immunohistochemical data show the tissue localization of CXCR5+ (dark blue) CD8+ (red) T cells (dark arrow) in the LNs from nine HIV-infected patients with different CD4+ T cell counts. The cell nuclei are stained light blue with hematoxylin. **(B)** Confocal microscopy of lymph nodes (LNs) stained with CXCR5+ (green) and CD8+ (red). The nuclei are stained using DAPI (blue). CXCR5+CD8+ cells are double stained in yellow (white arrow). **(C)** Statistical analysis of CXCR5+CD8+ T cells from three different patient groups. Each dot indicates one LN from one individual patient. The data represent three independent experiments with similar results ($n > 3$ for each group, Kruskal–Wallis H non-parametric test $^{***}p < 0.001$). **(D)** Immunohistochemical data of the same tissue of LNs with different markers: left-CD20 (dark blue) and CD8 (red), middle-CD20 (dark blue) and CXCR5 (red), and right-CXCR5 (dark blue) and CD8 (red). CD20+ cells are used for the identification of germinal centers (GCs; white dashed lines indicate GCs). **(E)** Representative flow cytometric data of programmed cell death 1 (PD-1) and CXCR5 staining gated on total CD8+ T cells and Pentamer+CD8+ T cells from the cells of LN.

T cells, the PD-1 pathway blockade inhibited the production of IFN-γ and TNF-α (**Figures 4A,B**) in CXCR5+CD8+ T cells, and the statistical analysis revealed a significant difference (**Figure 4B**). These results indicate that PD-1 expression exhibits opposing functions on CXCR5− and CXCR5+CD8+ T cells. In addition, CXCR5+CD8+ T cells produced more IFN-γ and TNF-α than CXCR5−CD8+ T cells after stimulation using HIV-1 overlapping peptides (**Figure 4B**). While for CD107a, the PD-1 blockade significantly increased the production of CD107a in CXCR5−CD8+ T cells, with no significantly changes in CXCR5+CD8+ T cells (**Figures 4A,B**). Consistent with previous reports (9), a significant fraction of CXCR5+CD8+ T cells produced granzyme B and perforin and granzyme B was significantly lower in CXCR5+CD8+ T cells compared with CXCR5−CD8+ T cells (**Figures 4C,D**).

To validate the function of PD-1 on CXCR5+CD8+ T cells, we purified CXCR5+ and CXCR5−CD8+ T cells from the PBMCs. As shown in **Figure 5A**, the purity of the individual populations was greater than 95%. In response to TCR stimulation, CXCR5+CD8+ T cells produced higher levels of IFN-γ, TNF-α, and CD107a than CXCR5−CD8+ T cells (**Figure 5B**); however, the PD-1 blockade exhibited the opposite effect on CXCR5+ and CXCR5−CD8+ T cells regarding the production of IFN-γ, TNF-α, and CD107a (**Figure 5B**). This finding was consistent with the data presented in **Figure 4**. In agreement with cytokine production, the killing capacity of the CXCR5+CD8+ T cells was greater than the CXCR5−CD8+ T cells (**Figure 5C**). The PD-1 blockade exhibited the opposite effect on CXCR5+and CXCR5−CD8+ T cells (**Figure 5C**). All these data provide evidence that PD-1+CXCR5+CD8+ T cells could be functional CTLs, rather than an exhausted CTL population during chronic HIV infection.

Compared CXCR5+CD8+ T and CXCR5− CD8+ T Cells between CRs and INRs

ART is associated with excellent efficacy for the clearance of peripheral HIV and the resultant immune reconstitution in the CRs. We analyzed CXCR5 expression on circulating total and HIV-specific Pentamer+ CTLs after ART. The number of CXCR5+CD8+ T cells and HIV-specific Pentamer+CXCR5- CD8+ T cells were no statistically significance between CRs and INRs (**Figure 6A**). Compared to INRs, ART result in the

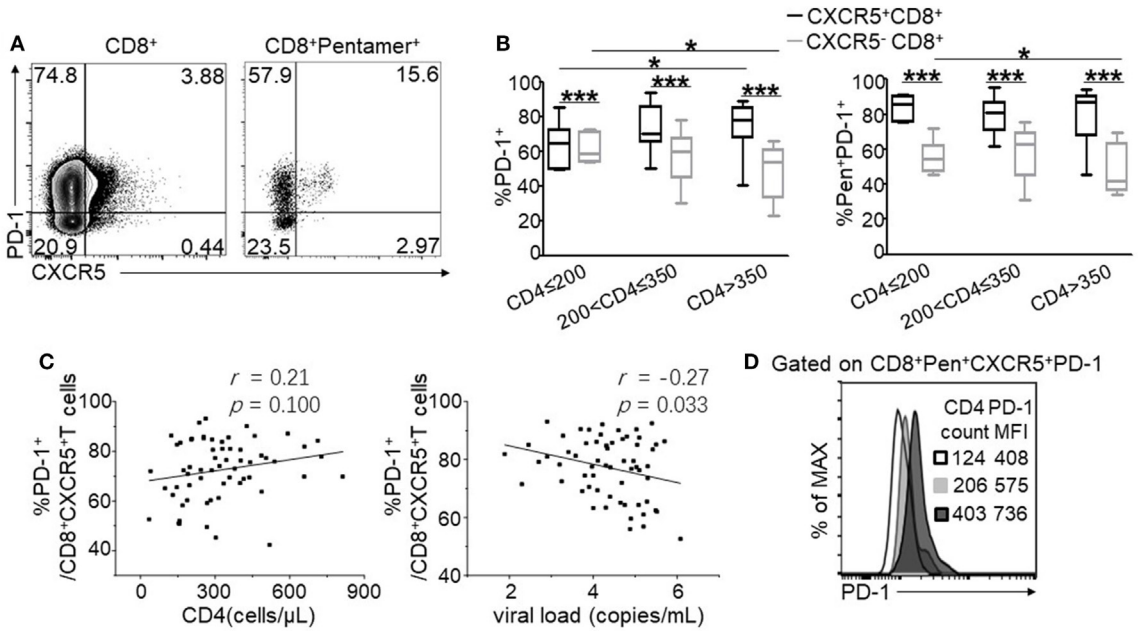

FIGURE 3 | Programmed cell death 1 (PD-1) is highly expressed on CXCR5+CD8+ T cells and negatively associated with HIV disease progression. **(A)** Representative flow cytometric data of PD-1 and CXCR5 staining gated on total CD8+ T cells and Pentamer+CD8+ T cells, respectively, from the peripheral blood. **(B)** Statistical analysis of PD-1 expression on CXCR5+ and CXCR5−CD8+ T cells in three groups categorized by CD4+ T cell counts. **(C)** Correlation between the percentage of CD3+CD8 +CXCR5+ PD-1+ T cells and CD4+ T cell counts (left, $p = 0.100$) or viral load (right, $p = 0.033$). **(D)** Representative data of PD-1 mean fluorescence intensity (MFI) on Pentamer+CXCR5+CD8+ T cells. The data represent three independent experiments with similar results ($n > 5$ for each group).

lower expression of PD-1 on total (**Figure 6B**, left) and HIV-specific Pentamer+CXCR5−CD8+ T cells (**Figure 6B**, right) in CRs; however, there were no difference of PD-1 expression on CXCR5+CD8+ T cells between INRs and CRs (**Figure 6B**). In addition, the fluorescence intensity of PD-1 was higher on CXCR5+CD8+ T cells in the CRs (**Figure 6C**). Functionally, the total population of CTLs from the CRs produced more IFN-γ and TNF-α in response to stimulation with HIV-derived overlapping peptides (**Figure 6D**). Thus, ART retained the quantity of circulating CXCR5+CD8+ T cells but failed to restore the function of HIV-specific CXCR5+CD8+ T cells in the INRs.

DISCUSSION

In this study, we found that HIV-induced CXCR5+CD8+ T cells correlated with immune control during chronic HIV infection. Unlike CXCR5−CD8+ T cells which used PD-1 as an exhaustion marker, CXCR5+CD8+ T cells exclusively retained high PD-1 expression. In addition, high PD-1 expression was associated with CXCR5+CD8+ T cells functionality. A PD-1 blockade inhibited rather than enhanced the functionality of CXCR5+CD8+ T cells. Thus, PD-1+CXCR5+CD8+ T cells may be regarded as a functional population during chronic HIV infection.

CXCR5+CD8+ T cells are induced during chronic HIV or SIV infections (6, 9). In this study, the correlation analysis between CXCR5+CD8+ T cells and disease progression in TPs revealed that CXCR5+CD8+ T cells, especially HIV-specific CXCR5+CD8+ T cells, was positively correlated with peripheral CD4+ T cell counts and negatively correlated with the viral load. CXCR5+CD8+ T cells mainly exist in lymphoid tissues and exhibited strong HIV-specific CTL function. In addition, lymphoid tissue is an important site for HIV replication (35). This may be the reason of negative correlation between CXCR5+CD8+ T cells and HIV disease progression. These findings indicated that CXCR5+CD8+ T cells may be involved in disease control and could be used as an immunological marker during a chronic HIV infection.

Moreover, the distribution of CXCR5+CD8+ T cells in the LNs of HIV-infected patients was analyzed using immunohistochemistry. Although it has been reported that antiviral CD8+ T cells have a limited capacity to migrate to the GCs of the lymphoid tissue in infected patients (36–38), we found that CXCR5+CD8+ T cells were found to be localized in and out of the GCs. Ayala VI (39) demonstrated that engineered CD8 T cells expressing human CXCR5 preferential localization within B-cell follicles. Petrovas et al. (22) observed higher frequencies of CXCR5hiCD8+ T cells in GCs compared to T cell areas. During chronic SIV infection, SIV-specific CXCR5+CD8+ T cells have been identified within the extrafollicular and intrafollicular regions of the lymphoid tissue (37). All these results indicate that CXCR5+CD8+ T cells could enter the GC during HIV infection.

Programmed cell death 1 is considered to be an exhaustion marker expressed on CD8+ T cells during chronic infection or tumor progression (10–12, 14–17); however, PD-1 expression on CXCR5+CD8+ T cells during chronic HIV infection remains poorly defined. In this study, we found that high expression of PD-1 was maintained on CXCR5+CD8+ T cells and HIV-specific

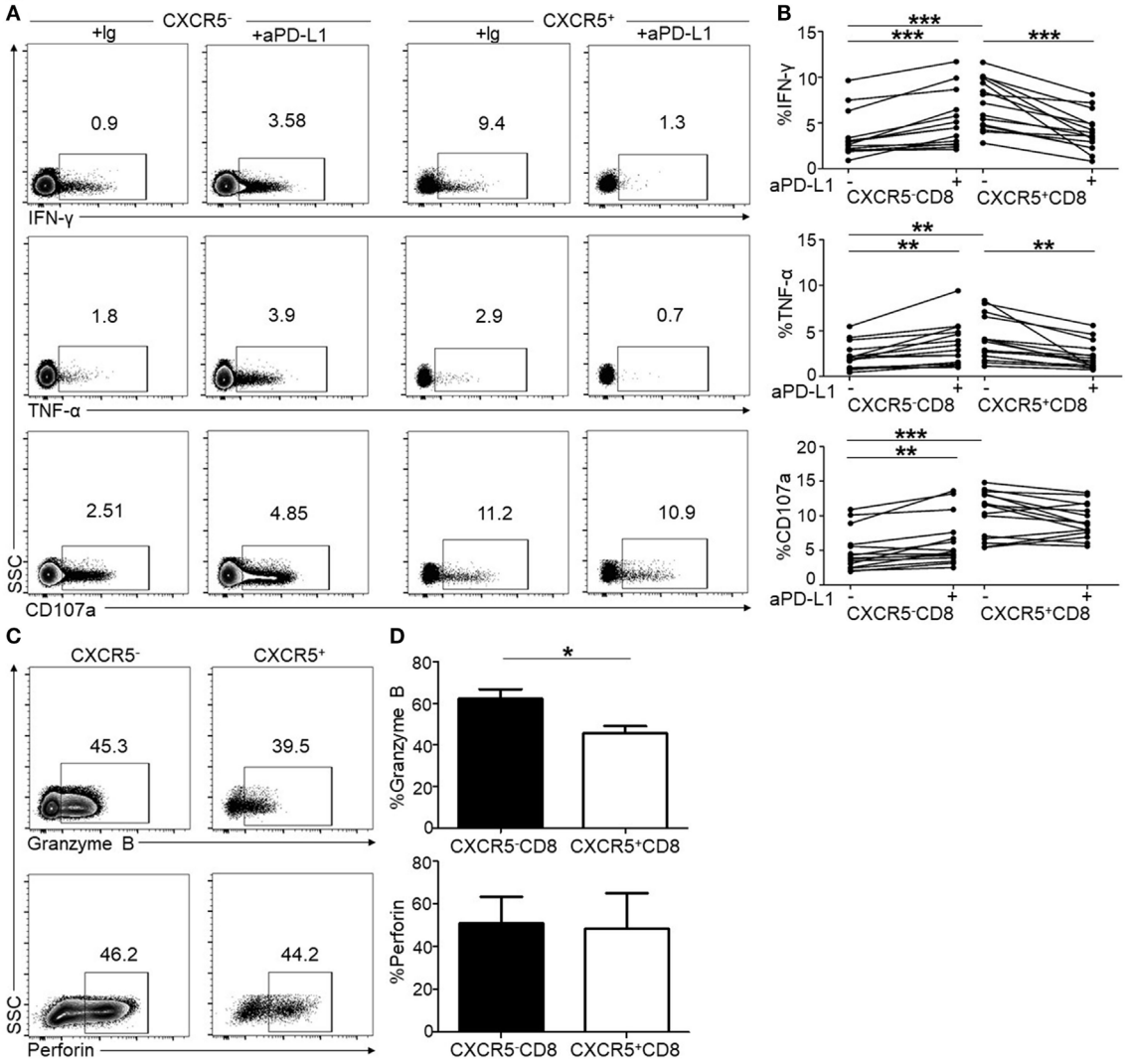

FIGURE 4 | Programmed cell death 1 (PD-1) blockade shows opposite effects on CXCR5⁻ and CXCR5⁺CD8⁺ T cells in peripheral lymphocytes. Peripheral blood mononuclear cells (PBMCs) from HIV-infected patients were stimulated for 8 h using HIV overlapping peptides with or without anti-PD-L1. The flow cytometric plots show intracellular IFN-γ **(A)**, TNF-α **(A)**, and CD107a **(A)** staining on CXCR5⁻ and CXCR5⁺CD8⁺ T cells populations, respectively. **(B)** Statistical analysis of intracellular IFN-γ, TNF-α, and CD107a staining of CXCR5⁻ and CXCR5⁺CD8⁺ T cells (a paired *t*-test was used, $^{**}p < 0.01$; $^{***}p < 0.001$). **(C)** The flow cytometric plots of intracellular granzyme B and perforin. **(D)** Statistical analysis of granzyme B and perforin staining of CXCR5⁻ and CXCR5⁺CD8⁺ T cells (a paired *t*-test was used, $^{*}p < 0.05$).

CXCR5⁺CD8⁺ T cells during chronic HIV infection. Moreover, the increased PD-1 expression on CXCR5⁺CD8⁺ T cells was associated with higher CD4⁺ T cell counts. Functionally, the CXCR5⁺CD8⁺ T cells produced more IFN-γ, TNF-α, and CD107a than CXCR5⁻CD8⁺ T cells in response to short-term TCR stimulation *in vitro*. This result is in agreement with other reports (6, 8, 22, 40). These findings indicate that CXCR5⁺CD8⁺ T cells are functional CTLs. Moreover, a blockade of the PD-1 pathway inhibited rather than enhanced the production of IFN-γ and TNF-α by CXCR5⁺CD8⁺ T cells, indicating that PD-1 expression was a functional marker for CXCR5⁺CD8⁺ T cells. Follicular TFHs are characterized by high expression of PD-1 which is also a critical functional molecule for TFHs (23–25). Both TFHs and CXCR5⁺CD8⁺ T cells are main located in lymphoid tissue, CXCR5⁺CD8⁺ T cells in peripheral blood have some common characteristics with CXCR5⁺CD8⁺ T in LNs, whether the microenvironment in lymph tissue leads to the different PD-1 profile between CXCR5⁺CD8⁺ T cells and CXCR5⁻CD8⁺ T cells worth further studying. In addition, Nakamoto et al. also found that HCV-specific CD8 T cells restoration was different in tissues and peripheral blood after PD-1 pathway blocking (41). However, Petrovas et al. (22) observed increased production of IFN-γ and TNF-a from CXCR5⁺CD8⁺ T cells after PD-1 pathway inhibition. There are some differences between Petrovas's study and this study: differences in patients, tissue, stimulus, and culture conditions. The functional changes of CXCR5⁺CD8⁺ T cells after

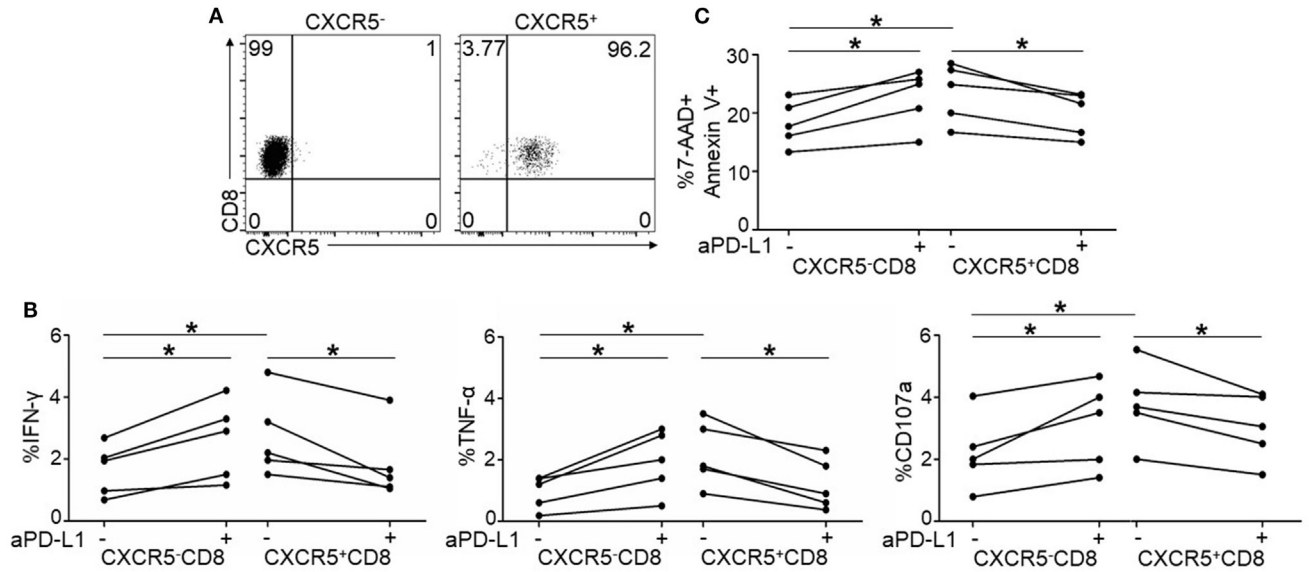

FIGURE 5 | Purified CXCR5+CD8+ T cells exhibit different response to programmed cell death 1 (PD-1) blockade from CXCR5−CD8+ T cells. **(A)** Representative flow cytometric data of FACS sorted CXCR5-(left) and CXCR5+CD8+ T cells (right). Data are representative of five individual samples. **(B)** Purified CXCR5− and CXCR5+CD8+ T cells from HIV-infected patients were stimulated for 8 h using HIV overlapping peptides with or without anti-PD-L1. Statistical analysis of intracellular IFN-γ (left), TNF-α (middle), and CD107a (right) staining on CXCR5− and CXCR5+CD8+ T cells (*$p < 0.05$). **(C)** Purified CXCR5− or CXCR5+CD8+ T cells from HIV-infected patients were co-cultured with Jurkat cells for 8 h with or without anti-PD-L1. Annexin V and 7-AAD staining was performed to show the killing functionality of the cytotoxic T cells (CTLs). Apoptotic Jurkat cells were compared between the different patient groups ($n = 5$ for each group).

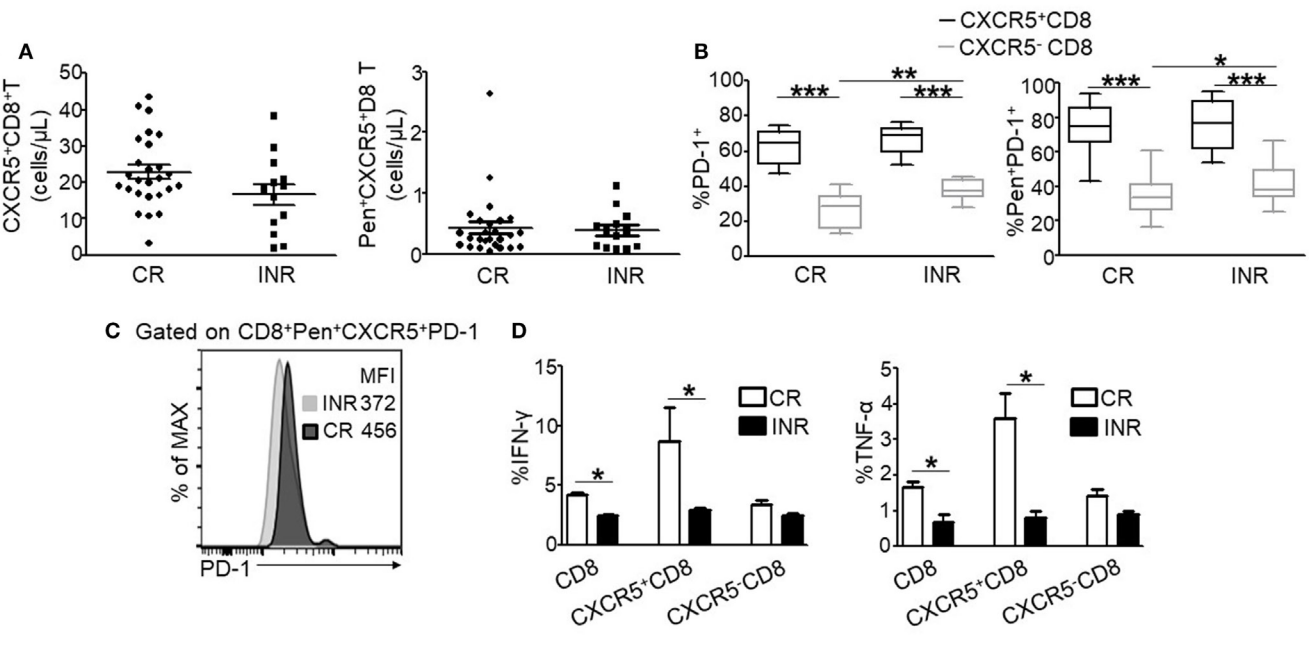

FIGURE 6 | ART treatment fails to restore the function of HIV-specific CXCR5+CD8+ T cells in immune non-responders (INRs) patients. **(A)** Comparing the number of CXCR5+CD8+ T cells (left) and Pen+CXCR5+CD8+ T cells (right) between the complete responders (CRs) and INRs. **(B)** Comparison of programmed cell death 1 (PD-1) expression on CXCR5− and CXCR5+CD8+ T cells gated on the total CD8+ (left) and Pentamer+ CD8+ T cell (right) populations, respectively ($n = 26$ for CRs; $n = 13$ for INRs). **(C)** Representative data of PD-1 mean fluorescence intensity (MFI) on Pentamer+CXCR5+CD8+ T cells from CRs and INRs. The data represent three independent experiments with similar results ($n > 3$ for each group). **(D)** IFN-γ (left) and TNF-α (right) production on the total, CXCR5−, or CXCR5+CD8+ T cells stimulated for 8 h using HIV-derived overlapping peptides *ex vivo* ($n > 8$ for each group). Each dot represents one individual patient. Statistical significance between two groups was determined by a Mann–Whitney non-parametric *U* test.

PD-1 blockade in HIV-infected patients need to be intensive studied in a large number of patients. In addition, in this study, we just detected the effect of blockaded PD-1 for a short time on HIV-specific CXCR5$^+$CD8$^+$ T cells, it is also an important issue to know the effect of blockaded PD-1for a long time on HIV-specific CXCR5$^+$CD8$^+$ T cells. In this study, we found that the ability of CXCR5$^+$CD8$^+$ T cells to produce perforin and granzyme B was lower than that of CXCR5$^-$CD8$^+$ T cells without PD-1 pathway blockade, and this result is consistent with some previous reports (8, 9, 21). Killing experiment showed that the purified CXCR5$^+$CD8$^+$ T cells had stronger killing ability to target cells than CXCR5$^-$CD8$^+$ T cells. Our results showed that the ability of CXCR5$^-$CD8$^+$ T cells secreting granzyme B was stronger than that of CXCR5$^+$CD8$^+$ T cells, which suggests that the killing function of CXCR5$^+$CD8$^+$ T cells may not be *via* granzyme B pathway.

Unlike TFH cells and PD-1$^+$CD4$^+$ T cells, which are the main HIV reservoirs during ART (38, 42, 43), CD8$^+$ T cells are not infected by HIV. Thus, functional PD-1$^+$CXCR5$^+$CD8$^+$ T cells may be utilized to clear the infected target cells in lymphoid tissues. HIV-specific CD8$^+$ T cells play an important role in eliminating latent HIV and HIV reservoirs (43). A PD-1 blockade may enhance the function of exhausted CXCR5$^-$CD8$^+$ T cells; however, PD-1 exhibited the opposite function on CXCR5$^+$CD8$^+$ T cells in this study. The mechanism of dichotomous functions for PD-1 on CXCR5$^+$ and CXCR5$^-$CD8$^+$ T cell needs further study. Of note, the PD-1 blockade increased the CXCR5$^+$CD8$^+$ T cell conversion into a CXCR5$^-$CD8$^+$ T cell (9, 21). Thus, these findings imply that a PD-1 blockade alone may be an inappropriate therapeutic strategy, and it is necessary to maintain the function of CXCR5$^+$CD8$^+$ T cells as well as the CXCR5$^-$CD8$^+$ T cell subset, as CD8$^+$ T cells expressing CXCR5 are redirected into LN follicles, which are important areas of HIV reservoir.

In this study, we first compared CXCR5$^+$CD8$^+$ T cells between CRs and INRs. Consistent with previous reports (11, 21), PD-1 expression on CXCR5$^-$CD8$^+$ T cells in INRs was higher than that in CRs, which was the opposite for CXCR5$^+$CD8$^+$ T cells. Functionally, HIV-specific CTLs, especially CXCR5$^+$CD8$^+$ T cells from CRs produced greater levels of IFN-γ and TNF-α in response to HIV peptide stimulation, indicating that ART failed to restore the function of HIV-specific CXCR5$^+$CD8$^+$ T cells in INRs.

In conclusion, we demonstrated that CXCR5$^+$CD8$^+$ T cells were negatively associated with HIV disease progression and PD-1$^+$CXCR5$^+$CD8$^+$ T cells were functional CTL population during chronic HIV infection. Furthermore, the functional PD-1$^+$CXCR5$^+$CD8$^+$ T cells failed to be restored in INRs compared to CRs. Our findings indicate that to specifically boost the function of PD-1$^+$CXCR5$^+$CD8$^+$ T cells represent a novel therapeutic strategy for AIDS patients.

AUTHOR CONTRIBUTIONS

Y-MJ, H-GY, LY, and F-SW conceived the study, designed the experiments, and analyzed the data; Y-MJ, H-GY, BT, S-JX, and LM performed the experiments; H-HH, BT, S-JX, LM, WX, RH, J-YZ, R-NX, LJ, MS, ZX, E-QQ, X-CW, and HW contributed to reagents and materials; and Y-MJ, H-GY, S-JX, and F-SW wrote the article. All authors read and approved the final manuscript.

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REVIEW
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Regulatory T Cells As Potential Targets for HIV Cure Research

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T regulatory cells (Tregs) are a key component of the immune system, which maintain a delicate balance between overactive responses and immunosuppression. As such, Treg deficiencies are linked to autoimmune disorders and alter the immune control of pathogens. In HIV infection, Tregs play major roles, both beneficial and detrimental. They regulate the immune system such that inflammation and spread of virus through activated T cells is suppressed. However, suppression of immune activation also limits viral clearance and promotes reservoir formation. Tregs can be directly targeted by HIV, thereby harboring a fraction of the viral reservoir. The vital role of Tregs in the pathogenesis and control of HIV makes them a subject of interest for manipulation in the search of an HIV cure. Here, we discuss the origin and generation, homeostasis, and functions of Tregs, particularly their roles and effects in HIV infection. We also present various Treg manipulation strategies, including Treg depletion techniques and interventions that alter Treg function, which may be used in different cure strategies, to simultaneously boost HIV-specific immune responses and induce reactivation of the latent virus.

Keywords: regulatory T cells, FoxP3, human immunodeficiency virus, simian immunodeficiency virus, lymph node, virus eradication, cytotoxic T lymphocytes

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INTRODUCTION

The human immune system walks a fine line between protection from pathogens and self-reactivity. These functions are mediated by both the innate and adaptive immune responses, such that all immune cells, from monocytes and natural killer cells to B and T lymphocytes, play integral roles in protection. Yet, a major function of the immune system, regulation, and self-tolerance, was not well understood for a long time. Gershon and Kondo in 1970 first described a population of thymus-derived lymphocytes, which were responsible for the induction of tolerance in bone marrow-derived lymphocytes (1). However, the mechanism of action and the cellular characteristics of these cells were not studied in detail until 1995, when Sakaguchi et al. reported that the $CD25^{hi}$ $CD4^{+}$ T cell subset has an immunoregulatory function and helps defend against the development of autoimmunity, rekindling the interest in this regulatory population (2). These cells have since been termed T regulatory cells (Tregs), and their immunosuppressive functions have been extensively investigated over the past 23 years. We now understand that Tregs are essential

for proper homeostasis of the immune system and regulation of self-tolerance. While clearly playing a significant role in the pathogenesis of HIV infection, there is still debate in the field of whether Tregs are a boon or bane to fighting the virus. More recently, Tregs were reported to be involved in the HIV reservoir seeding, maintenance, and control of reactivation. In this review, we discuss Tregs and their roles during HIV infection, with emphasis on their role in viral persistence, and strategies for Treg manipulation that may have an impact for an HIV cure.

TYPES OF Tregs

Based on their site of differentiation, Tregs can be classified into thymic Tregs (tTregs) and peripheral Tregs (pTregs). The differences in the differentiation of tTregs and pTregs have been described in detail by Lee et al (3).

Separation based on their immunophenotypes identified numerous distinct Treg subpopulations (4) (**Table 1**). Treg classification through other methods, such as mass cytometry, also showed that they form a very diverse population, with up to 22 different Treg subsets being identified (5). In this section, we only focus on the key Treg subsets for which both immunophenotypes and function were well characterized.

Forkhead box P3 (FoxP3) is the key marker and master regulator of Tregs (6). In fact, Tregs are defined to have a $CD25^{hi}$ $FoxP3^{+}$ $CD4^{+}$ phenotype. The importance of this protein was discovered when mutations in the *foxp3* gene that codes for FoxP3 were shown to cause the X-linked recessive disease, scurfy, in mice. Scurfy presents as lymphoproliferation leading to fatal autoimmunity, and mimics X-linked autoimmunity-allergic dysregulation syndrome in humans (7). Scurfy mice administered with stable Tregs, defined by FoxP3 expression and full suppressive functionality, did not develop any signs of the disease (8). FoxP3 expression can also be transiently induced following *in vitro* stimulation of nonsuppressive $CD25^{neg}$ $CD4^{+}$ T cells, which indicates that expression of FoxP3 alone is not responsible for the regulatory activity of T cells (52).

Thymic Tregs are defined by the expression of CD25 and FoxP3 on $CD4^{+}$ T cells. It has been shown that $CD25^{hi}$ $CD4^{+}$ Treg cells develop from self-reactive thymic cells that express a T cell receptor (TCR) with high affinity for self-antigens. Differentiation occurs as an alternative mechanism to apoptosis, such that self-antigen reactivity can induce an inhibitory response instead of an autoimmune response (53). Upon TCR interaction with these peptide-major histocompatibility complex (MHC) complexes, FoxP3 is induced in the immature thymocytes (54). However, FoxP3 expression is not sufficient to create a stable Treg. Demethylation of the FoxP3 locus in the Treg-specific demethylated region (TSDR) is required to generate stable tTregs (55). In addition, CpG hypomethylation of certain loci called "Treg cell representative regions" is imprinted in Tregs, also contributing to their stability (56). Interactions between B7 molecules (CD80 and CD86), expressed on the antigen-presenting cells (APCs), and CD28, on thymocytes, are co-stimulatory and are critical to the thymic development of Tregs, as evidenced by the severe decrease in Treg numbers in mice either deficient in CD28 or treated with a blocking anti-B7 antibody (15, 57, 58). Interleukin-2 (IL-2), the central cytokine involved in Treg biology, is also essential for tTreg maturation (59).

TABLE 1 | Key immunophenotypic markers/molecules and cytokines expressed by T regulatory cells (Tregs) and their function.

	Function in Tregs	Reference
Marker/molecule		
CD25	Receptor for IL-2, essential for Treg function and maintenance	(2)
FoxP3 (forkhead box P3)	Co-ordinates expression of various genes required for Treg activity	(6–8)
CD127 (Low)	Receptor for IL-7	(9, 10)
CTLA-4 (Cytotoxic T lymphocyte antigen-4)/CD152	Ablates CD28 costimulation by competitive binding to CD80 and CD86. Upregulation of IDO production by DCs	(11–14)
CD28	Development and maturation, activation induced Treg markers and expression of CCR6	(15, 16)
PD1 (Programmed cell death-1)	Binds to PD-L1, inhibits proliferation and effector responses of lymphocytes	(17–19)
ICOS (Inducible costimulator)/CD278	Controls expansion and maintenance of the $Foxp3^{+}$ regulatory T cells, and IL-10 production	(20–22)
LAG-3 (Lymphocyte activation gene-3)/CD223	Plays an important role during IL-27-mediated enhanced Treg function	(23)
GITR (Glucocorticoid-induced tumor necrosis factor receptor)/CD357	Differentiation of thymic Tregs (tTregs), and expansion of both tTregs and pTregs	(24, 25)
GARP (Glycoprotein A repetitions predominant)	Present on activated Tregs; promote activation and secretion of TGF-β	(26, 27)
TNFR2 (tumor necrosis factor receptor 2)/CD120b	Promotes sustained expression of FoxP3	(28)
Helios	Highly expressed on tTregs; enhances Treg function by increasing expression of other Treg functional molecules	(29–32)
CD39	Anti-inflammatory effect by hydrolytically cleaving ATP to AMP	(33, 34)
CD73	Anti-inflammatory effect by hydrolyzing AMP cleaved by CD39 to adenosine	(32, 35)
CCR4	Expressed on effector Tregs; required for recruitment to tissue, through CCL22	(36–38)
CCR6	Regulates migration to inflammatory tissue	(39)
CCR7	Required for migration to lymph nodes; limits Treg circulation back to the thymus	(40–42)
CXCR5	Expressed on Tfr cells; required for homing to the germinal centers	(43)
Cytokines		
IL-10	Secreted; anti-inflammatory	(44, 45)
TGF-β	Membrane bound and secreted; suppressive; important for Treg trafficking to the gut	(46–50)
IL-35	Secreted; suppressive	(51)

In addition to tTregs, it has become clear that *de novo* expression of FoxP3 can occur in non-Treg $CD4^+$ T cells, either *in vitro* or *in vivo*. Such induction of FoxP3 expression notably happens when naïve T cells are stimulated in the presence of transforming growth factor beta-1 (TGF-β1) (60), leading to the development of a subset of induced Tregs (iTregs). This subset has extensively been used to study the functions and characteristics of Tregs *ex vivo* (61); however, it is now recognized that these *in vitro*-induced iTregs may not accurately portray the characteristics of *in vivo*-induced, pTregs. Notably, full FoxP3 TSDR methylation does not occur in TGF-β-induced Tregs, leading to poorly suppressive and unstable Tregs (62). Conflicting reports have been published with regard to the contribution of retinoic acid (RA) to pTreg differentiation. RA from dendritic cells (DCs) was reported to be a key cofactor in generating pTregs in the gut (63, 64). However, supplementation by RA does not increase Treg frequency (65), which has cast doubt on the role really played by RA in pTreg differentiation. Interestingly, RA can phosphorylate AKT (protein kinase B) (66), thereby reducing its activity, and this pathway could be involved in pTreg differentiation, because a constitutively active AKT has been shown to impair *de novo* induction of $FoxP3^+$ cells (67). Another pathway involved in pTreg induction is antigen presentation by immature DCs. Notably, it has been shown that delivering peptides in subimmunogenic forms for a prolonged period of time can result in the induction of $CD4^+CD25^+$ Tregs from naïve T cells in peripheral lymphoid organs, even in the absence of a functional thymus (68).

Treg HOMEOSTASIS

It was thought that IL-2 is the most important Treg regulator, being required for both Treg maintenance and function (69, 70). More recently, it was shown that Tregs form two distinct populations, the $CD44^{lo}$ $CD62L^{hi}$ central Tregs, which actively recirculate through lymphoid organs and are sustained by paracrine IL-2, and the $CD44^{hi}$ $CD62L^{lo}$ $CCR7^{lo}$ effector Tregs, which are not found in the lymphoid tissue, do not require IL-2, and are instead maintained by inducible costimulator (ICOS) (71). *In vivo*, Tregs can proliferate in response to antigens, meaning that Tregs can dynamically respond to their environment (72). It has also been shown that B7/CD28 costimulation plays a critical role in maintenance of Tregs, as shown by experiments reporting a profound decrease in Tregs in B7/CD28-deficient mice (58, 73).

Tregs IN THE LYMPH NODES

L-selectin (CD62L) is thought to be crucial for the homing of Tregs to the lymph nodes (LNs). $CD4^+$ $CD25^+$ $CD62L^+$ Tregs more potently suppress the proliferative responses of $CD25^{neg}$ $CD4^+$ T cells than $CD4^+$ $CD25^+$ $CD62L^{neg}$ Tregs (74). CD62L-dependent homing induced survival and tolerance in a vascularized cardiac allograft mouse (75). It has therefore been postulated that Treg trafficking to the LNs may be dependent on CD62L (76). A $CD69^{neg}$ $CD25^+$ $CD4^+$ T-cell subset from the LNs was identified to efficiently suppress $CD57^+$ germinal center (GC)-Th cell-driven B cell production of immunoglobulins. These cells express CCR7 and efficiently migrate in response to CCL19, a chemokine expressed in the T cell zone of LNs. Furthermore, many of these $CD69^{neg}$ $CD25^+$ $CD4^+$ T-cells populate the T cell rich zone of the LNs; however, some are present in the GCs also (77).

A particular subset of Tregs, the T follicular regulatory cells (Tfr), discovered in 2011, are Tregs that have migrated into the LN, and thus share phenotypes with Tregs, such as FoxP3, CTLA-4 (cytotoxic T-lymphocyte antigen 4), and CD25 expression (78). Additionally, they undergo differentiation and share surface markers with T follicular helper cells (Tfh), such PD-1 and ICOS, and important for their localization in the GC s, Bcl-6, CXCR5, and CXCL13 (78). This phenotype allows them to modulate B cell and Tfh cell functions in the LN follicles and acts as immune regulators of the GC responses to stimulation (43, 78–80). Currently, CTLA-4 is the only molecule demonstrated to be necessary for full suppression (81, 82); however, IL-10 and TGF-β1 are theorized to play roles in the suppressive function (83). Although a small subpopulation of mature Tfr do not express CD25 (84), depletion of Tfr by anti-CD25 mAb still enhanced humoral responses, with significantly more Ab produced (85, 86).

Soon after their migration to the LNs, Tregs form long-lasting conjugates with DCs. This prevents the DCs from interacting with $CD25^{neg}$ $CD4^+$ T helper cells (87). Using a murine mathematical model, it has been shown that after occupying the LNs, Tregs do not recirculate, whereas naïve T cells do so readily (88).

MECHANISMS OF Treg SUPPRESSION

T regulatory cells produce multiple secretory cytokines that mediate their suppressive activities. The conventional dogma is that cell-to-cell contact is required for the Tregs to exert their suppressive activities (89). However, advances in cell culture capabilities have recently challenged this paradigm. A study using Treg separation from $CD4^+$ T cells with a 0.45-μm permeable membrane demonstrated that, while cell-to-cell contact in the presence of IL-10 and IL-35 appears to indeed be required for Treg activation, the suppressive capabilities of Tregs are not completely mediated by cell contact. Instead, the release of inhibitory factors, such as TGF-β, IL-10, and IL-35, plays a prominent role in Treg-mediated suppression (44, 45).

Transforming growth factor beta-1 is a cytokine secreted by Tregs, which is also present on the cell surface as a membrane bound cytokine. TGF-β1 suppresses non-Treg cells through interactions with the two heterodimer TGF-β receptors, TGF-βRI and TGF-βRII (90, 91). In fact, through the use of T cell-specific *Tgfb1* deletion and subsequent Treg cotransfer experiments in $Rag1^{-/-}$ mice, the inhibition of Th1 differentiation and colitis was shown to be dependent upon TGF-β1 production by Tregs (46). Additional studies with TGF-β1 blockades have further supported its role as a mediator of Treg suppressive function (47, 48). TGF-β1 primarily inhibits type 1 T-helper cell (Th1) responses by blocking differentiation through the inhibition of the master regulator T-bet. However, TGF-β1 is also able to directly suppress the effector functions of $CD8^+$ T cells through inhibiting cytokine and effector molecule secretion (49). Beyond direct suppression, TGF-β signaling is important for inducing

Treg trafficking to the gut, where they can then modulate gut Th17 cells and gut inflammation (50).

T regulatory cells also produce IL-10, which has been shown to be important in controlling inflammation, as disruption of IL-10 production caused colitis in mice. However, unlike TGF-β1, the lack of Treg-produced IL-10 does not cause systemic immunopathology, as demonstrated through Treg-specific IL-10 deletions by Cre recombinase. On the contrary, these mice present with contained pathology to the colon, lung, and skin, indicating a tissue-specific mechanism of IL-10 immune suppression (92). Nonetheless, IL-10 has been linked to Treg activation and their effector functions (45), thus playing a critical role in immune control.

The new IL-12 family heterodimer IL-35 (Ebi3-IL-12α) is an inhibitory molecule produced by Tregs, which is required for complete suppressive functionality in mice (51). In fact, both parts of IL-35, Ebi3 and IL-12α, are necessary to support T-cell proliferation, and recombinant IL-35 was sufficient for reduction of effector T-cell proliferation (51). Tregs are capable of inducing differentiation of naïve T cells to "iT(R)35" cells (93) through IL-10 and IL-35. These iT(R)35 cells have impressive suppressive capabilities originating from substantially increased IL-35 production, while they lack FoxP3 and do not produce TGF-β or IL10, making them a population distinct from tTregs (93). However, other studies questioned the importance of IL-35 and demonstrated that IL-35 is not constitutively expressed on human Tregs, while being shown to be produced by effector T cells (94).

On the cell surface, Tregs constitutively express CTLA-4, an inhibitory receptor that ablates CD28 costimulation by competitive binding of the B7-1 and B7-2 ligands (CD80 and CD86) on APCs (11, 12). Additionally, CTLA-4 also acts through upregulation of indoleamine 2,3-dioxygenase (IDO) production by DCs, inhibiting T cell expansion (95). The importance of this protein is clearly demonstrated by the observation that mice deficient in CTLA-4 die within 2–3 weeks from major organ lymphocytic infiltration and destruction, resulting from uncontrolled lymphocyte proliferation (13). A similar fatal autoimmune disease occurs if CTLA-4 is deleted from Tregs using Cre/lox with the FoxP3 promoter, due to loss of Treg suppressive function, particularly, lack of Treg-mediated DC CD80 and CD86 expression (14). Meanwhile, CTLA-4 blockade can induce autoimmune disease (96). Further support for the suppressive function of CTLA-4 through B7-1/B7-2 was obtained by demonstrating that reversal of the lymphoproliferative phenotype occurs after the administration of the CTLA4Ig, which mimics the ablation of CD28 costimulation by CTLA-4 (97). Imaging of conventional $CD4^+$ T cells, DCs, and Tregs in the LNs showed that CTLA-4 blockade increases the amount of $CD4^+$ T cell-DC interactions and T-cell activation through ablation of suppressive interactions of both B7-1 and B7-1 on DCs (98). However, CTLA-4 does not act exclusively through Tregs, being also expressed on conventional T cells, where inhibitory function can occur in *cis* by both the previously stated mechanism, as well as by signaling through the cytoplasmic region (99). CTLA-4 is also an important contributor to Treg survival, as the anti-CTLA-4 mAb Ipilimumab was found to have an additional function of targeting Tregs for death by $CD16^+$ nonclassical macrophages through antibody-dependent cell-mediated cytotoxicity (ADCC) (100).

An indirect Treg suppression mechanism is through consumption of IL-2. $CD4^+$ $CD25^+$ Tregs are able to bind IL-2, preventing non-Tregs from binding and thus inhibiting activation (101), while simultaneously depriving them of the necessary prosurvival signals to prevent apoptosis (102). This competition serves an additional function of enhancing Treg responses by priming them for IL-10 production after TCR stimulation (103, 104).

Extracellular adenosine triphosphate (ATP) is a known inflammatory signal that acts through the P2 purinergic receptors and is released from cells, which have a high intracellular concentration of ATP, during tissue damage [reviewed in Ref. (105)]. Tregs suppress the inflammatory responses to ATP through directly limiting the amount of extracellular ATP by hydrolysis of ATP to adenosine monophosphate (AMP) by CD39, which is highly expressed on the surface of $FoxP3^+$ Tregs (33) and is further upregulated during inflammation (106). Indeed, CD39 plays an important role during HIV infection, as suggested by the observations that $CD39^+$ Treg cells are inversely correlated with $CD4^+$ T cell counts (107) and polymorphisms that cause decreased expression of CD39 correlate with slower disease progression (107) and decreased suppression of effector T cells (106). Following hydrolysis, AMP is hydrolyzed to adenosine by CD73 on the surface of Tregs (108, 109), which is then shed from the plasma membrane (110). This further increases the suppressive nature of Tregs as adenosine is an anti-inflammatory molecule. *In vitro* experiments demonstrated that adenosine directly inhibits T cell activation and proliferation through binding to the receptor A_{2a}, preventing TCR-mediated IL-2R (111) and IFN-γ (112) expression. Adenosine further inhibits the Th1 response by decreasing TNF-α and IL-12 production by myeloid dendritic cells (mDCs) while simultaneously increasing IL-10 (113). Additionally, adenosine inhibits IFN-γ and IL-2 production of $CD4^+$ and $CD8^+$ T cells and is inversely correlated to gut inflammation and damage during SIV infection (34). Tregs also suppress T cells through cyclic AMP (cAMP). The binding of adenosine to receptors A_{2A} and A_{2B} induces adenylate cyclases, increasing the production of intracellular cAMP and suppressing the immune activation [reviewed in Ref. (114)]. Using gap junction inhibitors and cAMP antagonists, it was shown that Tregs transfer cAMP through gap junctions to suppress non-Tregs (115–117).

ROLE OF Tregs IN HIV/SIV INFECTION

Changes in Treg Frequency Throughout HIV/SIV Infection

Treg suppression of the cell-mediated immune response occurs early during the acute HIV/SIV infection, as reported in SIV-infected Rhesus macaques (RMs) (118). In fact, in HIV-infected individuals, the relative frequency of Tregs correlates with the viral load levels and disease progression (119–123), while being inversely correlated with the SIV-specific cytotoxic T lymphocyte (CTL) responses (118). Of note, $CD4^+$ T cell depletion

characteristic to acute HIV/SIV infection partially spares Tregs, as suggested by the observation that, in spite of the decrease in their absolute counts, Treg frequency increases during the acute HIV infection (121, 123–125). In HIV-infected subjects receiving ART, Treg frequency is partially restored (120, 126). Interestingly, elite controllers have higher Treg absolute counts, yet lower frequencies, in the peripheral blood and rectal mucosa compared to progressors (119, 127). The mechanism by which Tregs are spared relative to other $CD4^+$ cell subsets during HIV infection remains unclear. In the gut mucosa of SIV-infected RMs, non-Tregs were shown to have significantly higher apoptotic gene expression than Tregs, of which some were apoptotic genes associated with HIV production, supporting the concept that Tregs are relatively resistant to cell death mediated by SIV infection (128). This relative Treg resistance to HIV/SIV infection is further supported by the observations that the number of infected Tregs is similar to $CD4^+$ $CD25^{neg}$ cells (129) and that HIV gp120 binding to CD4 enhances Treg survival (122, 130).

Alternatively, the increases in Treg frequency may also be explained by increased conversion of peripheral conventional $CD4^+$ T cells to a pTreg phenotype. *Ex vivo* work performed with plasmacytoid dendritic cells (pDCs) and conventional $CD4^+$ T cells from HIV-infected individuals demonstrated enhanced induction of Treg differentiation when pDCs were stimulated with HIV (131). Similarly, tissue mDCs from SIV-infected NHPs were more efficient at converting non-Tregs to Tregs (132). Increases in the levels of TGF-β characteristic to pathogenic SIV infection of RMs (133) further substantiate the theory of increased pTreg conversion during HIV/SIV infection. This finding is important, because in progressors, Treg suppressive capacity is maintained throughout infection (134), with enhanced function in the LNs, where there is a HIV/SIV reservoir of importance, compared to the peripheral blood (135–137).

In the LNs, Tfr contribute to the impairment of Tfh function (138, 139). Tfh expand during HIV infection (140–142). Their increase is associated with B cell dysfunction (143), as documented by hypergammaglobulinemia, increased GC B cells, and decreased memory B cells (140–142, 144), a likely consequence of hyperactivation through chronic antigenic stimulation (144) and increased cytokine production (141, 142). During HIV infection, the Tfh/Tfr ratio increases and is associated with impaired somatic hypermutation and affinity maturation. These functions can be restored upon Tfr reconstitution (145). While the frequency of Tfr relative to total $CD4^+$ T cells increases during chronic SIV infection, the Tfr fraction of Tfh is decreased during both acute and chronic stages. Loss of Tfh proliferation control by Tfr during HIV/SIV infection has been examined as a possible explanation for Tfh expansion and may help explain the hyperactivation in the B cell follicles (146, 147). Other studies, however, reported opposite findings, showing that the Tfr/Tfh ratio increases postinfection and through expansion of the regulatory phenotype (139). These data are consistent with Tfh impairment, notably downregulation of ICOS and decreased expression of IL-21 and IL-4 (139), implicating Tfh inhibition in the aberrant humoral response. Thus, whether therapeutic targeting of Tfr in HIV/SIV infection is beneficial or detrimental is still up for debate, although their permissiveness to infection enhances the beneficial aspects of targeting Tfr (148).

Treg Suppression in HIV Infection

T regulatory cells are considered both beneficial and detrimental during acute HIV infection (**Figure 1**). Increased immune activation is a hallmark of HIV infection, and Tregs have been shown to control the activation status of HIV-infected $CD4^+$ T cells (118, 149–152). In the nonpathogenic SIV infection of African green monkeys, an increase in Tregs occurs early during the acute infection, as early as 24 h postinfection, concomitant with significant increases in TGF-β and IL-10 (153). In contrast, during the acute pathogenic SIV infection of RMs, there is only modest TGF-β induction and delayed increases in IL-10 (153). Additional support for the role of Tregs in acute infection was obtained by Cecchinato et al. who showed that CTLA-4 blockade early during the acute infection was detrimental to RMs, resulting in increased viral replication and decreased responsiveness to ART (154). These results may be due to essentially "adding fuel to the fire" by increasing immune activation and consequently expanding the target pool for SIV, although some of this may be due to non-Treg effects. *In vitro* studies have shown that, in addition to limiting the amount of susceptible cells, Tregs can limit the infection of conventional $CD4^+$ T cells through DC-$CD4^+$ T cell immunological synapses (117). Therefore, Tregs can help in preventing the deleterious pathogenic consequences of HIV/SIV infections by controlling the immune activation status of virus producing cells by shifting them into resting state. This thereby suppresses viral production, and prevents the spread of infection. The corollary of this paradigm is, however, that, by pushing infected T cells into a resting state, Tregs are promoting the generation of the latent HIV/SIV reservoir, the seeding of which starts as early as 3 days post-infection (155), which represents the ultimate obstacle for HIV cure research.

Meanwhile, Tregs also suppress the HIV-specific $CD8^+$ T cell responses (156), their frequency being inversely correlated with the SIV-specific CTL response (118) and T-cell activation (125). The disruption of the cell-mediated immune response against HIV by Treg-mediated suppression of $CD8^+$ T cytotoxic lymphocytes is therefore inhibiting viral clearance in infected individuals, likely leading to an increased viral replication. In fact, in controllers with the protective *HLA-B*27* and *HLA-B*57* alleles, the *HLA-B*27* and *HLA-B*57*-restricted $CD8^+$ T cells are not suppressed by Tregs, whereas the $CD8^+$ T cells with nonprotective HLA alleles are highly suppressed *ex vivo*, substantiating the role $CD8^+$ T cells in controlling virus (157). Jiang et al. infected humanized mice with HIV following Treg depletion and compared them with those that were not depleted of Tregs. They found that the Treg-depleted mice had lower levels of infection, as measured by peak viral loads and p24 intracellular staining in plasma and lymphoid tissues, such as spleen and mesenteric LNs (158), supporting a negative effect of Tregs on disease.

Of note, conflicting data were published with regard to the effect of Treg infection with HIV on their suppressive capabilities. Purified bulk Treg populations from HIV-infected individuals retain their suppressive activity (125, 149, 159). *In vitro,* HIV

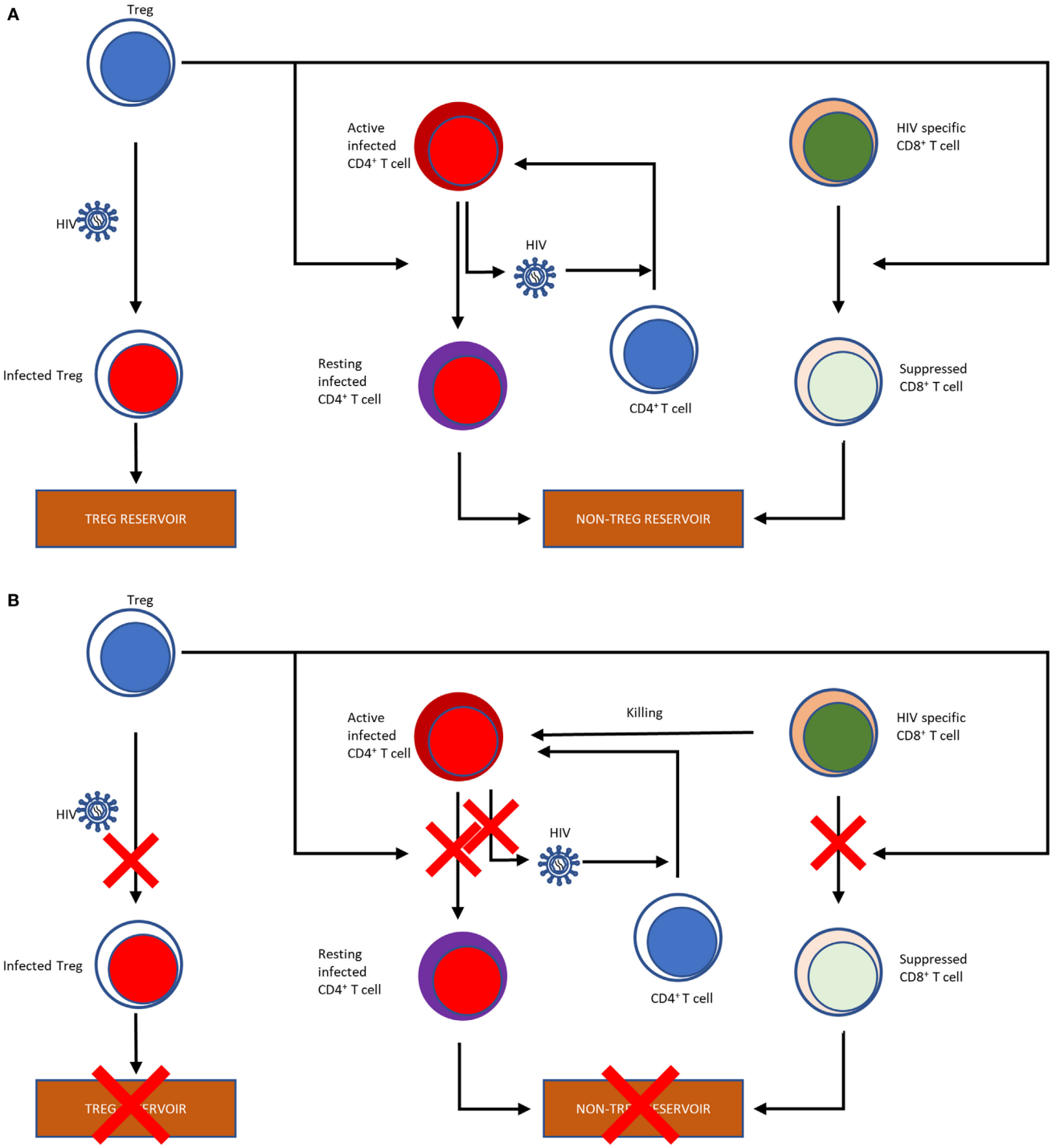

FIGURE 1 | Flow chart illustrating the effects of Tregs on the HIV reservoir **(A).** Tregs can be infected with HIV, thereby contributing to the HIV reservoir. Tregs reverse the activation status of the HIV-infected T cells into resting T cells, further contributing to the reservoir formation. Finally, by suppressing the HIV-specific $CD8^+$ T cells, which would otherwise kill infected cells, Tregs also shape the non-Treg reservoir. Potential effects of Treg depletion on the HIV reservoir **(B)**. Treg depletion obviously result in a reduction of the Treg reservoir through direct killing. Treg depletion also abolish their suppressive effects of the T cells, which may reverse their resting status, become activated and produce and release the virus. Finally, reversion of the suppressive effect of Tregs on HIV-specific $CD8^+$ T cells has the potential to boost their anti-SIV activity, which can also curtail the reservoir.

infection of Tregs has been reported to either have no effect on Treg functionality (160) or result in loss of functionality (161). However, when functionality was assessed on a per-cell basis, infected Tregs had a lower suppressive capacity and corresponding decreases in genes relating to suppressive function and increases in inhibitory genes compared to noninfected Tregs

(162), and this altered suppressive capacity may further contribute to the generalized immune activation in chronic infection.

Tregs AS AN HIV/SIV RESERVOIR

Suppression of the immune responses is not the only way that Tregs contribute to HIV disease. Treg infection with both SIV (118, 128) and HIV (160) occurs both *in vitro* (163) and *in vivo*. Indeed, when naïve T cells were transduced to express FoxP3, their susceptibility to HIV infection increased, and they produced infectious virus at levels comparable to memory T cells (163). In SIV-infected macaques, the fraction of mucosal Tregs containing SIV DNA is higher than that of the non-Tregs, but Tregs harbor less SIV RNA, which was interpreted as an indication that they are less susceptible to productive infection (128). Additionally, mucosal Tregs have a better survival rate than the non-Tregs, supporting increased infection rates without active replication (128). Similarly, in HIV-infected individuals, a larger proportion of Tregs contains HIV DNA than the non-Tregs and importantly, treatment with the histone deacetylase inhibitor (HDACi) valproic acid was able to reverse latency in resting Tregs from more patients than treatment of non-Tregs (164). Yet, when the comparisons are refined by comparing Tregs ($CD25^{+}$ $CD127^{neg}$) to effector memory T cells (TEM) ($CD25^{neg}$ $CD127^{+}$), the difference in the levels of integrated HIV DNA between the two cellular populations was no longer significant (160), an unsurprising, but notable result due to the inclusion of naïve T cells in the non-Treg group. Interestingly, when comparing the *in vitro* susceptibility to infection, Tregs were reported to be more susceptible to infection by CXCR4-tropic strains, while TEM were more susceptible to CCR5-tropic strains (160). Further substantiating Tregs as an important reservoir, replication competent virus has been reactivated from Tregs isolated from HIV-infected individuals on long-term ART (164–166).

As such, due to the increase in Treg frequency during HIV/SIV infection (118–126), a larger proportion of Tregs containing HIV/SIV DNA than non-Tregs (128, 164), better survival from SIV infection (128), and decreased suppressive activity of infected Tregs (161, 162), Tregs appear to be an important HIV reservoir. Together with their major role in shaping the viral reservoir, these data point to a key role for Tregs as targets in cure research strategies.

TARGETING Tregs AS A CURE RESEARCH STRATEGY

The suppressive function of Tregs during HIV infection has opened the forum to assess the benefit of manipulating Tregs for the HIV-infected subjects. However, this is not without its issues, as Tregs are also beneficial in some ways, particularly in suppressing general immune activation. The major problem with targeting Tregs is that the most typical marker for Tregs, the FoxP3 molecule is intracellular and, as such, it cannot be directly targeted *in vivo*. Multiple other targets have however been considered for *in vivo* Treg depletion strategies (summarized in **Table 2**).

TABLE 2 | Strategies for targeting T regulatory cells (Tregs) and/or their function.

Impact on Tregs	Target	Drug	Rationale	Expected Treg depletion/blockade	Reference	Potential complications
Depletion	CD25	IL-2 immunotoxin	Treg targeting through attachment to CD25, the receptor for IL-2 Treg killing through eEF-2 (Eukaryotic elongation factor 2) ribosylation by diphtheria toxin	Up to 75% depletion of circulating Tregs Up to 40% depletion of the lymph node (LN) Tregs	(158, 167, 168)	Autoimmunity, toxicity
		Daclizumab	Binds to CD25, preventing IL-2 binding and action. Il-2 is required for maintenance of Treg counts and function	Up to 50% depletion of circulating Tregs	(169–172)	
	CCR4	CCR4 immunotoxin	Targets Tregs by attaching to CCR4 (effector Treg marker) Treg killing through eEF-2 ribosylation by diphtheria toxin	Up to 40% depletion of circulating Tregs 9–22% depletion of the LN Tregs	(173, 174)	
		Mogalizumab	Targeting Tregs by attaching to CCR4 and causing antibody-dependent, cell-mediated cytotoxicity	Up to 80% depletion of circulating Tregs	(175, 176)	
		Cyclophosphamide	Treg depletion through DNA double strand breaks and decreased DNA repair. Treg sensitivity is due to decreased production of glutathione (required for detoxification of Cy active metabolites)	Up to 50% depletion of circulating Tregs Increased $CD8^{+}$ T cell and NK cell activation	(177, 178)	
Functional blockade	CTLA-4	Ipilimumab	Binds to CTLA-4 on Tregs, blocking it from inhibiting lymphocytes	Up to 75% decrease of circulating Tregs	(100, 154, 179, 180)	Autoimmunity, toxicity
	IDO	1-methyl-D-tryptophan	Inhibits IDO, blocking suppressive function	Increased expression of IFN-γ by the lymphocytes in the LNs, decreased plasma viral loads	(181, 182)	Autoimmunity

Targeting CD25 for Treg Depletion

Two drugs targeting Treg through their constitutive expression of CD25, Daclizumab and Ontak, have been used to deplete them (183, 184). Both compounds bind CD25, but act differently. For Denileukin difitox (ONTAK), which is IL-2 coupled with diphtheria toxin, the IL-2 identifies and binds the $CD25^+$ cells, allowing the diphtheria toxin to enter the cell and cause cell death by ADP-ribosylating host eEF-2 and preventing protein synthesis (185). Ontak has been used to treat $CD25^+$ cutaneous T cell lymphoma (186). Ontak has also been tested with relatively positive results in peripheral T-cell lymphoma, metastatic renal cell carcinoma, and unresectable stage IV melanoma (187–189). Other studies of Ontak administration to melanoma patients, together with a DC vaccine reported no peripheral Treg depletion; yet, this result may be due to the use of a very low dose (190). In the same study, *in vitro* assessments showed that, while the internalization of Ontak was observable in activated Tregs even at low concentrations, Ontak internalization in resting Tregs only occurred at very high concentrations (190). This may be a potential barrier to the use of Ontak to target the resting reservoir.

The second component, Daclizumab, is a monoclonal antibody to CD25, which prevents the interaction of IL-2 with its receptor. As such, Daclizumab may be used for Treg depletion, as IL-2 is essential for Treg development, maintenance, and function, as discussed above. It has been approved for the treatment of relapsing forms of multiple sclerosis (169, 170) and in adult T cell leukemia to induce remission (191). Daclizumab was also used in radio-immunotherapeutic approaches, after linking it with ^{90}Y, and its administration extended the length of remission in patients with adult T cell leukemia (171). This conjugated Daclizumab was also tested in other $CD25^+$ malignancies, with promising results, especially for patients with Hodgkin's disease (192). Finally, for a short time, it was used to prevent acute rejection in patients with kidney transplants (193).

In the context of HIV infection, these compounds look promising, with regards to reservoir control. Ontak administration to DKO-hu HSC mice, followed by infection with HIV-R3A, reduced the levels of Tregs in blood, spleen, and mesenteric LN and increased the expression of HLA-DR, a marker of immune activation, on $CD4^+$ and $CD8^+$ T cells. It nevertheless resulted in lower levels of HIV-1 present in the plasma and the lymphoid organs, during the acute stage of infection (158). Furthermore, Ontak administration to humanized NRG mice infected with HIV-1 and completely virologically suppressed by ART, resulted in viral reactivation in spleen and bone marrow. Cell-associated viral DNA levels did not change, indicating that the virions relapsed from the reservoir. The mice were maintained on ART, which prevented the reactivated virus to reinfect cells and, after virus control was achieved post-Ontak administration, the levels of cell-associated viral DNA were significantly decreased in the lymphoid tissue as compared to controls, with no significant change in total $CD4^+$ T cells in the spleen and bone marrow (194).

Ontak administration to chronically SIVsab-infected African green monkeys, resulted in a significant Treg depletion and induced significant $CD4^+$ and $CD8^+$ T cell activation (167). Finally, Ontak administration to SIVsab-infected RMs, a model of spontaneous complete control of HIV infection (195, 196), resulted in the depletion of 75–85% of the peripheral Tregs, an 8- to 10-fold increase in immune activation of the peripheral $CD4^+$ and $CD8^+$ T cells and a boost of SIV-specific T cells (168). Furthermore, a relatively robust virus reactivation was observed, with plasma viral loads reaching up to 10^3 viral RNA copies/mL (from below 5 copies/mL before treatment).

These results suggested that Treg depletion is a plausible strategy for reducing the HIV reservoir in circulation and lymphoid tissues, while boosting effective cell-mediated immune responses (168). Ontak was discontinued for clinical use due to the production issues related to difficulties in the purification from the bacterial expression system. Daclizumab has also been discontinued. However, a new bivalent IL-2 immunotoxin was developed that showed increased potency when compared to the Ontak-like monovalent version (197). When it was used in human $CD25^+$ HUT102/6TG tumor-bearing NSG mouse model, this bivalent immunotoxin was shown to significantly prolong survival of the mice in a dose-dependent manner (198).

Targeting CCR4 for Treg Depletion

T regulatory cells (Tregs) express a high level of CCR4 (199–201), which is the receptor for CC chemokines (MIP-1, RANTES, TARC, and MCP-1) and has been shown to be a coreceptor for HIV-1 (202). Wang et al. developed a diphtheria-toxin based anti-human CCR4 immunotoxin, which effectively binds to and cause protein synthesis inhibition in target cells. It prolongs the survival of tumor-bearing NOD/SCID IL-2 receptor $\gamma-/-$ (NSG) mice injected with human $CCR4^+$ acute lymphoblastic leukemia cells, indicating the efficacy of this drug (173). When the drug was tested in NHPs, it depleted ~80% of $CCR4^+$ $FoxP3^+$ and 40% of $FoxP3^+$ $CD4^+$ T cells in the peripheral blood. In the LNs, although there was a decrease of ~90% of $CCR4^+$ $FoxP3^+$ Tregs, overall $FoxP3^+$ $CD4^+$ T cells were decreased by only 9–22% (174). The anti-CCR4 monoclonal antibody, Mogamulizumab, also shows promise in treatment of peripheral T-cell lymphoma and cutaneous T-cell lymphomas like mycosis fungoides and Sezary syndrome, by depleting $CCR4^+$ malignant cells and $CCR4^+$ Tregs (175, 176).

Cyclophosphamide

Cyclophosphamide (Cy) is a well-established chemotherapeutic agent, which is widely used for the treatment of leukemias and lymphomas. In high doses, Cy acts as a nonselective cytoreductive agent, which directed its uses as part of a preparation regimen for allogeneic stem-cell transplantation (203) and in treatments for systemic lupus erythematosus (SLE) (204–206). In low, metronomic dosages, Cy retains its antitumor capabilities, with reduced side effects and improved clinical responses (207). In mice, low-dose Cy administration selectively and significantly depleted and reduced the functionality of Tregs (208, 209). *In vitro,* CTLs and T helper cells are more resistant to Cy cytotoxicity than Tregs (210). Treg selectivity has been attributed to decreased DNA repair, as demonstrated by the increased and sustained DNA intercross-linking, as well as increased and sustained phosphorylated histone 2AX. A different mechanism for sensitivity, decreased production of glutathione, a detoxifier

for Cy and its active metabolites, was also evoked. Indeed, Tregs have decreased ATP levels, which abrogate glutathione production, thereby inducing hypersensitivity to Cy (211). Interestingly, $CCR2^+$ Tregs are preferentially depleted in mice over $CCR2^{neg}$ Tregs. An analysis of the cell cycles demonstrated increased proliferation and activation in $CCR2^+$ Tregs (212).

Patients treated with a single dose of 300 mg/m^2 Cy experienced a ~20% Treg decrease sustained for 25 days and a decrease in proliferation marker Ki-67, further substantiating the loss of Treg homeostatic proliferation (177). Further studies of Cy in humans showed that end-stage cancer patients treated with metronomic dosing of 100 mg of Cy per day for 7 days for 4 weeks of on/off, such that the cumulative dose of ~777 mg/m^2 was split between 2 weeks with a week without treatment in between, had greater than 50% decrease in both relative frequency and absolute counts of Tregs at day 30 of treatment. Importantly, treatment caused an increase in $CD8^+$ T cell and NK cell cytotoxicity, a requirement for adequate clearance of infected cells during HIV cure approaches. Interestingly, when the dose was increased to 200 mg/day, the selective depletion of Tregs was ablated, underpinning the importance of the low dose for specific Treg targeting (178).

To be an effective therapy for HIV, Cy must be effective in depleting Tregs from the LNs, where there is a major viral reservoir. A study in mice showed that Cy treatment was beneficial in the LNs by selectively depleting the $CD8^+$ lymphoid-resident DCs while sparing the skin-derived migratory DCs and pDCs in the LNs and spleen. This selective depletion in turn boosted antigen presentation and cytokine secretion by the mDCs and pDCs, with a reduction in Treg suppressive capabilities. These results were confirmed by an adoptive transfer of $CD8^+$ DCs, which abrogated the immune enhancement (213). When patients were treated with a single IV low-dose of 300 mg/m^2 Cy, a less immunosuppressive environment compared to controls was observed in the LNs, including significant decreases in IL-10, IL-6, and VEGF (214). Altogether, these results demonstrate that Cy administration is effective in modulating Tregs from both the LNs and periphery.

Little is known about Cy as a therapeutic approach for HIV cure. In an HIV-positive patient with SLE, treatment with Cy induced an enormous burst in viral replication, with plasma viral loads peaking to $>1.3 \times 10^7$ copies/mL and quickly returned to below detectable levels (215). Using escalating doses up to 1.6 g/m^2, Bartlett et al., monitored the effects of Cy on HIV DNA in LNs and PBMCs and plasma viral loads. They found no significant difference in the HIV DNA burden of LNs and PBMCs *versus* the control group, but, of note, plasma viral loads were not suppressed in these patients, with two subjects out of five admitting to nonadherence to ART (216). Thus, it is possible that the increase in plasma viral loads and lack of viral DNA clearance may have been due to nonadherence.

Based on these data and the impressive benefits of Cy during various cancer treatments, low-dose Cy could be an effective therapy to decrease HIV reservoir, through its Treg-depleting effect. However, further studies are necessary to detail the potential of Cy to enhance HIV-specific CTL responses and/or reactivate latent HIV.

THERAPIES TARGETING Treg FUNCTION

Various therapies to affect Treg function have also been tested. $CTLA\text{-}4^+PD\text{-}1^{neg}$ $CD4^+$ T cells from multiple tissues are enriched for replication-competent SIV in infected RMs under ART, suggesting a potential therapeutic target for reservoir elimination (217). During HIV infection, CTLA-4 plays a role in suppression of HIV-specific T cells, with CTLA-4 blockade enhancing $CD4^+$ T cell functionality, i.e., IFN-γ production and cell proliferation (218, 219). In an HIV-infected individual treated with Ipilimumab (α-CTLA-4 mAb) for melanoma, plasma viral loads remained below the limit of detection using standard qPCR methods, whereas a general decline in plasma viral loads was seen when using the single copy assay, with an opposing increase in cell-associated unspliced RNA post-treatment, likely due to expansion of infected T cells (179). Additionally, in chronically infected RM given blocking CTLA-4 Ab while on ART, decreases in viral RNA was noted when therapy was interrupted, along with an increase in the SIV-specific immune response (180). However, when the same blockade was used in early infection with the pathogenic SIVmac251-infected RM model, it increased immune activation, viral replication, but did not augment SIV-specific responses, and abrogated responsiveness to ART (154). Thus, further studies need to be conducted to determine whether stand-alone CTLA-4 blockade can be used as a latency reversing strategy.

As mentioned earlier, Tregs also express PD-1, which affected their homeostasis (17, 220). PD-1 also is thought to participate in Treg suppression (221). Due to its major role in contribution to T cell exhaustion (222, 223), efficiency of PD-1/PD-L1 blockade is widely studied in HIV-1 infection. However, the lack of specificity to Tregs of these interventions puts PD-1 targeting out of the scope of this review [reviewed in Ref. (224)].

Indoleamine 2,3-dioxygenase has been observed to increase during HIV infection and may suppress the antiviral responses (225). Thus, IDO blockade has been attempted to enhance the antiviral response to HIV/SIV (181, 182). In SIV-infected macaques under ART, treatment with the IDO inhibitor 1-methyl-D-tryptophan (D-1mT) reduced plasma viral loads and SIV RNA in LNs (181). D-1mT combined with CTLA-4 blockade in SIVmac251-infected macaques under ART did not provide better control of viremia (182). Additionally, this treatment induced acute pancreatitis in all animals, whereas the same ART regimen given alone induced pancreatitis in only 10–20% of the animals (182). These data suggest either an exacerbation of ART toxicity, or more likely, the induction of auto-immune responses against pancreatic antigens. Whatever the underlying mechanisms, such findings are a cautionary warning of the potential risk of any Treg manipulation *in vivo*.

CONCLUSION

Treg suppression of virus-specific immune responses may limit the efficacy of virus reactivation strategies, which require effective killing of the reactivated HIV/SIV reservoir. As a result, Tregs may play a central role in shaping the HIV reservoir and compromising the HIV/SIV-specific immune responses. Future research should

focus on further refine the effects of various Treg manipulation techniques on the reservoir. Here, we have described several promising Treg treatments that may either suppress Treg activity or kill Tregs altogether. Treg depletion, which has the ability to directly target a small fraction of the reservoir, reactivate the virus, and boost cell-mediated immune responses, might be a desirable strategy for cure research. Although standalone Treg manipulations are promising, they can quite easily be added to other regimens. In the future, investigations into combining Treg therapies with the more traditional viral reactivation therapies, i.e., HDACis, PKA agonists, etc., or vaccinations may prove to be valid cure strategies.

AUTHOR CONTRIBUTIONS

Outlined the manuscript (AK, RS, IP, CC, CA); drafted the manuscript (AK, RS); reviewed the manuscript (IP, CC, CA); Overseen the process (CC, CA); prepared the figure (RS, AK). AK, and RS equally contributed to this manuscript. CC and CA are corresponding authors.

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Lymph Node Cellular and Viral Dynamics in Natural Hosts and Impact for HIV Cure Strategies

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Combined antiretroviral therapies (cARTs) efficiently control HIV replication leading to undetectable viremia and drastic increases in lifespan of people living with HIV. However, cART does not cure HIV infection as virus persists in cellular and anatomical reservoirs, from which the virus generally rebounds soon after cART cessation. One major anatomical reservoir are lymph node (LN) follicles, where HIV persists through replication in follicular helper T cells and is also trapped by follicular dendritic cells. Natural hosts of SIV, such as African green monkeys and sooty mangabeys, generally do not progress to disease although displaying persistently high viremia. Strikingly, these hosts mount a strong control of viral replication in LN follicles shortly after peak viremia that lasts throughout infection. Herein, we discuss the potential interplay between viral control in LNs and the resolution of inflammation, which is characteristic for natural hosts. We furthermore detail the differences that exist between non-pathogenic SIV infection in natural hosts and pathogenic HIV/SIV infection in humans and macaques regarding virus target cells and replication dynamics in LNs. Several mechanisms have been proposed to be implicated in the strong control of viral replication in natural host's LNs, such as NK cell-mediated control, that will be reviewed here, together with lessons and limitations of *in vivo* cell depletion studies that have been performed in natural hosts. Finally, we discuss the impact that these insights on viral dynamics and host responses in LNs of natural hosts have for the development of strategies toward HIV cure.

Keywords: HIV, SIV, natural hosts, lymph nodes, viral control, T cells, NK cells, inflammation

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INTRODUCTION

Combined antiretroviral therapy (cART) has transformed HIV infection from a lethal disease into a manageable chronic infection (1). Indeed, cART efficiently controls HIV replication leading to undetectable virus in blood and drastic increases in lifespan of people living with HIV (2). However, cART does not cure HIV infection as virus persists in cellular and anatomical reservoirs, from which the virus most often rapidly rebounds after cART interruption (3, 4). HIV probably rebounds from multiple sources (5). Virus-producing cells can be detected in SIVmac-infected macaques under suppressive cART in nearly every tissue, and in particular in the mucosal tissues and secondary lymphoid organs (6, 7). A major anatomical viral reservoir corresponds to lymph node

(LN) B cell follicles, where HIV-1/SIVmac replication persists in follicular helper T cells (T_{FH}) even in Elite controllers and cART-virologically suppressed individuals (8, 9). Surprisingly, T_{FH} cells expand during HIV-1 and SIVmac infections (10). Thus, lymphoid follicles have come to be considered as major sanctuaries for HIV/SIV (9). In parallel, HIV-1 and SIVmac might also persist in some $CD4^+$ T cells within the T zone of LN during cART (11). In this review, we will focus on the viral and host dynamics in LNs of natural hosts and discuss similarities and key differences with regard to HIV and SIVmac infections.

PRIMARY CHARACTERISTICS OF NON-PATHOGENIC SIV INFECTION IN NATURAL HOSTS

Natural hosts of SIV, such as African green monkeys (AGMs) (*Chlorocebus aethiops*), sooty mangabeys (SMs) (*Cercocebus atys*), and mandrills (*Papio sphinx*), generally do not progress to disease despite displaying persistently high viremia (12–16). The vast majority of the studies carried out on SIV infections in natural hosts have been performed using two species, SMs and AGMs (17). The comparison of the clinical, virological, and immunological parameters of infection in these species with that of HIV/SIVmac infections allowed advances in knowledge on the mechanisms linked to protection against AIDS. In particular, natural hosts rapidly resolve inflammation induced by SIV infection, and unlike pathogenic lentivirus infections do not develop chronic immune activation (see chapter below).

An important aspect of SIV infection in natural hosts is also their ability to preserve the function and structure of their tertiary and secondary lymphoid organs throughout the infection. Indeed, natural hosts avoid the widespread damage to the mucosal immune architecture that is observed in pathogenic infections (**Table 1**). While acute SIV infection leads to a rapid, near-complete loss of $CD4^+$ T cells in the intestine in both natural hosts and macaques, mucosal $CD4^+$ T cells partially recover in natural hosts, even if not to baseline levels (18–21). Furthermore, cART administration to SIV^+ SM induces a rapid and substantial recovery of mucosal $CD4^+$ T cells that is not typically observed in HIV infection (22). Moreover, despite high viremia and high-level replication in the gut (23), natural hosts, in stark contrast to non-natural hosts, preserve intestinal Th17 cells (24, 25), retain the structural integrity of the mucosal barrier (26), and do not exhibit leakage of mucosal lumenal microbiota (i.e., microbial translocation) into systemic circulation (27–29). With regard to LN during SIV infection in natural hosts, there is generally no sign of lymphadenopathia nor fibrosis and LN display a normal follicular dendritic cell (FDC) network (12, 30, 31) (**Table 1**). Another characteristic of natural hosts is the relatively low infection of central memory T cells (see below) (32). Natural hosts thus seem to have developed ways to protect the sites of education and memory of immune responses.

VIRAL DYNAMICS IN LNs DURING SIV INFECTION IN NATURAL HOSTS

Studies in SIVmac infection have shown that the viral seeding of LN occurs rapidly and progressively. One to three days after infection, some replicative viruses could already be detected in

TABLE 1 | Major similarities and differences between HIV/SIVmac infections and SIV infections in natural hosts at the level of lymph nodes (LNs).

LNs		Natural host (African green monkeys, sooty mangabeys)	Non-natural host (human/macaque)	Reference
Viral replication in LN (17, 12, 34)	Acute phase	High	High	(17, 33, 34)
	Chronic phase	**Low**	**High**	
Inflammation	Acute phase	**Rapid**	**Strong**	(35–39)
	Chronic phase	**No**	**Yes**	
	IFN-a	High in acute infection	High in acute infection	
	Interferon-stimulated gene	High in acute infection	**High in acute and chronic infection**	
	TGF-β and collagen deposition	**No**	**Yes**	
LN architecture	Lymphadenopathia	**No**	**Yes**	(12, 30, 40)
	Follicular dendritic cell network	**Preserved**	**Lost**	
	Fibrosis	**No**	**Yes**	
Location of SIV-infected cells	T cell zone	Yes	Yes	(11, 12, 41, 42)
	B cell follicles	**Rare/absent**	**Yes**	
	Virus trapping	**Rare/absent**	**Yes**	
SIV-infected cells	$CD4^+$ T_{CM}	**Low**	**High**	(43–47)
	T_{CM} PD-1^+CTLA4^+	nd	Yes	
	T_{FH}	**Rare/absent**	**High**	
	Plasmacytoid dendritic cell	Yes	Yes	
	macrophage	Yes	Yes	
Antiviral immune responses	HIV/SIV-specific T cell responses	Weak	Variable (strong in Elite controllers)	(17, 48–50)
	Follicular $CD8^+$ T cells	nd	Yes (rare)	
	Follicular NK cells	**Yes**	Yes (rare)	
	bNAb	nd	Yes (rare)	

The green and red colors highlight, respectively, major differences between SIV infection in natural hosts and HIV/SIV infections in non-natural hosts.

the draining LN and even in systemic LN (51). Of note, during the eclipse phase until peak viremia, productively infected cells are found essentially in the extra-follicular zone of LN (41). Only in later phases of primary infection, and in particular during chronic infection, viral RNA is found inside B cell follicles, where it replicates within T_{FH} cells (45). In addition, virus is trapped within the follicles by FDCs where it remains infectious for 9 months or more (30, 52, 53). The mechanism driving this shift from the T cell zone to the B cell follicles is incompletely understood.

During chronic HIV/SIVmac infections, virus replication in LN exceeds the levels in blood by several orders of magnitude. In ART-naive SIVmac infection, LN are estimated to support ~50% of viral burden, and be reduced to ~1% in the context of suppressive ART, with the remainder supported by mucosal tissue (6). In one SIVmac-infected macaque, the frequency of infected cells in LN was evaluated and appeared to be as high or slightly higher than in the gut (mean frequency ~8.7×10^5 $vRNA^+$ cells/g in LN and ~5.6×10^5 $vRNA^+$ cells/g in the gut) (6). ART administered for >20 weeks decreased the mean frequency of vRNA in LN by approximately 2 $\log_{10}$ in $SIVmac_{251}$-infected rhesus macaques (6).

The reason of the preservation of the normal architecture of LN in natural hosts might be associated with the significantly lower levels of viral replication in this tissue. Strikingly indeed, AGM and SM mount a strong viral control in LN shortly after peak viremia, which lasts throughout infection (12, 23, 43, 54–56). Thus, while during the first 2 weeks post-infection (p.i.), the number of productively infected cells as well as the copy numbers of cell-associated viral DNA and RNA are similar between SIV infection in natural hosts and macaques, major differences are observed after the viremia peak between natural and non-natural hosts (12, 49, 56). Thus in natural hosts, viral replication levels decrease drastically in LNs after peak viremia, whereas in pathogenic infections, after a moderate decrease, a relatively strong viral replication generally persists throughout the infection in absence of cART, leading to a difference of 2–3 log in the cell-associated viral RNA in LN during chronic infection between macaques and natural hosts. Viral RNA-producing cells as well as cell-associated viral RNA sometimes become even undetectable in LN of AGM, despite continuous high-level plasma viremia (12, 14, 33).

The anatomical distribution of virus replication in chronic infection is also very different between non-pathogenic and pathogenic infection. Indeed, in natural hosts, most virus is detected in the T cell zone, even if at extremely low levels, while in pathogenic HIV/SIV infection, most virus is present in follicles (**Figure 1**). Strikingly, in natural hosts, such as AGM and to a lesser extent in SM, viral RNA is generally absent in follicles. This is not a matter of the virus, as SIVsm and SIVagm infections of macaques lead to high SIV levels in follicles (57, 58). Natural hosts are thus characterized by a limited or absent replication in T_{FH} cells and frequent lack of FDC deposition of virus (35, 59). Understanding the underlying mechanisms of the strong viral control in LN in natural hosts might yield clues helpful for the development of strategies aiming the elimination of HIV reservoirs in follicles.

REGULATION OF INFLAMMATION IN LNs AND IMPACT ON SIV INFECTION IN NATURAL HOSTS

The deleterious impact of unabated inflammation in HIV infection has been well documented (35). This immune activation is positively correlated with HIV-1/SIVmac replication in both ART-naïve and ART-treated settings (60, 61). Among the myriad of detrimental manifestations due to the persisting inflammation that have been reported, a handful could be particularly influential in maintaining viral burden, namely: (i) recruitment of target cells, (ii) impairment/exhaustion of adaptive immunity, and (iii) the disruption of lymphoid structures. In this section, we will review existing data on inflammatory pathways differing significantly between natural hosts versus pathogenic SIV infection. These data will be reviewed in the context of the natural host's low-to-absent SIV burden in LN follicles. Hypotheses concerning the effect of non-natural inflammation in supporting LN SIV replication will be presented.

A longstanding observation in natural hosts is that they are devoid of the pan-lymphocyte activation and chronic inflammation seen in pathogenic HIV/SIV disease (62–64). The molecular and immunological distinctions of these species have been extensively characterized [reviewed in Ref. (35, 44, 65)]. Although natural hosts exhibit levels of immune activation similar to baseline during chronic infection, detailed longitudinal studies have demonstrated that rapid, early immune activation is evident, including elevated levels of IFN-a, $CD8^+Ki67^+$ T cells and PD1 expression in LN (63, 66, 67). The most striking confirmation is the massive upregulation of interferon-stimulated gene (ISG) expressions during acute infection in natural hosts (68, 69) in blood, LN, and gut. These ISGs include many antiviral restriction factors, such as MX2 and Tetherin. Of note, the upregulation of ISGs occurs very early, starting from days 1 or 2 p.i. in AGM, concomitantly with a very early transient increase in IFN-α (68, 70). By contrast, during SIVmac infection in macaques, it was reported that the expression of those ISGs encoding antiviral restriction factors was delayed and not upregulated before peak viral replication on day 10 (71). Thus, natural hosts seem to develop a more rapid antiviral innate response to SIV compared to non-natural hosts (66, 68, 70, 71). Subsequently, natural hosts rapidly resolve total ISG expression to baseline before the transition to chronic infection despite prevalent viremia. This downregulation of ISG expression in natural hosts is in stark contrast to HIV/SIVmac infections, in which ISG expression remains elevated indefinitely (72).

The observation that natural hosts resolve IFN-I related responses prompted a series of comparative studies into plasmacytoid dendritic cells (pDCs). pDC trafficking to LN has been reported for both natural and non-natural hosts. A peak of pDC accumulation in LN is observed approximately 7–14 days after SIV infection in macaques, SM, and AGMs concomitant with robust IFN-α and IFN-β *in situ* production by pDC in LN (66, 73–76). The trafficking of pDC to tissues during SIV infection differs in several aspects between natural hosts and non-natural infections: (i) in AGM, an early first peak of pDC in LN is observed around days 1–3 p.i. (66); (ii) pDC accumulate in the rectal mucosa in infected humans and macaques, but not

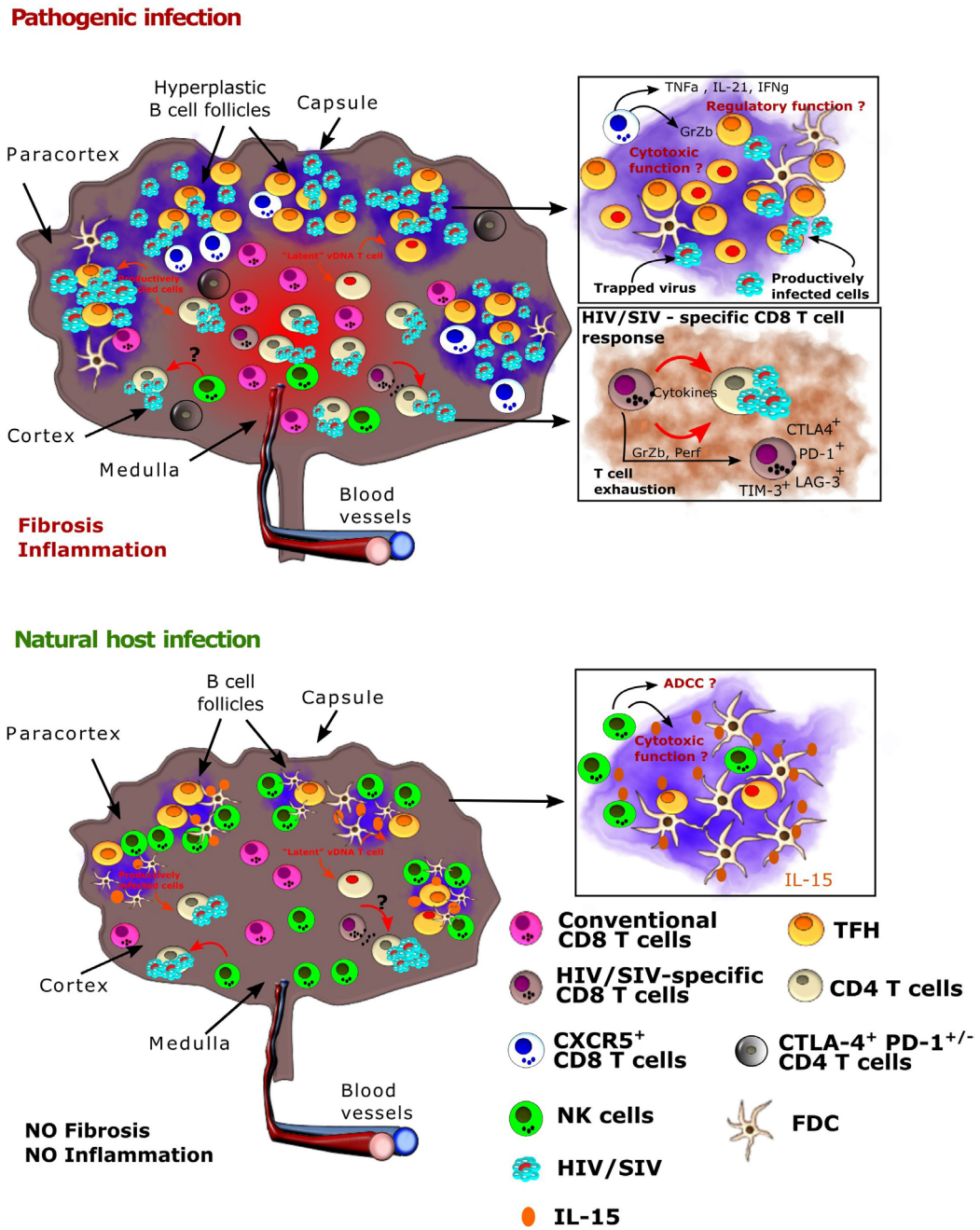

FIGURE 1 | Viral and host immune cell dynamics in lymph nodes (LNs) from natural hosts versus HIV-1/SIVmac infections. Schematic representation of a LN after HIV or SIV infection in pathogenic models (human, macaques, top) and natural hosts [African green monkey (AGM), sooty mangabey, bottom]. (*Top*) HIV-1 and SIVmac infection in, respectively, humans and macaques result in the formation of hyperplastic germinal centers in LNs with massive B cell proliferation. T_{FH} cells also expand during HIV-1 and SIVmac infections. Inflammation is uncontrolled and leads to collagen deposition and fibrosis. The follicular dendritic cell (FDC) network is disrupted on the long term. HIV-1 and SIVmac replicates in combined antiretroviral therapy (cART)-naïve individuals and animals in both T and B cell zones, but the viral burden is highest in the B cell zones (follicles). In the follicles, virus replication is concentrated within follicular helper T cells (T_{FH}). Virus is also trapped by FDC and remains infectious. On cART, virus persists mostly in T_{FH} cells in the follicles, where it is often outreach of conventional $CD8^+$ T cells and of optimal drug concentrations, as well as in $CTLA4^+CD4^+$ T cells within the T zone. The latter cells have a capacity for long survival. NK cells and conventional HIV/SIV-specific $CD8^+$T cells are often expressing immune checkpoint inhibitors. The presence of $CXRC5^+CD8^+$T lymphocytes has been described, but their role needs to be further studied. (*Bottom*) In natural hosts, virus replication is strongly controlled during the chronic phase of infection. Most follicles are exempt of virus. Conventional SIV-specific $CD8^+$ T cell responses are weak. NK cells play a major role in the control of viral replication in AGM LNs. Both the IFN-α and NK cell responses appear earlier than in SIVmac-infected macaques. NK cells accumulate in follicles in SIVagm-infected AGMs, which might be a direct consequence of a high production of IL-15 in the follicles. NK cell migration into B cell follicles in response to SIVagm infection is associated with the acquisition of CXCR5. $CXCR5^+$ NK cells express high levels of Fcγ receptors and of CD107a, which raises the question if they have the capacity to control SIVagm replication through antibody-dependent and/or -independent cellular cytotoxicity.

in SM, which has been attributed to heightened levels of α4β7 in SIVmac infection (77, 78), and (iii) pDC in LN during acute SIVmac infection are prone to apoptosis, while for natural hosts this is not known (39, 73). Both SM and AGM were demonstrated to retain intact sensing and IFN-α production in pDC in response to their native SIV (68, 79–81). Of note, pDC from AGM sense more efficiently SIVagm than SIVmac or HIV-1 viruses (81). Studies in natural hosts have revealed that SIV infection alters the capacity of viral sensing in cells other than pDC, which then can also produce IFN-I during acute infection (80). The contribution of pDC to IFN responses during chronic SIV infection remains unresolved, while some reports have not detected IFN-I in pDC during chronic infection (74), we have observed IFN-α transcripts in LN pDC as far out as 18 months post-infection (Bosinger, unpublished observations).

The consequences of unabated IFN production on immune function and viral reservoirs in HIV infection are under intense study. IFN-induced responses are clearly critical for the control of SIV in LN during acute infection, as antagonism of the IFN-α receptors (IFNAR) from before infection to early time points p.i. in macaques caused elevated levels of LN-associated SIV and plasma viremia (82).

The effects of IFN during chronic HIV infection are less clear. Mouse models have shown that persistent TLR and IFN signaling causes damage to the lymphoid structures (83). Several studies have demonstrated that irreversible fibrosis is evident in the LNs of SIV-infected macaques, but, interestingly, is absent in natural host infection (31, 84). The fibrosis in chronic HIV/SIV infection might be linked to persistent IFN-related inflammation, TGF-β produced by regulatory T cells (Treg) leading to collagen deposition, and/or other yet unknown factors (84). Disruption of IFN-I signaling in chronic infection appears to have indeed a beneficial effect on host immunity in certain settings. In the mouse model of lymphocytic choriomeningitis clone 13 infection, blockade of IFN-β signaling in chronic infection enabled spontaneous clearance of the virus (85–87). In a remarkable set of independent studies using ART-suppressed, HIV-infected humanized mice, disruption of IFNAR signaling reduced latent HIV levels and ameliorated systemic immune activation (88, 89). In both the LCMV and hu-mouse HIV datasets, IFN-blockade reduced expression of co-inhibitory molecules on $CD8^+$ T cells and improved cellular antiviral responses; thus, the mechanism of action was presumed to be alleviation of IFN-mediated exhaustion of T cell responses. These studies provide some rationale for IFN blockade to be applied as a therapy to lower the reservoir, but this hypothesis would first need validation of efficacy and safety in pre-clinical studies. Taken together, the observations that (i) SIV natural host species avoid long-term ISG expression and (ii) *in vivo* antagonism of type I IFN signaling can improve antiviral immunity and reduce reservoir levels in the hu-mouse model suggest that the overall contribution of IFN in chronic HIV/SIV infection is harmful by maintaining high levels of immune activation and contributing to immune dysfunction. However, exogenous administration of IFN-α to ART-suppressed, HIV-infected patients have shown in some cases clinical benefit in terms of reduced levels of cell-associated HIV DNA (90–92). Thus, the contribution of IFN-α to chronic inflammation and viral persistence during ART-treated HIV/SIV infections is still unclear. Injection of exogenous IFN-α into SIV-infected AGM and SM have not been able to reproduce the phenotype of widespread immune activation observed in non-natural hosts (70, 93). However, the injections of exogenous recombinant IFN-α induced a rapid state of tolerance *in vivo* to this molecule. It is not excluded that one might need to treat for long periods of time with intermediate breaks to see an effect on chronic inflammation. The other possible explanation is that IFN-I levels are not different between pathogenic and non-pathogenic infections and/or that IFN-I are not the major culprits of the persistent ISG expression (68, 70). Other factors, such as IFN-γ, might contribute to ISG upregulation (68, 94). Collectively these comparative studies in distinct models indicate that IFN-I signaling is (i) beneficial during acute infection, (ii) a major contributor to early immune activation, (iii) alone insufficient to cause chronic immune activation, and (iv) its impact is highly context dependent.

Several other factors have been put forward to explain the absence of chronic inflammation in natural hosts. For example, by sequencing for the first time the genome of the SM, a mutation was uncovered in the gene encoding TLR4, the primary receptor for LPS, that yields a truncated protein and attenuated signaling (95). Intriguingly, this mutation was also observed in the *TLR4* gene of the two other natural host species (AGM, mandrills) (95). This mutation might contribute to a lower monocyte/macrophage activation in natural hosts.

The maintenance of viral replication in LNs could impact systemic inflammation, due to the sheer immune "traffic" and recirculatory nature of immune cells. From this point of view, the fact that natural hosts strongly control viral replication in LN might contribute to their capacity to resolve inflammation. In this light, understanding the mechanisms by which HIV/SIV replication could be controlled in the LN is likely to be critical not only for viral eradication strategies but also for therapies aiming at reversing immune activation.

TARGET CELLS FOR SIV IN LNs FROM NATURAL HOSTS AS COMPARED TO PATHOGENIC HIV AND SIVmac INFECTIONS

Reducing the persistent HIV/SIV reservoir remains an essential milestone for the achievement of a functional cure for HIV-1 infection; however, this goal has been significantly hindered by poor means for identification of the $CD4^+$ T cell subsets that harbor replication-competent virus, as well as by the anatomic location of these cells in sanctuaries for HIV. Several key differences in the nature of cells targeted by SIV in natural versus non-natural hosts have been identified, raising the fascinating hypothesis that the type of infected $CD4^+$ T cells, even more than the quantity, could contribute to the different capacity to control immune activation and disease progression between the two hosts.

Central Memory $CD4^+$ T Cells (TCM)

In vivo and *in vitro* comparative studies showed that the frequency of SIV-infected TCM in SM is significantly lower as compared

to both CD4+ T effector memory cells of SM and CD4+ TCM of macaques in both blood and LN (32, 59). Thus, SM are partially protecting the important CD4+ TCM cell subset from SIV infection. In line with this relative preservation from viral infection, CD4+ TCM cells are more preserved in SIV-infected SM compared to SIV-infected rhesus macaques (46). CD4+ TCM cells are long-lived, self-renewing cells able to replenish the pool of non-self-renewing, shorter lived CD4+ effector memory cells, thus their maintenance is key for the homeostasis of the overall CD4 T cell compartment and immune memory. Remarkably, a low contribution of infected CD4+ TCM to the overall viral reservoir has similarly been described in (i) long-term non-progressors with protective HLA alleles (96); (ii) viremic non progressors, i.e., rare HIV-infected individuals who maintain high CD4+ T cell levels despite uncontrolled viremia (97); and (iii) post-treatment controllers, i.e., patients with a durable control of viremia after ART-interruption (98). With a distinct strategy, AGMs have also evolved to protect memory CD4+ T cells from viral infection. Indeed, CD4 molecules get downregulated from the surface of the CD4+ T cells when the latter get activated. Of note, these cells maintain their T helper functional activity (99).

The mechanisms of TCM protection are not clear. It has been suggested that CCR5 plays a role. Thus, CD4+ T cells from natural hosts express less CCR5 in blood, LN, and mucosae compared to humans and macaques (100, 101). It also has been shown that *in vitro* stimulation of SM CD4+ T cells, particularly the TCM, fail to upregulate CCR5 (32). CD4+ TCM cells expressing low levels of cell-surface CCR5 are less susceptible to SIV infection when compared to TCM of macaques both *in vivo* and *in vitro* (46, 102). However, SIV from natural hosts can also efficiently use other coreceptors than CCR5 to infect primary CD4+ T cells and other factors might as well be implicated in the relative preservation of TCM to infection in natural hosts (103). LN comprises a higher fraction of TCM compared to mucosal tissues, the latter containing higher proportions of effector cells in mammals (104). Thus it is possible that in natural hosts, the lower ratio of TCM infection is related to the control of viral replication in LN, whereas the predominant virus replication in the gut would explain why most virus infects CD4+ effector T cells in natural hosts. Altogether, the viral tissue distribution could thus in part also explain the lower frequency of infection rate in TCM compared to CD4+ effector T cells in natural hosts.

Follicular Helper T Cells (T_{FH})

T_{FH} correspond to a subpopulation of memory CD4+ T cells expressing high levels of CXCR5 and PD-1 residing within the follicles of secondary lymphoid organs. They impact the activation, differentiation and survival of B cells. Several studies explored the frequencies, function, and infection rate of T_{FH} cells in HIV-infected humans or SIV-infected macaques. They revealed that T_{FH} cells are infected at high frequencies in chronic infection. Despite the high rate of HIV/SIVmac replication in T_{FH} cells, these cells expand during HIV and SIVmac infections (45, 59, 105, 106). More recently, it was shown that T_{FH} cells constitute an important source of persistent replication-competent virus in ART-treated, aviremic individuals (8). By contrast, a low infection rate of T_{FH} cells has been described during non-pathogenic infection of SM (59) and AGM (49), where follicles often remain virus free. LN T_{FH} cells showed lower levels of Ki-67 expression than non-T_{FH} memory CD4+ T cells and fewer of the T_{FH} cells expressed CCR5, but this was similar between macaques and natural hosts (59). Phenotypic studies on T_{FH} cells in natural hosts are though limited so far and whether T_{FH} cells in LN expand differently during SIV infection in natural hosts needs to be further investigated.

CD4+PD-1+CTLA-4+ T Cells

The contribution of T_{FH} cells to the persistent reservoir progressively decreases with increased length of cART (8, 107), suggesting that other cell subsets, apart from T_{FH} cells, can contribute to the magnitude of the pool of latently infected cells. In a recent study, it was found that PD-1+ cells, the subset that contributes most to T_{FH} cells, were indeed the dominant contributors to the viral DNA pool in the B cell follicles in the LN in ART-treated SIV-infected macaques; however, CTLA-4+PD-1− memory CD4+ T-cells, a subset comprised predominantly of Tregs, were identified as a previously unrecognized component of the SIV reservoir (11). These cells are significantly enriched in SIV DNA in multiple tissue compartments, including the blood, LN, spleen, and gut and have been shown to harbor replication-competent and infectious virus (11). CTLA-4+PD-1− cells localized in the extrafollicular zones of the LN in ART-treated SIV-infected macaques and HIV-infected humans. Therefore, in addition to PD-1+ T_{FH} cells, HIV-1 and SIVmac are able to establish and maintain viral persistence through the specific targeting of another CD4+ T cell subset, CTLA-4+PD-1− cells. These cells seem to have long living capacities (11). Further studies are needed to determine if the rare SIV-producing cells in the T zone of natural host's LNs correspond, at least partially, to these CTLA-4+PD-1− cells.

Plasmacytoid Dendritic Cell

Unlike humans' and macaques' pDC, pDC from natural hosts display substantially lower CD4+ and CCR5+ surface expression (80). The lowered SIV receptor/coreceptor expression however does not affect the ability of SIVagm to infect pDCs. Indeed, high rates of pDC infection were detected in the spleen of AGM, to a similar high rate as pDC infection by HIV in cART-naïve humans (81).

POTENTIAL IMMUNE-MEDIATED MECHANISMS FOR VIRAL CONTROL IN LN: THE ROLE OF CD8+ T AND NK CELLS

There are several clear lines of evidence that CD8+ T cells play an important role for the overall control of HIV-1/SIVmac replication (108, 109). Some of the most convincing evidences have been obtained in macaques and include a temporal correlation between the rise of SIV-specific CD8+ T cells and post-peak viremia decline, as well as the increase of viremia after *in vivo* depletion of CD8+ cells (110). Of note, most *in vivo* depletion studies used monoclonal antibodies that did not discriminate between CD8+ T and NK cells, and thus in some of these studies, the contribution of NK cells remained undetermined. Nonetheless, the role of CD8+ T cells in viral control is undeniable and is evident in

HIV controllers and rhesus macaques with specific MHC alleles (111, 112).

$CD8^+$ T cells in LN are generally located in the T cell zones. Early studies have revealed massive infiltrations of activated $CD8^+$ T cells into B cell follicles in progressors, but this could be due to the disruption of the FDC network in late stage disease (113–116). Nevertheless, the magnitude of fully cytolytic $CD8^+$ T cells was significantly higher in LN compared to blood (117), and HIV-1-specific $CD8^+$ T cells are preferentially located in LN compared to blood, including a subset of responses that is present solely in secondary lymphoid organs (118). This preferential location of HIV-1-specific $CD8^+$ T cells in the LN was also observed in chronically infected individuals on cART (118). These migrating $CD8^+$ T cells localize to the extra-follicular zones of the LNs, where most of endogenous HIV-1-specific CTL were also observed, far from sites of virus replication inside the follicles (117). After *in vivo* depletion of $CD8^+$ cells in SIVmac-infected macaques, the frequency of SIV-infected cells in extra-follicular regions increased and reached levels similar to that in B cell follicles (9) confirming that $CD8^+$ T cells exert control of viral replication predominantly in the T cell zones. Until recently, it was considered that $CD8^+$ T cells generally do not migrate into the B cell follicles and it was further suggested that antiretroviral drugs inefficiently diffuse into or are unequally distributed within LN (84), collectively making follicles a prime sanctuary for HIV/SIV replication. Nonetheless, a small proportion of $CD8^+$ T cells expressing $CXCR5^+$ has been recently described in both SIVmac and HIV-1 infections (119–121). The levels of these $CXCR5^+CD8^+$ T cells in LN were higher in HIV-infected individuals compared to healthy donors, and they were detected in close proximity to viral RNA^+ cells, probably starting from primary infection on (119, 122). The frequency of SIV-specific $CXCR5^+CD8^+$ T cells correlated negatively with that of SIV infection in T_{FH} cells and viremia, suggesting a role of $CXCR5^+CD8^+$ T cells in viral control (50). However, other studies highlighted a regulatory phenotype of $CXCR5^+CD8^+$ T cells with poor capacity of viral control which could further impair germinal center function in HIV infection (120, 123). Unfortunately, little is known about these recently described follicular $CD8^+$ T cells, and whether the contrasting results are due to the presence of distinct $CXCR5^+CD8^+$ T cell subsets, differences in the infection models studied or other yet unknown factors.

In natural hosts, the contribution of $CD8^+$ T cells to controlling SIV replication may be comparatively small. Indeed, although SIV-specific $CD8^+$ T cell responses were observed for SIV-infected SM and AGM, their magnitude and breadth were similar or even lower than those generally observed in HIV-1 and SIVmac progressive infections both in blood and LN (124–127). However, it has been suggested that these responses appear temporally earlier in LN of natural hosts compared to pathogenic species and that this confers an advantage (56). Of note, these cells do not seem to migrate into follicles. $CD8^+$ T cells from natural hosts were indeed found to be exclusively located in the T cell zones both in non-infected and SIV-infected animals (12). In line with this, $CD8^+$ T cells in LN from AGM do not upregulate CXCR5 in response to SIV infection (49). To further address the question of the role(s) played by $CD8^+$ T cells during natural host's SIV infection, *in vivo* cell depletion experiments have been conducted. Administration of anti-$CD8^+$ and anti-$CD20^+$ antibodies during the first 2 weeks of $SIVagm_{ver90}$ infection in pig-tailed macaques (pathogenic infection) and AGM (non-pathogenic infection), led to dramatically different results in the two species (128). In pig-tailed macaques, a one-log increase in peak viremia and four-log increase in set-point viremia were observed following antibody administrations. Moreover, these animals rapidly progressed toward disease and displayed CMV reactivation. By strong contrast, in AGM, depletion of $CD8^+$ and $CD20^+$ cells did not modify peak viremia and the animals displayed only a minor delay in post-peak viremia decline compared to control animals, and all animals remained clinically healthy (128). In another study, treatment of SIVsm-infected SM using a CD8α-specific Ab (OKT8F) led to a profound depletion of $CD8^+$ cells in both blood and tissues such as LN, but only minor changes in plasma viremia (129). Similar results were also observed in AGMs in which $CD8^+$ cell depletion during the acute phase led only to a delay of 5–10 days in the post-peak viral decline (130). By contrast, virtually all $CD8^+$ *in vivo* depletion studies conducted in non-natural host models during acute or chronic SIV infection have reported significant increases in viral loads and rapid disease progression (110, 131–133). Altogether, these data highlight that while CTL responses can play a large role in HIV controllers, they may contribute only modestly to the control of viral replication in LN in natural hosts. Thus, while $CD8^+$ T cells might still be involved to some extent in the control of viral replication in the T cell zone, they most likely do not represent the major cellular component of viral control in LN follicles during SIV infection in natural hosts.

As an alternative to $CD8^+$ T cells, multiple lines of evidence pointed toward a role of NK cells in the control of SIV replication in LN of natural hosts. Upon SIV infection, AGM temporarily display high levels of IFN-α and IL-15 in the plasma (70). These cytokines are known to activate NK cells and enhance their cytotoxic profile (134, 135). Plasma IFN-α levels correlated indeed with activation and cytotoxic activity (CD107a) of NK cells and plasma IL-15 with the proliferation (Ki-67) of NK cells in LN during acute SIVagm infection (70). During the acute phase of SIVagm infection, $CD107a^+$ NK cells increased to higher levels in LN than in blood (70). Studies in SM demonstrated a more rapid activation of NK cells compared to macaques (136, 137). These previous studies raised the hypothesis that NK cells may play a role in LN viral control in natural hosts. It was subsequently shown that upon SIVagm infection, NK cells change their distribution within LN and migrate into follicles, where they accumulate (49). The increase of NK cell numbers in follicles was associated with a high production of IL-15 within follicles, presented in membrane-bound form by FDC and antigen-presenting-like cells (49). By contrast, the number of functionally competent NK cells in LN decrease in macaques in response to SIV infection (49, 138). The pattern of LN homing receptors (CX3CR1, CD62L, CXCR3, CCR7) were similar on NK cells from SIV-infected AGM and MAC and do not explain the higher levels of NK cells in LN of AGM as compared to MAC (49). It is more likely that in SIVagm infection, the IL-15 in the follicles enhances the survival of NK cells. Interestingly, SIVagm-infected AGM showed high levels

of CXCR5$^+$ NK cells in LN (49). This suggests that migration of NK cells into AGM follicles was CXCR5-mediated. The presence of CXCR5$^+$ NK cells was observed in secondary lymphoid organs (LN, spleen), but not in blood or gut of SIV-infected AGM. Thus, the CXCR5 expression on NK cells during SIVagm infection was tissue-specific. Of note, this enrichment of CXCR5$^+$ NK cells in secondary lymphoid organs was not observed in SIVmac-infected macaques. Strikingly, IL-15-mediated depletion of NK cells in chronic SIVagm infection led to high viral replication in the follicles as well as in the T zones (49). These results indicate that T_{FH} cells are not resistant to SIV infection in AGM and clearly reveal a crucial role for NK cells in the viral control within LN of a natural host.

CONCLUDING REMARKS

Herein, we summarize current knowledge on differences in LN of non-natural versus natural hosts. The remarkable control and clearance of virus from lymphoid follicles in natural hosts is associated with multiple differences compared to pathogenic infection: (1) LN architecture is preserved; (2) inflammation is controlled; (3) FDC network is maintained intact; (4) rapid mobilization of innate antiviral responses; (5) viral replication is strongly controlled; (6) T_{FH} are particularly spared from virus; (7) NK cells migrate into follicles; and (8) high IL-15 production within follicles (**Table 1**). Collectively, natural hosts have developed mechanisms of protection for the most vulnerable lymphoid CD4$^+$ T cell subsets: CD4$^+$ TCM, T_{FH} cells in LN, and Th17 cells in gut (35, 139). Better preservation of these cells likely influences the preservation of intact lymphoid structures, immune competencies, and immune memory (49, 55). As a control model for lentivirus infections, we must ask how we might exploit the knowledge garnered from natural host research. Given the IL-15-dependent accumulation of NK cells (and potentially CD8$^+$ T cells) in natural hosts into follicles, this could be envisioned therapeutically to recapitulate virus clearance in pathogenic hosts and HIV patients. Multiple oncology studies are now exploring the utility of IL-15 superagonists and heterodimers to expand both CD8$^+$ T cells and NK cells and recent studies evaluated these molecules in the SIV macaque model (140–143). Additional cytokine therapeutics (i.e., IL-21 and IFN-α) could also be attractive targets to mimic or induce the conditions in natural hosts that are conducive to virus clearance in the LNs. Recently, the use of NKG2A inhibitors has also been suggested as an attractive approach in HIV cure strategies (144). Many open questions remain, including delineation of factors responsible for the high IL-15 production in LN follicles, the maintenance of an intact FDC network, the upregulation of CXCR5 on NK cells in LN and the very rapid innate antiviral responses in natural hosts. The remaining gaps in the knowledge base will require future studies to understand how natural hosts reduce inflammation and how they protect LN architecture. Such ongoing studies are hoped to direct future strategies aimed at granting permissive entry of relevant effector cells into the highly restricted lymphoid follicles, thus creating a unique opportunity for reservoir clearance and representing a further step toward HIV remission and cure. Altogether, studies in natural hosts of SIV continue to reveal clues highly relevant for understanding and managing HIV infection in humans.

AUTHOR CONTRIBUTIONS

NH, SB, MP, RR, and MM-T wrote the review. NH designed the figure and the table. RR and MM-T edited the text. MM-T composed and oversaw the chapters.

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REVIEW
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Visualizing the Immune System: Providing Key Insights into HIV/SIV Infections

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Immunological inductive tissues, such as secondary lymphoid organs, are composed of distinct anatomical microenvironments for the generation of immune responses to pathogens and immunogens. These microenvironments are characterized by the compartmentalization of highly specialized immune and stromal cell populations, as well as the presence of a complex network of soluble factors and chemokines that direct the intra-tissue trafficking of naïve and effector cell populations. Imaging platforms have provided critical contextual information regarding the molecular and cellular interactions that orchestrate the spatial microanatomy of relevant cells and the development of immune responses against pathogens. Particularly in HIV/SIV disease, imaging technologies are of great importance in the investigation of the local interplay between the virus and host cells, with respect to understanding viral dynamics and persistence, immune responses (i.e., adaptive and innate inflammatory responses), tissue structure and pathologies, and changes to the surrounding milieu and function of immune cells. Merging imaging platforms with other cutting-edge technologies could lead to novel findings regarding the phenotype, function, and molecular signatures of particular immune cell targets, further promoting the development of new antiviral treatments and vaccination strategies.

Keywords: HIV, lymph nodes, mucosa, immune cells, T cells, imaging

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INTRODUCTION

Investigation of the human immune system in the context of infectious diseases has been accomplished primarily based on studies utilizing circulating cells. However, use of such biological material may not capture the *in vivo* timing or mechanisms governing the initiation and development of immune responses to pathogens at important anatomical sites, such as secondary lymphoid organs, mucosal-associated lymphoid tissues (MALTs), and mucosae. Therefore, the need for comprehensive analysis of tissues central to disease pathogenesis, and interactions between theses tissues, is of great importance. The application of multidimensional methodologies, like polyparametric flow cytometry, has provided critical information regarding the phenotype and functionality of tissue-resident immune cells, especially T and B cells (1–5). Despite their analytical power, these methodologies cannot address the tissue distribution/localization of lymphoid populations *in vivo*, as well as the anatomical context in which their highly dynamic interactions occur. On the other hand, tissue investigation using histopathological assays, like immunohistochemistry, has provided critical information regarding the impact of HIV/SIV on the organization of the human immune system at a tissue level (6–14).

Imaging technologies are continuingly advancing, with new hardware (i.e., new types of cameras, laser lines, hybrid detectors, etc.) and software, improving the quality of images obtained at the

level of acquisition, segmentation, and deconvolution of cells. Furthermore, the availability of steadily increasing antibody specificities and appropriate labels/probes further facilitates the application of imaging technologies to biological material. The introduction of advanced imaging technologies, such as multispectral confocal (15) and multiphoton microscopy, as well as imaging mass cytometry, positron emission tomography (PET), and magnetic resonance imaging (MRI) (16–18), opens new opportunities for the investigation of molecular and cellular events at dimensions that range from the nanoscale to the entire body and for visualizing the dynamic changes occurring in living tissues and individuals. Furthermore, the availability of technologies like stimulated emission depletion microscopy (STED) can provide unprecedented resolution (~20–50 nm) using light microscopy (19) for the detailed analysis and quantification of molecular dynamics at a subcellular level (20). Therefore, the application of cutting-edge imaging technologies can provide substantial novel insights into host–pathogen interactions that are simply not feasible with other approaches (**Table 1**), which may be critical for the development of vaccines, especially those aiming to elicit broadly neutralizing antibodies, as well as for the discovery of novel immunotherapy targets to eliminate HIV.

WHY DO WE NEED TISSUE IMAGING?

Secondary lymphoid organs (i.e., lymph nodes and spleen) and MALT create an extended tissue network that provides a unique microenvironment for pathogen capture, antigen presentation, and induction of adaptive immune responses (21–23). The *ex vivo* and *in vitro* analysis of cells derived from such tissues using powerful methodologies like polyparametric flow cytometry and sequencing of sorted cell subsets has provided important information about the character and molecular profile of cells involved in the development of these responses (4, 15, 24, 25). The application of imaging technologies, however, can provide relevant information about cell populations in their "natural environment" and with respect to their spatial positioning, displacement, surrounding cells, and milieu microenvironment. Furthermore, estimating the possible role of parameters, like cell shape and polarization (26), in the biological process under investigation is impossible for cells removed from their natural tissue microenvironment. To this end, the combination of *ex vivo* organ culture models (27) with imaging analysis and whole-body *in vivo* studies would significantly increase our knowledge about the role of particular cells and soluble factors in HIV/SIV pathogenesis.

The compromised immune response against pathogens in subjects with genetic defects that affect the architecture and development of follicles demonstrates the importance of tissue integrity for an effective response against pathogens (28–30). It is well established that HIV/SIV infections are associated with extensive changes/damage of tissue architecture, especially in LNs and gut mucosa (31). Stromal cells, like fibroblastic reticular cells (FRCs) and follicular dendritic cells (FDCs), represent critical elements of the lymphoid tissue architecture, which are significantly affected by HIV/SIV (32–35), and because of their biology and function forming extended interdigitating networks within the follicular (FDC) (36) and extra-follicular (FRC) (37) areas, their isolation and *in vitro* analysis is challenging. Thus, imaging these stromal elements in their native intact tissue environments, with 3D volumetric analysis, will likely be essential to fully understand the importance of these networks in HIV/SIV infections. A comprehensive understanding of tissue perturbations in terms of cellularity and architecture will further elucidate defects in adaptive cellular responses and in the generation of antibody responses with functionalities that effectively control the virus, including broadly neutralizing antibodies.

The development of effective adaptive immune responses against pathogens is a multistep process that requires the orchestrated function of several cell types and soluble factors within the LN environment. A critical aspect of this process is the compartmentalization of immune cell subsets with different origins or maturation status, as well as the presence of chemokine gradients that direct this compartmentalization and trafficking of cells between and within areas of LN. For example, the development of high-affinity, antigen-specific B-cell responses requires interactions between CD4 + T cells and B cells in the follicle. The identification of human follicular helper CD4 + T cells (Tfh) revealed a highly specialized CD4 + T-cell subset with a unique phenotypic, functional, and molecular signature (38–40). Still, Tfh cells represent a heterogeneous cellular population with different combinations of expressed surface receptors, such as PD-1, CD150, and CD57 (4, 41, 42). Likewise, follicular B cells represent a diverse population with different phenotypic profiles depending on their localization in germinal cell areas [light and dark zone (43, 44)]. Analysis of these populations based on their phenotype using flow-cytometry assays has been particularly informative with respect to their relative frequencies and dynamics in human and animal disease models. However, their phenotype does not always indicate their localization within tissue microenvironments. For example, although the dark zone is the site where B-cell division takes place, many proliferating (Ki67 +) B cells can be found in the light zone, and under physiological conditions, Tfh subsets have a distinct localization pattern (**Figure 1**). This type of imaging analysis can provide additional unique information regarding the juxtaposition/clustering of Tfh cells and B cells, the "polarization" pattern of germinal centers, as well as the distribution of Tfh cells, B cells, and FDCs within these LN follicular areas. Investigation of the impact that HIV/SIV infection has on the microanatomy of tissue environments could provide information about the cellular and molecular mechanisms mediating the development of humoral responses, as well as the local interplay between the host and virus during HIV/SIV disease progress.

The introduction of novel imaging technologies could generate new perspectives regarding the role of particular immune cell

TABLE 1 | Importance of performing tissue imaging.

- Cells in their natural environment
- Tissue architecture, stromal cells
- Compartmentalization of immune reactivity (immune cells, soluble factors)
- Complexity of local immune dynamics
- Displacement of cells and duration of local interactions
- Dynamics and mechanisms of virus transmission
- Generation of new questions
- Tissue pathology and damage

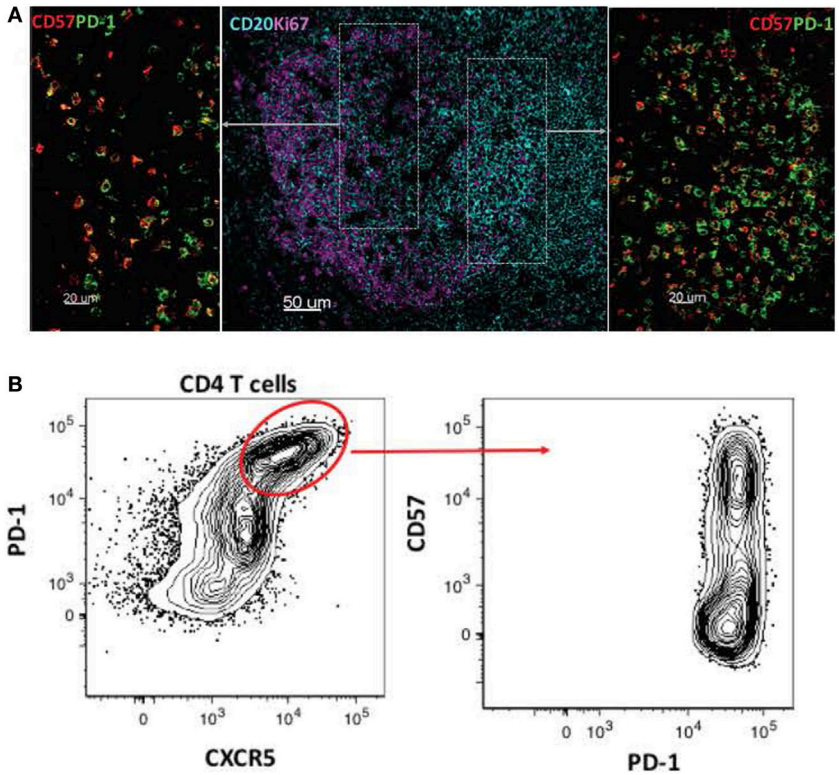

FIGURE 1 | Heterogeneity of follicular cell populations. **(A)** Confocal images showing the relative distribution of proliferating B cells (CD20hi/dimKi67hi, CD20/cyan, and Ki67/magenta) and CD4 Tfh subsets (PD-1hiCD57hi, PD-1hiCD57lo, PD-1/green, and CD57/red) in a tonsillar follicular area. **(B)** Flow-cytometry plots showing the phenotype of tonsillar Tfh subsets based on the combined expression of PD-1, CXCR5, and CD57 surface receptors.

subsets. Traditionally, the quality of CD8 + T cells in HIV/SIV infection has been evaluated based on (i) their capacity to produce multiple cytokines (poly-functionality) (45), (ii) the expression of an "exhausted" phenotype related to their function and survival/proliferative capacity (46–48), and (iii) their potential for killing infected targets, mainly through perforin/granzyme protein expression (49, 50). However, an effective CTL response requires trafficking of activated and differentiated CD8 + T cells in areas with HIV/SIV-infected cells, followed by their ability to sense, efficiently engage the infected cells and perform their CTL effector function. Development of imaging-based methods allowing for the evaluation of such biological processes/steps (10) could provide a comprehensive analysis of CTL responses in HIV/SIV infection and contribute to a holistic view of the efficiency of adaptive responses needed for virus elimination.

WHAT CAN WE LEARN FROM HIV/SIV INFECTIONS?

The Host: Lymph Nodes

Classic immunohistochemistry studies have provided valuable information regarding the impact of HIV/SIV infection on the structure of tissues, such as LNs and gut mucosa. Early histological studies revealed lymphoid tissue pathologies (i.e., follicular hyperplasia, follicular lysis, and depletion and fibrosis) that are hallmarks of HIV infection (31, 51). Further work demonstrated a process of progressive deposition of fibrotic collagen, beginning early after HIV infection, driven by TGFβ regulatory CD4 + T cells (34, 52, 53), leading to the loss of stromal cells, like FRCs, and CD4 + T-cell populations (34). Additional significant changes take place in the follicular areas, manifested as enlarged/less-defined follicles and germinal centers, with presumably an important effect on Tfh cell dynamics.

Follicular Helper CD4 + T Cells (Tfh)

Follicular helper CD4 + T cells represent a highly differentiated CD4 + T-cell subset with a unique phenotype (4, 40, 41, 54, 55) and molecular signature (4, 39), which provides critical help to follicular B cells during the development of B-cell responses against pathogens and immunogens (44). The chronic phase of HIV/SIV infection is characterized by accumulation of Tfh cells, at least in a group of individuals (4, 56, 57). Furthermore, SIV infection has a significant impact on the gene signature of Tfh cells, characterized by increased expression of IFNγ- and TGFβ-related genes (4). Imaging studies have facilitated the characterization/localization of Tfh cells within the follicular areas during HIV/SIV infection, based on the expression of surface receptors like PD-1 and CXCR5 (4, 8, 13, 58, 59). Furthermore, imaging analysis has revealed that Tfh cells populate different areas of the follicle (marginal zone, surrounding GC, or mainly within the light zone) (**Figure 1**) (4, 8, 58), presumably exposed to different

local signals. Given the dramatic effect of HIV/SIV infection on the LN structure and follicular organization, imaging provides critical information about the distribution of Tfh cells within the follicular areas, as well as their proximity and engagement with B cells. The cellular and molecular mechanisms regulating the dynamics of Tfh cells during HIV/SIV infection are not well understood (4, 60). To this end, tissue imaging can contribute valuable information regarding:

1. The heterogeneity of follicular cells (Tfh subsets like Th1-like Tfh cells (61), dark zone vs. light zone B cells, etc.) and how these populations are impacted during different stages of infection.
2. The possible role of local immune activation/inflammation on T- and B-cell dynamics during HIV/SIV disease progression.
3. The impact of follicular damage/alteration (i.e., loss of FDC) on Tfh and B-cell dynamics.
4. The possible role of locally expressed cytokines (i.e., IL-21, TGFβ, IL-10) or chemokines (i.e., CXCL-13) on Tfh, B-cell dynamics.
5. The distribution of Tfh-infected cells and the dynamics of local HIV/SIV replication.
6. The impact of LN pathologies (i.e., fibrosis, etc.) on LN function (i.e., antigen capture, vaccine responses, etc.).
7. The impact of cART and HIV cure strategies on the FDC reservoir.

Follicular CD8 + T Cells

Chronic HIV/SIV infections are characterized by accumulation of CD8 + T cells in the LN and particularly in the follicle, a process referred to as "follicular lysis" (15, 24, 62). Imaging studies have shown that trafficking of virus-specific CD8 + T cells into the follicular area is relatively compromised (63–65). Flow-cytometry-based assays have shown that similar to Tfh cells, follicular CD8 + T cells are characterized by low expression of CCR7 and upregulated CXCR5, and have a unique transcriptional profile (15, 24). What regulates the trafficking of CD8 + T cells, particularly the cytotoxic effector cells, into the LN and distinct microenvironments, like follicles, is not well understood. It was recently shown that expression of viral proteins *per se* may not represent the main force behind this trafficking (15). Imaging analysis can provide critical information regarding the role of local inflammatory cells/signals as mediators of CD8 + T-cell trafficking in the follicular areas during HIV/SIV infection, potentially leading to novel targets for the *in vivo* manipulation of LN CD8 + T-cell dynamics. FRCs provide the cellular network for trafficking of T cells in the T-cell zones (37). Chronic HIV/SIV infection is associated with significant damage to both FRC (34) and follicular structures (31, 33). Whether this tissue damage creates an environment where T-cell trafficking becomes highly stochastic and/or dysfunctional is not known. Thus, imaging studies could be highly informative in addressing these unresolved questions, for example, by assessing the relationship between the magnitude of FDC changes, follicular lysis, and altered chemokine gradients on one hand with follicular CD8 + T-cell enrichment on the other hand.

Innate Immunity Cells

Innate immune cells play an important role in HIV/SIV infections and disease at multiple levels, including (i) virus capture and dissemination (66), (ii) expression of pro- and anti-inflammatory mediators (i.e., IFNα/β, TNFα, IL6, IL10, etc.) (67–71), and (iii) expression of pro-inflammatory chemokines (i.e., IP-10, MCP, MIP-1α/β, etc.) (72, 73). Flow-cytometry studies have shown an increased recruitment of hyporesponsive monocyte/macrophages and plasmacytoid dendritic cells early after SIV infection that could affect the ability of IFNα production in the LN (74–76). Complementary to flow-cytometry data, imaging studies have revealed an accumulation of monocytic-lineage cells in areas surrounding the follicle and in close proximity to CD8 + T cells in chronic HIV infection (15) as well as in pathogenic SIV infection of rhesus macaques but not in non-pathogenic SIV infection in nature hosts (i.e., sooty mangabeys) (77). Furthermore, pharmacological manipulation of monocyte activation results in reduced recruitment of activated monocytes to the LN and reduced viral replication (78). More recently, tissue imaging has shown that infected macrophages could contribute to the rapid disease progression in SIV-infected non-human primate (NHP) infants (79). While monocytes/macrophages can clearly become infected with HIV/SIV, the relative contribution of infected monocytes/macrophages as long-lived viral reservoirs *in vivo* is still an open question. Novel, high-resolution imaging approaches allowing for the simultaneous detection of viral RNA and DNA could shed light upon this issue. Furthermore, it is not known if the viral dynamics of infected monocytes/macrophages occurs in a similar fashion in LNs from different anatomical sites, for example, comparing axillary and mesenteric LNs or MALT (80), and warrants further investigation.

Natural killer (NK) cells play an essential role in antiviral immunity, but knowledge of their function in secondary lymphoid organs is incomplete. Contrary to SIV-infected macaques, *in situ* approaches demonstrated that NK cells in secondary lymphoid organs from chronically SIVagm-infected African green monkeys (AGMs) were frequently CXCR5 + and entered and persisted in lymph node follicles where they seem to play a major role in viral reservoir control (81). The relative positioning/compartmentalization of innate cells and associated soluble factors could inform on the role of these cells in the generation and maintenance of effective adaptive immune responses during HIV/SIV.

The Host: Mucosa

Mucosal barriers are the body's first defense against external pathogenic threats. Although they represent the boundary between the external environment and the host, mucosal surfaces are often the sites of pathogen transmission (82). In the context of HIV infection, mucosal surfaces represent the major routes of transmission, with the most relevant mucosal tissues being the genital mucosa and gastrointestinal tract (82). Imaging studies utilizing SIV NHP models have been absolutely instrumental in dissecting key aspects of HIV-1 transmission across mucosal surfaces and the early events surrounding mucosal infection, including (i) understanding the unique cellular composition and characteristics of different mucosal tissues and their susceptibility to viral transmission, (ii) defining the early host–viral dynamics within mucosal tissues, including characterizing the principal target cells *in vivo*, and (iii) demonstrating the process and principal pattern of viral dissemination and establishment (83–86).

Disruption of the intestinal barrier and subsequent microbial translocation and inflammation is one of the hallmarks of HIV/SIV pathogenesis and disease progression (87–89). Damaged epithelial integrity (90, 91), as well as the loss of relevant cells from the gut mucosa (92–94), has been associated with HIV/SIV pathogenesis. Imaging studies have been instrumental in investigating the impact of these tissue perturbations in the context of HIV/SIV infections. Besides the documentation of the magnitude of barrier damage, imaging studies have shown: (i) the possible role of gut macrophages with respect to their phagocytic activity (80, 91) or capacity to produce pro-inflammatory cytokines (95) in chronic immune activation and progression to AIDS, (ii) that blocking microbial translocation can reduce viral replication and dissemination in LNs (96), and (iii) that barrier damage and microbial translocation differentiate pathogenic and non-pathogenic SIV infections (91). Furthermore, tissue imaging has been very informative concerning the verification of animal models for SIV pathogenesis––such as the use of pigtail macaques (97) or experimental colitis as an alternative model to investigate the impact of barrier integrity in SIV pathogenesis (16). Similar to LNs, imaging studies will be instrumental in our understanding of the local interplay between the virus and innate/adaptive immunity.

The Host: Other Tissues

Besides lymphoid organs, other tissues have also been shown to play a role in the pathogenesis of HIV/SIV infections. Imaging studies have been instrumental for our understanding of the CD3 + (98) and CD8 + T cell (99), NK (100) as well as myeloid (Kuppfer) cell (101) dynamics in liver during SIV infection. *In situ* hybridization imaging assays have also shown insufficient viral replication in liver (99, 101). Besides the liver, RNA *in situ* hybridization imaging has been widely used for the detection of cells harboring transcribed virus in several tissues including the following:

(1) adipose tissue and specifically in the stromal vascular fraction (6);
(2) lungs, where macrophages represent a main source of virus production in infant NHP (79, 102) with lung tissue damage associated with infection of interstitial rather than alveolar macrophages (103); and
(3) brain (104) in line with other assays showing that CNS macrophages represents a latent reservoir in cART-treated animals. Confocal imaging of protein markers has revealed the heterogeneity and possible role of monocytes/macrophages, especially recently infiltrating cells, in HIV/SIV encephalitis (105, 106). Increased frequency of perivascular proliferating macrophages (107) could account for the accumulation of macrophages in SIV-infected animals. CNS lesions, found in monkeys receiving cART, were associated with inflammation dominated by lymphocyte and low levels of SIV RNA in the brain (108). Complementary to these imaging studies, use of laser capture microdissection revealed a compartmentalization of viral sequences in brain from animals infected with a neurotropic virus (109). In addition to tissue imaging assays, MRI-based methodologies have been widely used for the study of HIV/SIV neuropahtogenesis (110–113) as well as the *in vivo* viral dynamics in the brain of experimental models (17).

Host–Virus Interplay

A major obstacle for HIV eradication is the establishment of long-lived viral reservoirs, particularly in "immunologically privileged" areas, like B-cell follicles (114, 115). Therefore, the molecular characterization of cells contributing to these reservoirs, as well as their tissue topology, is of great importance in the development of novel strategies for virus reactivation and elimination. Sensitive PCR-based assays have contributed significantly to our knowledge regarding the dynamics/kinetics of virus replication, the efficacy of cART, and the characterization of cell subsets harboring actively transcribed or latent virus (116–119). Early studies have shown sequestration of viral RNA in follicles using *in situ* hybridization techniques (120). More recently, novel next-generation *in situ* hybridization platforms have been developed with great potential for the comprehensive analysis of viral reservoirs at a tissue level. These platforms allow for the detection of viral RNA (RNAscope) and/or viral DNA (DNAscope) (114, 121, 122). Merging this technology with multispectral confocal microscopy will allow for a comprehensive analysis of (i) the viral reservoir with respect to relevant molecular markers of cells harboring the virus, (ii) the local microenvironment (surrounding immune cells, inflammatory cells, cytokines/chemokines), and (iii) virus dynamics (based on the simultaneous detection of viral RNA, DNA, and viral particles).

In addition to identification of individual cells harboring virus at a tissue level, imaging assays have contributed significantly to our understanding of viral dynamics *in vivo*. Confocal imaging has provided important information regarding viral transmission across and infection in the female reproductive tract (86), as well as revealed that Th17-lineage CD4 + T cells as a preferential target for the virus early after vaginal inoculation (12). Application of technologies like whole-body immune-PET has provided additional insight into the distribution of virus among different organs in chronic SIV infection (123), as well as the impact of antiretroviral or immune-based treatments on viral dynamics (123, 124). Non-invasive whole-body imaging, although of relatively low resolution, provides a "real time" and non-invasive monitoring of viral or relevant immune cell dynamics and could guide the performance of tissue imaging assays for a high-resolution analysis of related cells. Additional information can be obtained from whole-body PET-TDM for drug distribution dynamics (125) allowing the identification of pharmacological sanctuaries, drug interactions and helping the optimization of drug delivery use and drug design.

Which Imaging Platform?

Today, several imaging technologies and platforms are available for tissue analysis. Several factors should be taken into consideration regarding the choice of the most relevant platform, including the following:

(i) The scientific question under investigation: tissue cell composition and viral reservoirs [light microscopy, confocal

microscopy, ion beam imaging (126), Laser Capture Microdissection (127)], subcellular structure and virus–host protein interactions (confocal microscopy, electron microscopy, high-resolution optical imaging technologies), or assessment of cellular and viral dynamics at organ or whole-body level [MRI (17), PET scan (123), confocal endoscopy].

(ii) The requirement for high-resolution, "volumetric" analysis or live imaging (two-photon microscopy) to address the biological process under investigation. The introduction of the two-photon intravital microscopy in the NHP SIV model could revolutionize the field of HIV/SIV pathogenesis and vaccine development by providing real time, *in vivo* measurements of immune cell trafficking, tissue cell dynamics, and interactions between host cells and virus.

(iii) The ability to simultaneously use multiple probes (dimensionality), allowing for the comprehensive analysis of several cells, proteins, and RNA/DNA sequence within the same imaged plane (confocal or imaging mass cytometry). Although high-resolution technologies like electron microscopy-based platforms or super resolution confocal microscopy can provide unprecedented information for the tissue, cell structure, and molecular dynamics, they are lacking their capacity to simultaneously use multiple probes, at least in their current form.

(iv) The potential for "fusion" with other high-throughput platforms. Although current confocal microscopy assays can visualize several probes simultaneously (15), the selection of probes/antibodies is hypothesis-driven. Merging multiplexed confocal microscopy assays with technologies like tissue imaging mass cytometry (128) could provide unique information at multiple levels, including unbiased pathway analysis, discovery of novel therapeutic molecular targets, and pharmacokinetics of antiretroviral regimens.

Modern imaging technologies allow for the acquisition of high-dimensional data. To foster new discoveries derived from such data sets, the development and application of sophisticated algorithms is required. Accurate tissue reconstruction (using advanced 3D tomography algorithms) (129, 130), quantitative analysis of imaging objects using platforms like histocytometry (15, 131), algorithms allowing for the fusion of imaging with other high-throughput platforms (128), as well as modeling tissue cell and virus dynamics based on imaging data could significantly improve our understanding of the highly complex tissue immunobiology, especially during HIV/SIV infection.

Although tissue imaging is a powerful tool, we should keep in mind that there are also limitations that could lead to misinterpretation of tissue immune dynamics. Limited access to tissue material, especially from human subjects, represents a major limitation for tissue analysis. Collecting images from one or two random tissue sections could potentially lead to inaccurate measurements ("sampling error"). Ideally, application of novel, large-volume imaging techniques, like optically cleared tissue imaging (132), could overcome the "sampling error" limitation. However, the need for multiple assays and measurements from usually limited tissue material precludes these types of imaging applications, especially when human tissues are under investigation. Therefore, one should be very cautious with the interpretation of imaging data generated from limited tissue sections. One common practice for the validation of imaging data could be their comparison to data derived from other types of assays, such as flow cytometry.

Future Directions

Imaging studies have significantly improved our understanding of cellular and molecular mechanisms for HIV/SIV pathogenesis both in humans and NHP SIV models (133). Several imaging studies have validated SIV infection of NHPs as models for HIV pathogenesis, including the illustration of early events resulting in HIV/SIV transmission (86, 134, 135), the documentation and role of gut mucosal barrier damage in HIV/SIV pathogenesis (89, 91), as well as the impact of infection in secondary lymphoid tissues (31). Particularly for lymph node dynamics, a similar profile for follicular CD4 + (4, 56, 57) and CD8 + T cells (15, 24) has been shown in infected humans and NHPs, while *in situ* hybridization assays have established the importance of these sites for virus persistence (64, 122). Given the difficulty in obtaining human tissues from different anatomical sites, the performance of imaging studies in SIV models will continue to provide unpresented information regarding the anatomical compartmentalization of these immune dynamics.

We are witnessing a boom of imaging technologies that expand our capacity for comprehensive spatial analysis of tissue cells and molecules with high definition. Given the complexity of tissue immunobiology, the performance of different imaging-based assays, as well as their merging with other high-throughput assays, is of great importance for the generation of high-dimensional data. Besides the characterization of virus and cells at the tissue level, imaging technologies could prove useful in the analysis of other biological parameters, such as metabolic status, monitoring of therapeutics (pharmacokinetic studies), or novel immunotherapies (i.e., administration of multi-specific antibodies). Although HIV/SIV infections lead to major changes in tissue architecture, imaging the immune system in infected humans and NHPs can potentially provide insight into the overall anatomy and organization of the immune system in disease and contribute to the generation of a human cellular atlas.

AUTHOR CONTRIBUTIONS

All authors have contributed equally to this work.

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REVIEW
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The B-Cell Follicle in HIV Infection: Barrier to a Cure

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The majority of HIV replication occurs in secondary lymphoid organs (SLOs) such as the spleen, lymph nodes, and gut-associated lymphoid tissue. Within SLOs, HIV RNA$^+$ cells are concentrated in the B-cell follicle during chronic untreated infection, and emerging data suggest that they are a major source of replication in treated disease as well. The concentration of HIV RNA$^+$ cells in the B-cell follicle is mediated by several factors. Follicular CD4$^+$ T-cell subsets including T-follicular helper cells and T-follicular regulatory cells are significantly more permissive to HIV than extrafollicular subsets. The B cell follicle also contains a large reservoir of extracellular HIV virions, which accumulate on the surface of follicular dendritic cells (FDCs) in germinal centers. FDC-bound HIV virions remain infectious even in the presence of neutralizing antibodies and can persist for months or even years. Moreover, the B-cell follicle is semi-immune privileged from CTL control. Frequencies of HIV- and SIV-specific CTL are lower in B-cell follicles compared to extrafollicular regions as the majority of CTL do not express the follicular homing receptor CXCR5. Additionally, CTL in the B-cell follicle may be less functional than extrafollicular CTL as many exhibit the recently described CD8 T follicular regulatory phenotype. Other factors may also contribute to the follicular concentration of HIV RNA$^+$ cells. Notably, the contribution of NK cells and γδ T cells to control and/or persistence of HIV RNA$^+$ cells in secondary lymphoid tissue remains poorly characterized. As HIV research moves increasingly toward the development of cure strategies, a greater understanding of the barriers to control of HIV infection in B-cell follicles is critical. Although no strategy has as of yet proven to be effective, a range of novel therapies to address these barriers are currently being investigated including genetically engineered CTL or chimeric antigen receptor T cells that express the follicular homing molecule CXCR5, treatment with IL-15 or an IL-15 superagonist, use of bispecific antibodies to harness the killing power of the follicular CD8$^+$ T cell population, and disruption of the follicle through treatments such as rituximab.

Keywords: B cell follicle sanctuary, follicular dendritic cell, gamma delta T cells, NK cells, cytotoxic T-cell (CTL), HIV cure research, T follicular helper cell subsets, regulatory T cells

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THE B-CELL FOLLICLE IN UNTREATED AND TREATED DISEASE

HIV and SIV RNA$^+$ cells are concentrated in B-cell follicles of secondary lymphoid organs (SLOs) during chronic untreated infection prior to the development of AIDS (1–3). In chronic HIV disease, a follicular CD4$^+$ cell is ~30–40-fold more likely to harbor HIV RNA than an extrafollicular CD4$^+$ cell (2, 4). The follicular concentration of RNA$^+$ cells has only been examined in lymph nodes (LNs) during HIV infection, but it has also been observed in other SLOs such as the spleen,

and gut-associated lymphoid tissue (GALT) during chronic SIV infection of rhesus macaques (3). Notably, in advanced disease, the follicular concentration of virus-producing cells wanes, and lymphoid tissue architecture is increasingly disrupted demonstrating follicular depletion and involution (3, 5). During antiretroviral therapy (ART), the frequency of HIV RNA$^+$ cells in LNs and B-cell follicles is reduced and lymphoid architecture is at least partially restored (6). However, RNA$^+$ cells are still detected in B-cell follicles during ART in both HIV and SIV infection (7–9). It remains controversial whether HIV RNA$^+$ cells found in SLOs during ART are the result of successful new infection or reactivation of latently infected cells. It has been reported that ART concentrations are suboptimal in LNs, which may allow continued cycles of replication (10). However, evidence of ART resistance mutations arising from SLOs of well-suppressed patients is lacking, as might be expected with suboptimal ART concentration (6, 11, 12). Regardless, after ART cessation, viral rebound occurs in SLOs in both HIV and SIV infection (13, 14). Given the concentration of HIV/SIV RNA$^+$ cells in the B-cell follicle during ART, it seems likely that the HIV reservoir in B-cell follicles contributes to viral rebound post-ART cessation.

SUSCEPTIBILITY OF FOLLICULAR HELPER T-CELLS TO INFECTION

The dominant follicular CD4$^+$ T-cell subset in B-cell follicles is T-follicular helper cells (TFH). TFH are essential for the development of germinal center (GC) reactions and the subsequent affinity maturation, class switching, and differentiation into memory subsets of B-cells (15). TFH are defined by expression of the master transcription factor BCL-6, constitutive expression of the follicular homing receptor CXCR5, and production of IL-21, which mediates B-cell class switching (16–21). Interestingly, during HIV infection, TFH cells expand numerically, but have diminished function (22, 23). TFH also serve as a major reservoir of HIV and SIV RNA$^+$ and DNA$^+$ cells during chronic infection (2, 4, 22, 24). TFH are more susceptible to HIV infection than extrafollicular (EF) CD4$^+$ T-cell subsets *ex vivo* (25–27). It should be noted that the relative susceptibility of TFH to HIV infection has mainly been determined by spinoculation. While spinoculation is an efficient way to facilitate HIV/lentiviral infection, it is not necessarily representative of *in vivo* infection pathways. During chronic HIV infection, TFH reside in close proximity to follicular dendritic cells (FDCs) decorated with HIV-immune complexes (ICs), which may serve as a major route of infection *in vivo*. Importantly, TFH are also more susceptible to FDC-bound HIV-IC-mediated infection than EF subsets (28).

T-follicular helper cells can be further subdivided into a GC-localized subset defined by high expression of PD-1, and a non-GC subset defined by intermediate or low expression of PD-1. GC TFH are even more susceptible to HIV infection than non-GC TFH *ex vivo* (25). This finding was supported with the observation that *in vivo* HIV RNA$^+$ cells are more concentrated in GCs than non-GC areas of the B-cell follicle, or EF regions (25). More recently, a T-follicular regulatory subset of CD4$^+$ T cells (TFR) was discovered in humans (29–31). TFR limit the GC reaction and antibody production to prevent autoimmunity (31). Chronic HIV and SIV infection cause TFR to expand numerically (32). *Ex vivo*, HIV infection causes TFR to increase expression of regulatory molecules and more potently inhibit TFH function (32). It is therefore likely that HIV-related TFR expansion contributes to poor TFH function in HIV-infected individuals. TFR are also more susceptible to R5-tropic HIV infection *ex vivo* and contain the highest percentage of HIV RNA$^+$ cells compared to EF, TFH, and EF Treg CD4$^+$ subsets (26).

Several mechanisms have been proposed to be related to increased permissivity of TFH and TFR. TFH have been shown to have increased expression of the antiapoptotic protein BCL-2 when infected with R5-tropic HIV compared with EF CD4$^+$ T-cell subsets (33). Furthermore, the TFH master transcription factor BCL-6 mediates constitutively diminished expression levels of interferon-stimulated genes important in antiviral immunity (27). The enhanced susceptibility of TFR compared to TFH to HIV R5-tropic infection is likely partially mediated by higher levels of CCR5 expression and an enhanced proliferative state (26).

TFH Memory

It is well established in peripheral blood that CD4 central memory (CM) and transitional memory subsets contain the majority of proviral HIV DNA during ART (34). During chronic untreated HIV and SIV infection memory, TFH in LNs are enriched for DNA$^+$ cells (22, 35). Recently, it was reported in HIV infection that during ART, PD-1$^+$ memory TFH are the major reservoir of cells harboring replication competent virus (36). Similarly, during SIV infection, ART results in the concentration of SIV DNA$^+$ cells in PD1$^+$ CTLA-4$^+$ TFH in the B cell follicle. Interestingly, in the T cell zone, ART resulted in the concentration of SIV DNA in PD-1$^-$ CTLA4$^+$ cells (37). The extremely low number of memory SIV DNA$^+$ cells that could be isolated prevented detailed quantification of the relative contribution of PD-1$^+$ CTLA-4$^+$ and PD-1$^-$ CTLA-4$^+$ memory CD4 T cells to the pool of replication competent SIV. However, replication competent virus was detected in PD-1$^+$ CTLA-4$^+$ memory CD4 T cells in seven of seven animals and in PD-1$^-$ CTLA-4$^+$ memory CD4$^+$ T cells in six of seven animals (37). Taken together, these data suggest that memory PD-1$^+$ TFH contain a large reservoir of replication competent HIV and SIV during ART.

Interestingly, a recent study demonstrated that peripheral blood TFH (pTFH) constitute the major reservoir for replication competent HIV from peripheral blood CM CD4$^+$ T cells of patients on ART (38). Furthermore, pTFH were more susceptible than non-pTFH peripheral blood CD4 T cell subsets to HIV infection *ex vivo* (38). The existence of pTFH seems like an oxymoron, given the close association of TFH with the B-cell follicle. However, pTFH express CXCR5 and are capable of stimulating B cell antibody production *via* IL-21, in a manner analogous to TFH (39). How TFH and pTFH are related in origin and function in healthy or HIV-infected individuals remains largely unknown. In mice, some pTFH appear to traffic to GCs and appear similar to TFH post immunization, suggesting that cells may be able to transition between TFH and pTFH (40).

Impairments in Humoral Immunity Mediated by TFH Dysfunction

The majority of HIV-infected individuals fail to produce broadly neutralizing antibodies (bnAbs) (41). Most bnAbs show evidence of somatic hypermutation (42). Given the critical importance of TFH to the GC reaction and resulting somatic hypermutation, it seems likely that HIV infection in the B cell follicle and the resulting changes in frequency and function of TFH and TFR contributes to the inability of most individuals to produce bnAbs. This hypothesis is supported by the observation that the induction of bnAbs in HIV-infected individuals correlated with the frequency of pTFH (43). Additionally, in rhesus macaques vaccinated with HIV envelope trimers, the quality of TFH in GCs was associated with the production of bnAbs (44). Furthermore, in SHIV-infected rhesus macaques, TFH frequencies were associated with more IgG^+ B cells in GCs and bnAb production (45). Finally, it is well established that HIV-infected individuals have poor antibody responses to vaccines (46). It was recently demonstrated in patients on ART that pTFH function correlated with the ability to respond to the H1N1/09 influenza vaccine (47). Taken together, these data suggest that defects in TFH, mediated by direct infection and/or TFR expansion, may cause systemic defects in humoral immunity.

CTL CONTROL

There is extensive evidence that effective cytotoxic T-cell (CTL) responses are critical to controlling HIV and SIV infection. The decline in viremia that occurs following acute infection correlates with the rise of HIV-specific CTL (48). Additionally, several MHC class I alleles are strongly correlated with long-term non-progression and CTLs from long-term non-progressors show enhanced antiviral activity compared to viremic individuals (49, 50). Finally, $CD8^+$ T cell depletion during SIV infection results in a spike in viremia (51, 52). Thus, CTL are essential for control of HIV replication, although in most instances unable to fully suppress it.

Early in the epidemic, it was recognized that increased numbers of $CD8^+$ cells, many of which are virus-specific CTL, are found within secondary lymphoid tissues including B cell follicles in HIV/SIV-infected individuals compared to uninfected individuals (53–57). Indeed, infiltration of $CD8^+$ T cells into B cell follicles was suggested to be a hallmark of HIV infection (58). Nevertheless, frequencies of virus-specific CTL within B cell follicles have been shown to be consistently lower than outside of the follicles (3, 4, 59–61). During acute infection before the virus-specific CTL response has matured, frequencies of HIV RNA^+ cells per mm^2 tissue are similar in extrafollicular and follicular areas. Only during chronic infection, after the maturation of the CTL responses does the concentration of HIV RNA^+ cells in the B cell follicle become apparent (3). CD8 depletion of chronically infected SIV-infected rhesus macaques demonstrated that most of the increase in SIV RNA^+ cells occurred in the extrafollicular zones, with relatively smaller increases within the follicles (62). Collectively, these findings suggest that CTLs are highly effective in suppressing HIV/SIV replication in EF areas, but not follicular areas and that the latter are relatively immune privileged sites.

While it is clear that there are significantly fewer $CD8^+$ T cells in follicular areas, it is unclear whether the $CD8^+$ T cells that do localize to B cell follicles have effective cytolytic potential. There is some evidence that $CD8^+$ T cells that localize to B cell follicles are on average less cytolytic than others. In SIV infection, follicular $CD8^+$ T cells were often in contact with Tregs, and many others expressed PD-1, suggesting they may be functionally exhausted (62). In disaggregated human tonsil cells, ~90% of $CD8^+$ T cells with a follicular phenotype ($CXCR5^+CCR7^-$) appear to be part of the newly described $CD8^+$ follicular Treg subset, This subset is defined phenotypically as $CD44^{hi}CXCR5^{hi}$, and functionally by expression of IL-10, TGF-β, and Tim-3 and low levels of perforin (63) Follicular $CD8^+$ Tregs have been demonstrated in mouse models to inhibit TFH expansion, antibody production, and autoimmunity (64, 65). $CD8^+$ follicular Tregs suppress TFH-mediated IL-21 and antibody production in response to *ex vivo* HIV infection (63). However, it is important to note that $CD8^+$ follicular Tregs have some ability to inhibit HIV replication in TFH *ex vivo*, through unknown mechanisms (63). Additionally, other reports have found that follicular CD8 T cells have potent cytolytic potential against HIV RNA^+ cells. It was recently reported by He et al. that in peripheral blood from HIV-infected individuals $CXCR5^+$ $CD8^+$ T cells expressed lower levels of exhaustion markers including Tim-3 and higher levels of IFN-γ than $CXCR5^-$ $CD8^+$ T cells (66). Importantly, the frequency of $CXCR5^+$ $CD8^+$ T cells inversely correlated with viral load. Additionally, in the LNs of HIV-infected individuals $CXCR5^+$ $CD8^+$ T cells expressed higher levels of perforin than $CXCR5^-$ $CD8^+$ T cells (66). This may suggest that although $CXCR5^+$ $CD8^+$ T cells are a relatively rare population, they may be more potently cytolytic than $CXCR5^-$ $CD8^+$ T cells. Interestingly, Petrovas et al. also found that follicular $CD8^+$ T cells from LNs of HIV-infected individuals had higher cytolytic activity that extrafollicular $CD8^+$ T cells (67). However, they also reported that follicular $CD8^+$ T cells had lower polyfunctional cytokine expression and higher expression of PD-1 than extrafollicular $CD8^+$ T cells (67).

In summary, the role of follicular $CXCR5^+$ $CD8^+$ T cells in HIV infection is still unknown. Some studies have suggested that follicular $CXCR5^+$ $CD8^+$ T cells are potently cytolytic, while others have suggested that the majority of follicular $CXCR5^+$ $CD8^+$ T cells are part of a $CD8^+$ Treg subset that exhibit poor cytolytic control of HIV producing cells and may contribute to defects in humoral immunity. The ability of follicular $CD8^+$ T cells to control HIV infection is an area requiring further elucidation.

The mechanisms mediating poor CTL accumulation and possibly poor cytolytic activity in B cell follicles remain poorly understood. However, it is known that for B cells and TFH to localize to the B cell follicle, they must express the follicular homing molecule CXCR5, which is attracted by its ligand CXCL13, which is expressed in B cell follicles. Furthermore, to migrate to the follicle TFH must downregulate CCR7, whose ligands CCL19 and CCL21 lie in the extrafollicular regions (68). Very few HIV- and SIV-specific CTL exhibit the follicular homing phenotype $CXCR5^+CCR7^-$ during chronic infection (3) (and unpublished observations). The mechanisms mediating $CXCR5^+CCR7^-$ CTL

development are still poorly understood. However, this phenotype was recently demonstrated in mice to be partially dependent on BCL-6, which is traditionally thought of as the CD4+ TFH master transcription factor (61).

FOLLICULAR DENDRITIC CELLS

Follicular dendritic cells accumulate ICs bound to Fc and complement receptors on their cell surface. FDC-bound ICs act as long-term storage for antigen, which is critical to the development of high affinity antibodies including neutralizing antibodies (69). During chronic HIV infection, antibody and/or complement bound HIV-ICs accumulate on FDCs. HIV-ICs bound to the surface of FDCs are known to be present in LNs, GALT, and spleens and are a reservoir of HIV virions of considerable magnitude (70–72). The total amount of HIV RNA stored on FDCs has been estimated to be ~10–40 times greater than the amount of HIV RNA in infected lymphocytes in untreated disease at steady state (73). Additionally, the FDC HIV reservoir persists even during ART, although at diminished levels (9, 73, 74). The necessary time for infectious HIV-IC to be cleared from FDCs during ART is a question critically important to researching a cure. To our knowledge, no reports have demonstrated FDC networks completely free of HIV virions. Years or decades of ART may be necessary to clear HIV-ICs. Alternatively, the presence of low numbers of RNA+ cells in SLOs during ART may continually reseed FDCs with HIV-ICs at a low rate and prevent FDC networks from ever being completely cleared of HIV-ICs during ART.

HIV virions bound to FDCs have been shown *ex vivo* to be potently infectious (28, 75, 76). FDC-bound HIV virions are difficult to neutralize and can remain viable *ex vivo* for at least 25 days (75, 76). In an elegant study from Dr. Burton's laboratory, mice were passively immunized with a non-neutralizing anti-gp41 antibody to allow the formation of ICs upon viral challenge. Upon challenge with HIV, HIV-ICs decorated FDCs in the LNs of these mice (75). Since murine CD4+ T cells are non-permissive for HIV infection, the FDCs could not be continually reseeded with HIV-ICs. The FDCs were then isolated at different times post challenge and co-cultured with susceptible human CD4+ T cells. The murine FDCs were able to retain infectious virions for at least 9 months (75). This duration of infectivity is remarkable considering that the infectivity of HIV virions is limited by gp120 shedding, mediated by the non-covalent nature of the gp120 gp41 complex and/or by interaction with soluble CD4 (77, 78). The shedding of gp120 by HIV virions is reduced when the virions are in ICs and bound to FDCs, but not individually (79). This suggests that the combination of IC formation and binding to FDCs preserves and protects HIV virions and prevents gp120 shedding. However, the mechanisms of this protection remain completely uncharacterized. One recent report *in vitro* suggests that FDCs endocytose HIV-IC into non-degradative compartments and that this may contribute to protection of the virions (80). It should be noted that several studies using electron microscopy from HIV-infected lymphoid tissue have reported qualitatively that HIV virions reside mostly on the surface of the FDCs (56, 72, 81, 82). However, to our knowledge, the amount of HIV-ICs on the surface of FDCs versus the amount of HIV-ICs in endosomes within FDCs has not been quantified. Additionally, even if the majority of HIV-IC are on the FDC surface at steady state *in vivo*, it is possible that HIV virions are occasionally endocytosed and recycled to the cell surface.

Follicular dendritic cells may also contribute to HIV pathogenesis by release of cytokines. TNF-α produced by FDCs results in a significant increase in HIV replication when co-cultured with infected CD4+ T cells (28). Additionally, exposure of FDCs to HIV *in vitro* results in the production of cytokines that are unfavorable to B cell survival and antibody production (83).

γδ T CELLS

γδ T cells are thymus derived cells that create TCRs by VDJ recombination, although with a much reduced diversity compared to αβ T cells (84). γδ T cells have been implicated in tumor immunity as well as microbial immunity (85). The precise ligands of γδ TCRs and the manner in which these ligands are presented is an area of intensive study. However, evidence is mounting that non-peptide pyrophosphate molecules, commonly called phosphoantigens, are a ligand of some γδ TCRs, although likely not the only class of ligand (86, 87). Phosphoantigens are produced in high concentration by both prokaryotes and transformed eukaryotic cells (86).

In adult humans, between 0.5 and 16% of CD3+ cells in peripheral blood are γδ T cells (84). In humans there are two major subsets of γδ T cell, Vδ1 and Vδ2. In uninfected humans, the Vδ2 subset is predominant, but during chronic HIV infection, the Vδ2 subset is depleted and the Vδ1 subset is expanded, becoming the predominant subset (88). Recently, it was reported that Vδ2 γδ T cells contain a reservoir of replication competent virus in patients on ART (89). In 14 of 18 patients tested, replication competent virus was recoverable from Vδ2 γδ T cells from peripheral blood (89). Freshly isolated γδ T cells from healthy adults have no or very low CD4 expression, which seemingly should preclude them from HIV infection. However, it has been demonstrated *in vitro* that immune activation can induce CD4 expression and thus susceptibility to infection. In support that this may occur *in vivo*, γδ T cells from patients acutely infected with HIV express considerably more CD4 (range, 9.5–15.9% CD4+) compared to uninfected patients (<0.3%) (89).

The ability of γδ T cells to contribute to HIV control is unclear. Several studies have demonstrated lytic properties against HIV-infected cells *in vitro* (90, 91). However, the *in vivo* significance of γδ T cells to control HIV remains less clear. One study reported that in SIV infection a higher percentage of γδ T cells in blood and the endocervix correlated with lower viremia (92).

Almost nothing is known about the ability of γδ T cells to be productively infected or to control HIV infection in SLOs during treated or untreated HIV/SIV infection. However, it is worth noting that γδ T cells have been reported to be able to adopt a TFH-like phenotype *in vitro*. These γδ T cells express BCL-6 and CXCR5 and are capable of stimulating antibody production in B cells (93, 94). In human, tonsils ~50% of Vδ2 γδ T cells express CXCR5 and appear to be able to stimulate antibody production, suggesting that the TFH-like phenotype of γδ T cells may exist *in vivo* (95). It remains unknown if these TFH-like γδ T cells play any role in HIV control or persistence. However, it is tempting to

speculate that TFH-like γδ T cells may have increased susceptibility to HIV infection similar to TFH αβ T cells. The role of γδ T cells in HIV persistence and control in SLOs and B cell follicles in particular is an area ripe for further study.

NK CELLS

NK cells are an innate immune cell subset with potent antiviral activity. NK cells are generally categorized as either $CD56^{bright}CD16^{-}$ or $CD56^{dim}CD16^{+}$ (96). $CD56^{bright}CD16^{-}$ NK cells are often poorly cytolytic but can secrete inflammatory cytokines such as interferon-γ and TNF-α. $CD56^{dim}$ $CD16^{+}$ NK cells express high levels of perforin and granzyme-B and are highly cytolytic (96). NK cells can lyse virally infected cells by two main mechanisms: lysis of cells expressing stress receptors and/or expressing low levels of MHC class I or lysis of cells *via* antibody-dependent cell-mediated cytotoxicity (ADCC). In the former case, a delicate balance of activating (i.e., NKG2D) and inhibitory receptors [i.e., killer-cell immunoglobulin-like receptors (KIRs)] determines the cytolytic potential of NK cells. During HIV infection, Nef mediates MHC-I downregulation to avoid CTL killing (97). This can predispose HIV-infected cells to NK cell-mediated lysis, as MHC-I interacts with inhibitory KIRs. In the case of ADCC, antibody binding to Fc receptors (i.e., CD16) on NK cells results in perforin and granzyme release and cytotoxicity.

In both healthy and chronically HIV-infected humans, NK cells represent a very small percent of cells in the LN (<3%) (98). However, during chronic HIV infection, the $CD56^{dim}CD16^{+}$ subset becomes depleted and a novel $CD56^{-}CD16^{+}$ subset emerges (99). $CD56^{-}CD16^{+}$ NK cells appeared to be poorly cytolytic and possibly anergic (99). Similar observations have been made in SIV infection. During chronic SIV infection, the frequency of NK cells in LNs was approximately equal to naïve animals. However, during chronic infection, the percent of $CD56^{-}CD16^{+}$ NK cells was higher. As in HIV infection, $CD56^{-}CD16^{+}$ NK cells generated during SIV infection had limited cytotoxic activity and expressed a marker of anergy/exhaustion (100). These data suggest that LN NK cells may become exhausted/anergic during chronic infection.

Recently, it was reported that NK cells enter the B cell follicle and control SIV infection in African green monkeys (AGMs) (101). Unlike rhesus macaques, AGMs usually do not develop SAIDs, exhibit effective control of SIV in SLOs, and do not accumulate SIV virions on FDCs (102–104). During SIV infection, rhesus macaques exhibited a gradual decline in NK cell frequencies in LNs, while AGMs NK frequencies stayed relatively constant (101). Importantly, the numbers of NK cells in B cell follicles increased during SIV infection of AGMs, but not of rhesus macaques (101). SIV DNA levels had a strong negative correlation with frequencies of NK cells in the LN in AGM, but not in rhesus macaques. In AGM, NK cell localization to B cell follicles appeared to be associated with IL-15 production by FDCs. IL-15 is known to be essential for NK cell survival. Furthermore, anti-IL-15 treatment of AGMs resulted in near total depletion of NK cells from blood and LNs. This treatment also resulted in a large increase in plasma viremia and RNA^{+} and DNA^{+} cells in LNs including in the B cell follicles, suggesting that NK cells were critical to controlling SIV replication in the B cell follicle. However, it is important to note that anti-IL-15 treatment likely affects more than just NK cells. Notably, some decrease in blood $CD8^{+}$ T cells was observed after anti-IL-15 treatment (101). So, while not completely definitive, this report provides strong evidence that the effective control of SIV infection in AGMs may be largely mediated by the ability of NK cells to efficiently infiltrate B cell follicles.

CURE STRATEGIES AND THE B-CELL FOLLICLE

A wide variety of strategies have been proposed to cure HIV. In this review, we will discuss many of the prevailing cure strategies and their potential to clear the HIV reservoir in the B cell follicle (**Table 1**). While clearing HIV reservoirs in the B-cell follicle is likely necessary for the development of an effective cure, it may not be sufficient. Other anatomical sites have been proposed to be sites of persistence, most notably the central nervous system (105). However, strategies to target these reservoirs are out of the scope of this review.

A minority population of HIV-infected individuals, known as elite controllers, are able to suppress HIV replication effectively and maintain undetectable viral loads for years, although virus can usually be identified in them using sensitive techniques (106). It has been demonstrated that the early initiation of ART after primary infection can lead to effective long-term control of viral replication without continuing ART (107, 108). However, this effect is only seen in a subset of patients and is dependent on beginning treatment very soon after initial infection, and is usually not durable over more than a few years (109).

Furthermore, these patients still have a readily detectable HIV reservoir and are therefore considered to have a functional and not sterilizing cure (108). The only known person to have a possible sterilizing cure with no detectable reservoir is Timothy Brown (a.k.a. the Berlin patient). After being diagnosed with acute myeloid leukemia, the Berlin patient received chemotherapy and whole body irradiation followed by a stem cell transplant from a donor who was homozygous for the CCR5 delta32 allele (110, 111). Initial reports failed to detect any HIV DNA or RNA in the Berlin patient (110, 111). However, one report has suggested that he may still harbor extremely low levels of HIV RNA and DNA. It is important to note that the levels of HIV RNA and DNA were so low, it is possible they were false positives (112). Regardless of the true nature of the Berlin patient's cure, his case stands as an important milestone in HIV cure research. Importantly, bone marrow transplant patients who did not receive CCR5 homozygotic donor cells have all relapsed, although in some instances the timing of the relapse was delayed relative to what is seen in HIV-infected individuals who stop ART (113). Residual latently infected $CD4^{+}$ T cells are frequently invoked as the source of these relapses; however, the role of FDC-bound virions cannot be excluded, particularly since they are radiation resistant (114, 115).

Given that CTL are critical for controlling viremia, several proposed cure strategies revolve around altering or expanding CTL. One proposal is to isolate CTL from infected patients and

TABLE 1 | Summary of HIV cure strategies discussed in this review.

Cure strategy	Pros	Cons	Engineering to target B cell follicle	Ability to clear HIV on follicular dendritic cell (FDC) network
CTL expanded *ex vivo*	May not require transduction	Cost of leukapheresis; risk of emergence of escape variants	Cytokine treatment or transduction to express CXCR5	Likely ineffective due to lack of presentation of HIV peptides on MHC-I
Chimeric antigen receptor T cells	Ligands are not MHC-I dependent, low risk of escape variants, demonstrated long-term persistence and safety	Cost of leukapheresis; requires transduction of large numbers of lymphocytes	Cytokine treatment or transduction to express CXCR5	Unknown, but possible due to MHC-I independence
Immunotoxins	Ligands are not MHC-I dependent	Development of anti-toxin immune responses could reduce efficacy of multiple doses	Form immunotoxin-immune complexes to localize to surface of FDCs	Unknown, but possible due to MHC-I independence
Broadly neutralizing antibodies	High neutralization potential	High risk of escape variants and have proven ineffective at producing durable reductions in viremia	Transduce CXCR5$^+$ cells to express the antibodies *in vivo*	Unknown, but possible due to MHC-I independence
Bispecific antibodies	High neutralization potential also can function as a latency reversal agent (LRA)	Risks of escape variants and expensive to produce *ex vivo*	Transduce CXCR5$^+$ cells to express the antibodies *in vivo*	Unknown, but possible due to MHC-I independence
Rituximab	Demonstrated ability to destroy B cell follicles	May induce immunodeficiency	N/A	Would likely destroy most or all FDCs
Histone deacetylase inhibitors and protein kinase C agonists	May be combined with other strategies to improve efficacy	Have failed to produce durable remission in cure trials when used alone. Some may inhibit CTL responses	N/A	Likely ineffective because FDCs are not infected
Recombinant IL-15/ ALT-803	Acts as an LRA and can increase CTL responses. May be combined with other strategies to improve efficacy	Multiple doses in quick succession have diminishing effects	N/A	Unknown, LRA activity ineffective as FDCs are not infected, but proinflammatory effects could have some effect on FDC-bound HIV

The pros, cons, potential B cell follicle engineering strategies, and ability to clear HIV particles on the FDC network are listed. N/A = not applicable.

expand HIV-specific CTL *ex vivo* using HIV peptides and then reinfuse these back into patients. Several versions of this strategy have been tried over the years, but have failed to yield large or durable reductions in viremia (116–118). One problem associated with expanded CTL is that escape mutants can arise (117). Attempts to circumvent this problem by expanding polyclonal CTL against multiple HIV epitopes have been attempted, but to date have been unable to achieve durable reductions in viremia (116, 119). A similar strategy is to use chimeric antigen receptor (CAR) T cells that target HIV moieties. Several CARs have been developed that use the CD4 ectodomain and/or anti-ENV single chain antibody fragments to bind HIV envelope (120–122). CAR T cells have several potential advantages over *ex vivo* expanded CTL. CAR T cells do not need to recognize their ligands in the context of MHC, meaning that Nef-mediated downregulation of MHC-I should not affect CAR T cell killing. Additionally, CAR T cells can target highly conserved regions of HIV envelope such as the CD4 binding site, making escape mutations less likely. In a clinical trial, a CD4 ectodomain-based CAR resulted in only minor reductions in HIV reservoir size (123). However, an encouraging observation with these studies was that the CAR T cells were able to persist in many patients for over 10 years, demonstrating that durable engraftment of CAR T cells is achievable (124). Additionally, many improvements have been made in HIV-specific CAR T cells since those studies were undertaken, leaving CAR T cell therapies as a very promising avenue to be explored.

Critically, in the case of either *ex vivo* expanded CTL or CAR T cells, penetration into the B cell follicle will likely be poor without efforts to target these cells to the B cell follicle. In order to target CTL or CAR T cells to the B cell follicle, it may be necessary to treat the cells with cytokines or transduce them to constitutively express the follicular homing molecule CXCR5. One recent study has demonstrated the ability of TGF-β to induce CXCR5 expression in CTL (125). Transduction with CXCR5 has been demonstrated to allow the B-follicular localization of CTL in rhesus macaques, but it remains undetermined if they retain effective antiviral responses and will be able to clear the B-follicular HIV reservoir (126).

A similar strategy is to use HIV-specific immunotoxins, formed by either an anti-ENV single-chain antibody fragment or the CD4 ectodomain conjugated to the translocation and effector domain of a bacterial toxin, such as *Pseudomonas aeruginosa* exotoxin A (127). An anti-HIV immunotoxin in combination with ART was shown to be very effective at reducing HIV RNA$^+$ cells in bone marrow–liver–thymus (BLT) humanized mice (128). However, BLT humanized mice do not effectively form B cell follicles or GCs during immune reconstitution (129). To date, no strategies to target anti-HIV immunotoxins to the B cell follicle have been reported. Therefore, it remains an open

question if immunotoxins can effectively deplete HIV-infected cells in the B cell follicle. However, it is known that HIV-ICs will accumulate on FDCs in B cell follicles and remain potently infectious long term (75). Perhaps treatment of immuntoxins with non-neutralizing antibodies, would result in the decoration of FDCs with viable immunotoxin-ICs that would retain the ability to clear HIV-infected cells, but this remains untested.

Broadly neutralizing antibodies have been proposed and tested for HIV cure, with the idea that immune cells, mainly NK cells, would kill infected cells through ADCC against HIV envelope protein. Previous attempts have shown reductions in viremia, but failed to garner durable responses (130). Now attempts are being made to engineer bispecific antibodies to increase the number of strains that can be neutralized (131). Recently, a bispecific antibody that targets CD3 and gp120 (CD3/VRC07) has been developed (132). The CD3/VRC07 antibody can both act as a latency reversal agent (LRA) by stimulating latently infected T-cells through CD3 binding and facilitate ADCC against cells expressing newly synthesized gp120. Furthermore, the CD3/VRC07 antibody was recently demonstrated to be able to induce follicular $CD8^+$ T cell-mediated killing of infected cells *ex vivo* (67). Again, it remains unclear how effective any antibody-based strategy would be against the HIV reservoir in the B cell follicle. However, CXCR5 expressing lymphocytes could be transduced to secrete the bispecific antibodies to help localize the effects to the follicle. Given the recent demonstration of highly effective control of SIV infection in B cell follicles by $CXCR5^+$ NK cells in AGMs and the high ADCC potential of NK cells, combining strategies to localize NK cells to B cell follicles with anti-HIV antibodies may prove highly effective.

Another potential strategy to deplete the HIV reservoir in the B-cell follicle is to destroy B cell follicles with B cell targeting agents such as rituximab. Rituximab is an anti-CD20 antibody fragment that induces apoptosis in B cells. Depletion of B cells with rituximab in rhesus macaques prior to and during infection with SIV resulted in no statistically significant differences in viral load during either acute or during chronic infection (between 0 and 240 dpi), although viral loads trended lower in rituximab treated macaques (70). Interestingly, in follow-up studies (>400 dpi) SIV RNA was undetectable in 6 of 7 rituximab treated animals, while SIV RNA was detectable in four of four control animals (70). These data may suggest that rituximab treatment can contribute to control of viremia. However, it is important to note that B cell depletion was incomplete in three of seven animals, and these animals had no delay in seroconversion (70). Additionally, the animals were infected with an SIV strain that is highly prone to neutralization. Importantly, animals with complete B cell depletion showed no evidence of SIV virions accumulating on FDCs in LNs or Peyer's patches at 28 dpi (70). This demonstrates the potential of rituximab to prevent the accumulation of SIV-ICs on FDCs. However, anti-FDC stains were not reported and as the B cell depletion greatly inhibited the production of antibodies, IC formation was also likely inhibited. Therefore, it is unclear if existing FDCs were eliminated or if they couldn't bind SIV virions due to lack of IC formation. It also remains unknown what effect rituximab treatment will have on SIV/HIV-ICs already bound to FDCs. Finally, none of the animals were ART treated. Any cure strategy utilizing rituximab would likely occur during ART. It is also important to consider that a possible complication of rituximab treatment would be immunodeficiency and/or immunopathologies.

Latency Reversal Agents

All the cure strategies discussed so far rely upon active transcription and translation of viral products. Therefore, any successful cure strategy will almost certainly need to be combined with an effective LRA. An in-depth discussion of the wide variety of potential LRAs is outside the scope of this review. However, three promising candidates worth mention are protein kinase C (PKC) agonists, histone deacetylase inhibitors (HDACi), and recombinant IL-15 (or an IL-15 superagonist such as ALT-803).

One of the most promising classes of LRAs is PKC agonists. PKC agonists are believed to cause latency reversal by promoting NF-κB nuclear translocation and binding to HIV LTR (133, 134). PKC agonists can effectively activate HIV transcription across a wide variety of HIV latency models (135). Importantly, in a model of *ex vivo* latency reversal from resting CD4 T cells from infected patients, only the PKC agonist Bryostatin 1 was able to induce HIV mRNA transcription (136). Recently, it was demonstrated that a synthetic analog of Bryostatin 1 could induce HIV activation in the humanized BLT mouse model (137). However, as previously mentioned, the BLT mouse model does not effectively recapitulate B cell follicles and GCs (129). In summary, PKC agonists are a promising class of LRAs to be used in cure strategies. However, to our knowledge, the ability of PKC agonists to activate HIV reservoirs in the B cell follicle remains untested.

One of the most extensively investigated strategies to date to activate and eliminate the latent HIV reservoir has been the use of HDACi. Sung et al. demonstrated that HIV-specific $CD8^+$ T cells effectively kill cells in which latent virus has been reactivated by HDACi *in vitro* (138). Nevertheless, although increases in vRNA have been documented in ART-treated humans who received HDACi, no decreases in latently infected cells have been observed *in vivo* (139–143). Similarly, in ART-treated SIV-infected rhesus macaques, treatment with the HDACi romidepsin resulted in reproducible induction of plasma viremia, but no delays in rebound viremia upon cessation of ART (144). Although some have suggested that the failure of HDACi to diminish the latent reservoir or delay rebound viremia is due to the lack of effector virus-specific $CD8^+$ T cells (145), CD8 T cell depletion in ART-treated rhesus macaques has demonstrated that they contribute to virologic control even during ART (146). Alternatively, the failure of HDACi to diminish the latent reservoir may be due to the failure of virus-specific $CD8^+$ T cells to access B cell follicles in large numbers, where the majority of $vRNA^+$ cells are located in treated disease (60). Therefore, while HDACi have been demonstrated to be ineffective in diminishing the latent reservoir alone, HDACi may be more effective when combined with other cure strategies described in this review.

Both recombinant IL-15 and the IL-15 superagonist ALT-803 have been demonstrated to have latency-reversing properties (147). Importantly, ALT-803 also enhances CTL function against productively infected cells (147). Some other potential LRAs, including a subset of HDACi, inhibit CTL effector

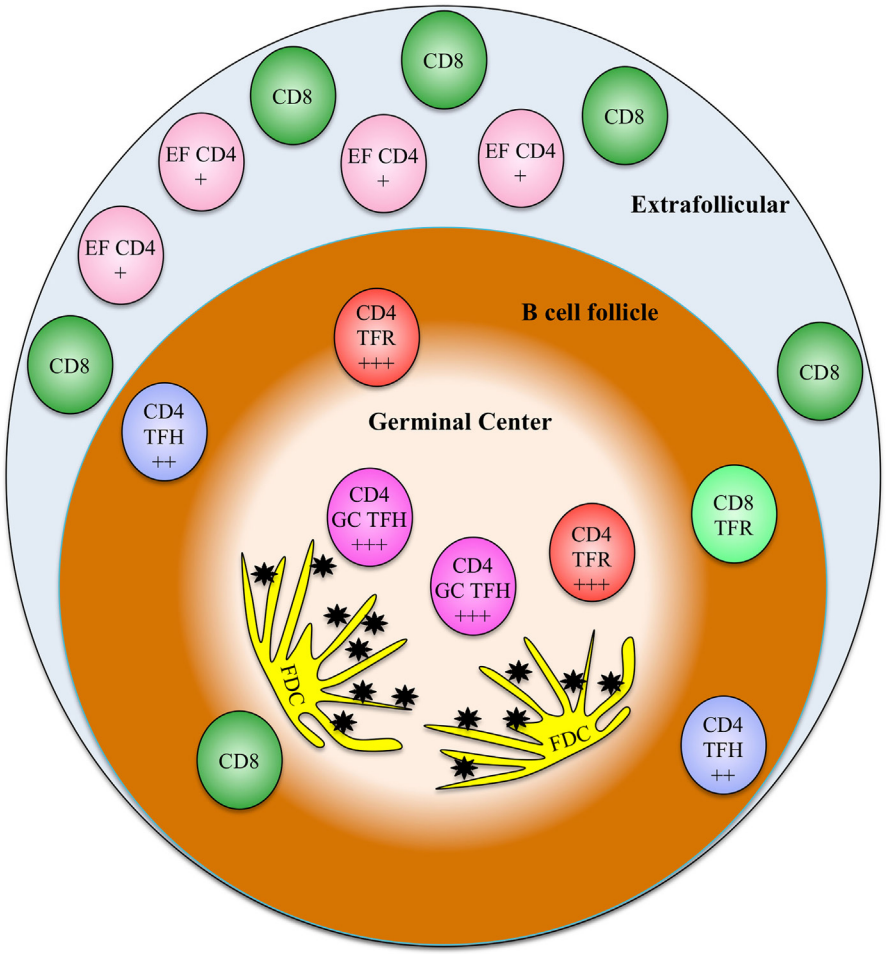

FIGURE 1 | A model showing the relative frequencies and localizations of various relevant T cell types discussed in this review. The relative susceptibility of CD4 subsets to HIV infection is indicated on each cell type (+ indicates somewhat susceptible, + + + indicates highly susceptible). EF CD4, extrafollicular CD4 T cells; TFH, T follicular helper cells; GC TFH, germinal center T follicular helper cells; TFR, T follicular regulatory cells; FDC, follicular dendritic cells, black stars represent extracellular HIV immune complexes.

functions (148). Although, it is worth noting that the *in vivo* significance of HDACi-mediated CTL inhibition is a subject of continuing debate (149). It was recently reported that in SIV-infected rhesus macaques, administration of ALT-803 resulted in potent (~2 log) reductions in viremia (150). However, these reductions were transient and repeated doses in close succession (2 weeks apart) had diminishing effects on viral replication. A repeat dose given after a long break (29 weeks later) demonstrated renewed reductions in viral replication (150). These data strongly suggest that ALT-803 has potent antiviral effects *in vivo*, but is ineffective alone in controlling replication and its antiviral effect has limited durability. The lack of CTL inhibition, combined with the importance of IL-15 in mediating follicular SIV control by NK cells in the AGM model makes ALT-803 a particularly promising candidate as an LRA (101). However, it remains to be seen what effects ALT-803 will have on the follicular reservoir and if targeting ALT-803 to the B cell follicle will be necessary.

Clearing the HIV Reservoir on FDCs

It is unclear how any of the cure strategies described above will affect the FDC-bound HIV reservoir. CTLs expressing TCRs against HIV peptides would presumably be totally ineffective against FDC-bound HIV as the FDCs themselves do not become infected and thus likely do not express HIV peptides on MHC-I. A more promising avenue is CAR T cell therapies because they are independent of MHC-I presentation. However, the stability of FDC-bound HIV virions may suggest that they are largely shielded from sCD4-mediated gp120 shedding. If true this could mean that CD4-based CAR T cells may be unable to bind FDC-bound HIV virions. However, anti-CD4 antibodies prevent infection of CD4 T cells by FDC-bound HIV-ICs *ex vivo*, suggesting that the CD4 binding site on gp120 must become at least temporarily exposed on FDCs (76). Furthermore, even if CAR T cells can initiate an immune response against FDC-bound HIV virions, it is unclear how effective this would be in removing the reservoir. Given the dendritic morphology of

FDCs, it remains unknown if a CTL attack on the extremities of these dendrites will kill the FDC or just damage a single dendrite. For all of these reasons, it remains an open question whether CD4 CAR T cell therapies will remove or significantly reduce the FDC-bound HIV reservoir. These problems may also apply to strategies that rely on ADCC.

It is equally unclear if immunotoxins will be able to kill FDCs decorated with HIV-ICs. If it is true that HIV-ICs are endocytosed by the FDC, then it is possible that immunotoxins could effectively kill FDCs by delivering the toxic moieties directly to the cytoplasm of the cell. However, these endocytic compartments, if they exist *in vivo*, are believed to be non-degradative and thus presumably non-acidic. Many bacterial toxins require endosomal acidification for membrane insertion and intoxication (151, 152). Furthermore, many bacterial toxins, such as PE, require retrograde trafficking to the endoplasmic recticulum (ER) and it is unclear if HIV-IC recycling endosomes could be diverted to the ER or any other subcellular compartments (152). For these reasons, it remains unclear if immunotoxins can efficiently kill FDCs by binding to HIV-ICs on the cell surface.

It may be possible to specifically dislodge HIV-ICs from FDCs by targeting the Fc and complement receptors on the FDC surface that bind HIV-ICs. Treatment with the ectodomain of the complement receptor fused to an Fc domain (CD21-Fc) was able to significantly reduce the number of virions bound to FDC *in vitro*, but as yet remains untested in any *in vivo* model (80). It is possible that specific clearing of HIV-ICs from the surface of FDCs or targeted killing of FDCs coated with HIV-ICs will prove unachievable *in vivo*. If this is the case, total depletion of the B cell follicle and FDCs by rituximab or some other agent may be necessary to remove the FDC-bound HIV reservoir.

CONCLUDING REMARKS

The susceptibility of follicular $CD4^+$ T cell subsets, poor follicular CTL accumulation and possibly function, a large extracellular FDC-bound viral reservoir, and possibly other factors all promote the B cell follicle as a critical sanctuary for HIV replication and persistence (**Figure 1**). HIV replication in the B cell follicle also likely mediates defects in humoral immunity that promote systemic defects in anti-HIV immunity. Targeting follicular reservoir of virus will likely be essential to suppression or eradication of HIV. Thus, a better understanding of the mechanisms mediating HIV persistence in the B cell follicle is critical to development of an effective HIV cure strategy.

AUTHOR CONTRIBUTIONS

MB and EC composed and edited this review. PS contributed to the editing of this review.

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MINI REVIEW
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Follicular Dendritic Cells of Lymph Nodes as Human Immunodeficiency Virus/Simian Immunodeficiency Virus Reservoirs and Insights on Cervical Lymph Node

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A hallmark feature of follicular dendritic cells (FDCs) within the lymph nodes (LNs) is their ability to retain antigens and virions for a prolonged duration. FDCs in the cervical lymph nodes (CLNs) are particularly relevant in elucidating human immunodeficiency virus (HIV)-1 infection within the cerebrospinal fluid (CSF) draining LNs of the central nervous system. The FDC viral reservoir in both peripheral LN and CLN, like the other HIV reservoirs, contribute to both low-level viremia and viral resurgence upon cessation or failure of combined antiretroviral therapy (cART). Besides prolonged virion retention on FDCs in LNs and CLNs, the suboptimal penetration of cART at these anatomical sites is another factor contributing to establishing and maintaining this viral reservoir. Unlike the FDCs within the peripheral LNs, the CLN FDCs have only recently garnered attention. This interest in CLN FDCs has been driven by detailed characterization of the meningeal lymphatic system. As the CSF drains through the meningeal lymphatics and nasal lymphatics *via* the cribriform plate, CLN FDCs may acquire HIV after capturing them from T cells, antigen-presenting cells, or cell-free virions. In addition, CD4+ T follicular helper cells within the CLNs are productively infected as a result of acquiring the virus from the FDCs. In this review, we outline the underlying mechanisms of viral accumulation on CLN FDCs and its potential impact on viral resurgence or achieving a cure for HIV infection.

Keywords: cervical lymph nodes, follicular dendritic cells, T follicular helper cells, central nervous system, human immunodeficiency virus, simian immunodeficiency virus, viral reservoirs, combined antiretroviral therapy

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INTRODUCTION

The cervical lymph nodes (CLNs) are a group of lymph nodes (LNs) in the neck region that are located adjacent to the cervical region of the spinal cord and in close proximity to the sternocleidomastoid muscle. Depending on the location of the CLNs, they may be classified as (a) superficial anterior CLNs, (b) superficial posterior CLNs, (c) superior deep CLNs, or (d) inferior deep CLNs. The glymphatics and meningeal lymphatics system is a functional waste pathway in the vertebrate central nervous system (CNS) (1, 2). The glymphatics and meningeal lymphatic system connects the CNS with the CLNs (3–10). More importantly, T cells and antigen-presenting cells (APCs) migrate along with the cerebrospinal fluid (CSF) as it drains along the nasal lymphatic path through the cribriform plate and eventually access the CLNs (**Figure 1A**) (3). Within the LN, there is a

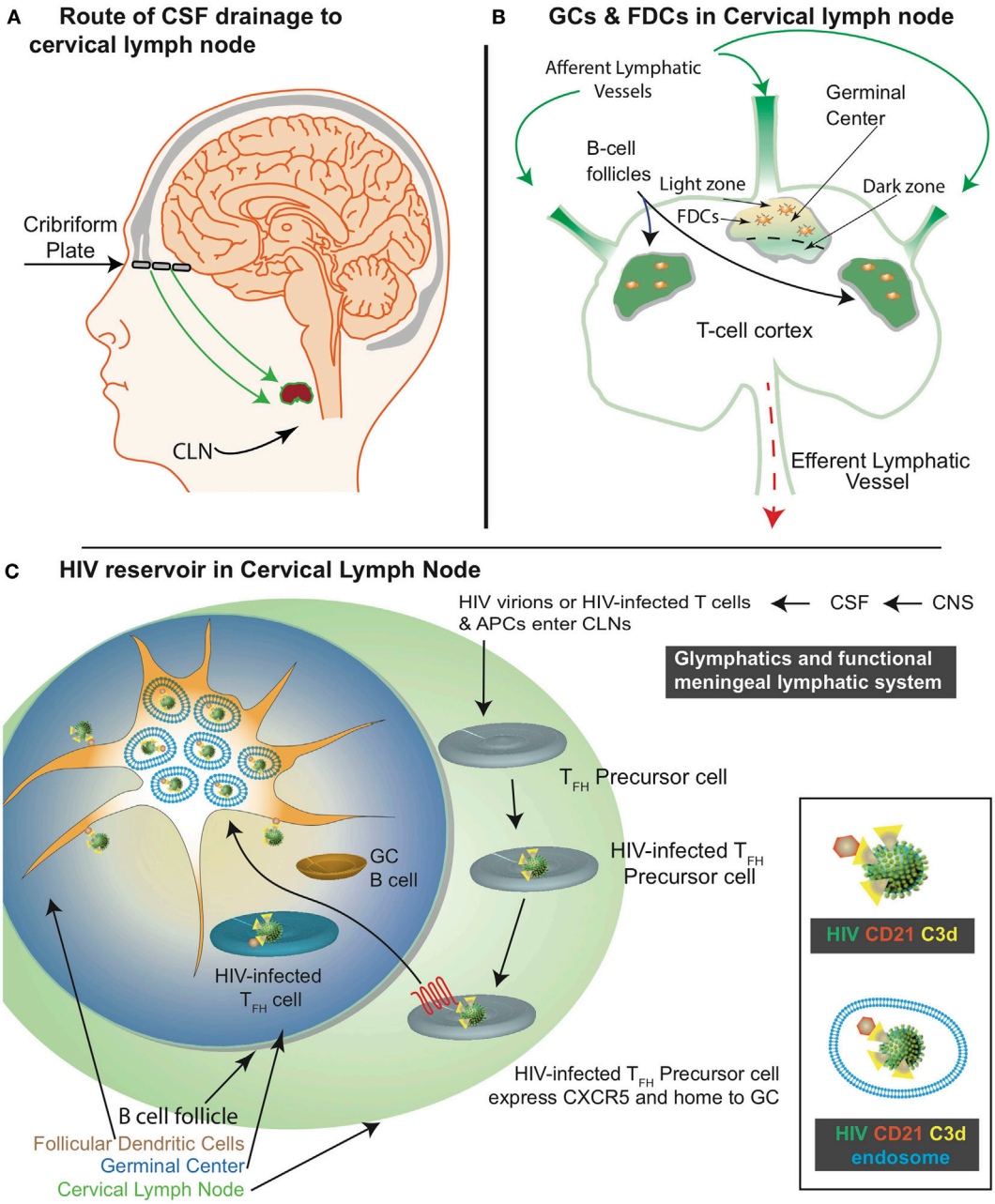

FIGURE 1 | Schematic representation of the central nervous system (CNS)-associated meningeal lymphatic system and the human immunodeficiency virus (HIV) reservoir in the cervical lymph nodes (CLNs). **(A)** The functional meningeal lymphatic vessels drain cerebrospinal fluid (CSF). T cells and antigen-presenting cells migrate with the CSF along the nasal lymphatic pathways through the cribriform plate to access the CLNs. **(B)** CSF enters the CLN *via* the afferent lymphatic vessel and exits through the efferent lymphatic vessels. Germinal center (GC) is located within the B-cell follicle. The follicular dendritic cells (FDCs) are located within the light zone of the GC. **(C)** Within the CLNs, HIV infects T follicular helper precursor cells, which subsequently express CXCR5 and migrate to the light zone. As depicted in the inset, CD21 interacts with C3d on HIV surface. This interaction results in HIV acquisition by the FDCs. Majority of the FDC associated HIV cycles through the endosomal compartment.

network of stromal cells that includes the follicular dendritic cells (FDCs). FDCs were first identified as "antigen retaining reticular cells" (11). Subsequently, FDCs have been recognized for their unique ability to retain antigens for a prolonged duration (12). This property of FDCs is critical for several immune functions, including germinal center (GC) formation and long-term immune memory. FDCs develop from perivascular precursors of stromal cell origin, which are seeded throughout the body. Their maturation requires lymphotoxin alpha and tumor necrosis factor alpha (TNF-alpha) signaling *via* B cells (13). FDCs are found within the

B-cell follicles (BCFs) where GCs develop as a result of a T cell-dependent antibody response (14). As the BCFs mature within the GCs, FDCs migrate into the light zone (**Figure 1B**).

Antigen acquisition, processing, and retention by FDCs impact the immune response. FDCs retain antigens for months to years (15, 16). However, there is inadequate experimental data demonstrating prolonged antigen retention by FDCs (17). In fact, most predictions are extrapolations based on decay rates. In addition to prolonged antigen retention, FDCs can also similarly retain human immunodeficiency virus (HIV)-1 (**Figure 1C**) (18). The FDC microenvironment is highly favorable for HIV infection (17). There is evidence in support of combined antiretroviral therapy (cART)-mediated viral clearance (19). Of note, there is a study (20) that conflicts this observation. As such, further investigations are necessary to understand if cART diminishes FDC-associated viral reservoir. Nonetheless, FDCs are considered a lymphoid tissue viral reservoir responsible for residual ongoing viremia (21) as well as, a source of viral resurgence upon cessation of cART (22). Of note, HIV retained by FDCs represents a divergent viral archive (23). The CLN FDCs like FDCs within the peripheral LNs also constitute a HIV/simian immunodeficiency virus (SIV) reservoir. In this review, we discuss how CLNs acquire, accumulate, and transmit HIV. In addition, we present some recent advances in FDC-related HIV research (**Table 1**).

TABLE 1 | Advances in follicular dendritic cell (FDC)-related human immunodeficiency virus (HIV) research.

Major findings	Reference
1. Simian immunodeficiency virus (SIV) accumulation in rhesus macaque cervical lymph node (CLN) FDCs and transmission to T follicular helper (T_{FH})	(24)
2. Enrichment of SIV DNA in CTLA-4 + PD-1-memory cells in lymph nodes	(25)
3. Engineering unselected CD8 T cells to express CXCR5 directs them into viral sanctuaries	(26)
4. Identification of a specialized group of CXCR5 expressing cytotoxic T cells that selectively entered B cell follicles and eradicated infected T_{FH} cells and B cells	(27)
5. T_{FH} are a source of replication competent HIV during latency	(28)
6. HIV-exposed FDCs show an increased production of inflammatory cytokines	(29)
7. RNAscope- and DNAscope-based characterization of HIV/SIV lymphoid reservoir	(30)
8. Combined antiretroviral therapy (cART) interruption results in widespread resurgence of rebounding/founder HIV variants	(22)
9. Productive SIV infection is restricted to CD4 + TFH cells in Elite controller macaques and not typical progressors	(31)
10. Trafficking of conventional DCs into germinal center (GC) of CLNs	(32)
11. SIV-infected GC T_{FH} derived from T_{FH} precursor cell subsets	(33)
12. Persistent viral replication in lymphoid tissue due to suboptimal drug penetration	(34)
13. FDCs as a source of low-level viremia	(21)
14. FDCs increase HIV transcription and production by a soluble tumor necrosis factor-alpha-mediated mechanism	(35)
15. FDC-trapped virus was replication competent and demonstrated greater genetic diversity than that of virus found in most other tissues and cells	(23)
16. Anti-CD21 mABs decreases HIV trapping by lymph node cells	(36)
17. Species-specific colocalization of osteopontin with the FDC network in lymphatic tissues in HIV-1 and simian immunodeficiency virus infections	(37)
18. FDC–virus interactions stabilize the virus particle, thus contributing to the maintenance of infectivity	(38)
19. FDCs serve as a reservoir of infectious virus and render surrounding GC T cells highly susceptible to infection with X4 isolates of HIV-1	(39)
20. FDC microenvironment is highly conducive to active HIV infection	(17)
21. FDC-associated virus accumulates soon after infection and cART does not diminish the FDC HIV reservoir	(20)
22. HIV-1 binds to B cells with CD21 receptor	(40)
23. FDCs accumulate HIV for a prolonged duration	(18)
24. FDCs-associated HIV is rapidly cleared with potent antiretroviral therapy	(19)

CNS AND CLN FDCs ARE IMPORTANT COMPONENTS OF HIV NEURO-IMMUNOPATHOGENESIS

Human immunodeficiency virus neuroinvasion occurs very early during infection (41, 42) with transmigrating infected monocytes/macrophages (43) and CD4+ T cells (42). In SIV/SHIV macaque models, SIV neuroinvasion occurs within a few days to weeks (44). HIV cannot be eliminated from the CNS as infected monocytes or microglia have a long lifespan and low turnover (45). These monocytes and microglia within the CNS support latent HIV infection (46–49). Since, there is suboptimal penetration of cART (50, 51) across the blood–brain barrier (BBB) resulting in establishment of reservoir within the CNS.

Besides the CNS, HIV persists within the LNs, spleen, gut-associated lymphoid tissue, reproductive organs, and lungs (52, 53). LNs are a known reservoir of persistent HIV/SIV viral infection under suppressive cART (34, 54–58). Several unique characteristics of the LNs contribute to the ability of HIV to persist in this tissue. For example, LN tissue has a slower decay rate than in the peripheral blood (34). Additionally, the LN follicles contain FDCs that capture HIV virions on their cell surface in immune complexes (24). FDCs in the peripheral LNs have also been characterized as another viral reservoir site (21, 35, 59). Importantly, HIV-susceptible T follicular helper (T_{FH}) cells are located in close proximity to FDCs, which within peripheral LNs have been shown to trap virions in their native non-degraded state for months to years (60–63) with a half-life of approximately 2–3 months (23, 64). While FDC-trapped virus does not replicate or evolve; however, it can infect nearby trafficking cells (23, 24). Even during cART, replicating virus persists and replenishes trapped stores of HIV (22, 54).

Until the description of glymphatics and the functional meningeal lymphatic system, CNS was considered to be immune-privileged (5). With the elucidation of structural and functional features of this CNS-associated lymphatic system (2, 3, 6), it is now

well established that the CNS undergoes constant immune surveillance in the meningeal compartment. The meningeal lymphatic system, along with glymphatics presents a unique connection between the CNS and CLNs. HIV may pass with CSF as virions, infected T cells, or APCs through the cribriform plate along the nasal lymphatic pathway and access the CLNs. Lymph entering the CLNs through the afferent lymphatics is channeled through the subscapular sinus into the medulla. The fibroblastic reticular cell (FRC) conduits access afferent lymph and traverse BCFs, where they intersect FDCs. FRC conduits continue into the cortex where they end at high endothelial vessels or the medulla (65).

Recent focus directed at better understanding of the meningeal lymphatic system has tremendously enhanced our understanding of immune surveillance in the CNS (2–9). Lymphatic vessels were first identified in the dura mater of rats (7). In some studies of the meningeal lymphatics (3), the system has been described as part of the CNS, while others have drawn opposing conclusions (9). This is not surprising since lymphatic vessels are component of the surrounding connective tissue that is included in the CNS. However, lymphatic vessels can absorb CSF from adjacent subarachnoid space and brain interstitial fluid *via* the glymphatics. Further detailed investigations are required to fully understand the functionality of CSF drainage, and how it might impact HIV accumulation within CLNs (1).

Circulating conventional DCs (cDCs) are known to traffic into the CNS in response to neuroinflammation (66–73) during HIV/SIV infection (74). Within CNS, cDCs act as both "*carrier and bearer*" of HIV and contribute both to neuropathology as well as CNS reservoir. Recent studies suggest that cDCs may capture HIV within the CNS and deliver it to different compartments of CLNs including FDCs (32, 60, 61, 75). The CLN FDCs would create a viral repository where virus can remain bound for prolonged duration (63). The immune cell retrograde transport studies (3, 6, 76, 77) provide clues that cDCs upon encountering HIV virions within the brain would migrate along the meningeal lymphatic vessels to draining LNs (CLNs, near the brain stem) *via* glymphatics delivering HIV particles to different CLN compartments including FDCs as shown for peripheral LNs (60, 61, 75). However, CLNs are the major site for systemic activation of CNS-specific T cells. They receive input from the CNS in the form of antigens and cDCs (78). Within CLNs, HIV viral particles may be transmitted to CD4+ T cells or trapped on the FDC network, thereby stabilizing and protecting HIV and creating a long-term reservoir of infectious HIV (21, 34, 38, 54). In addition, FDCs activate CD4+ T cells within GCs and increase virus production in these cells even in the presence of cART (35, 39, 79–81). Assessing the involvement of CLNs in HIV neuropathogenesis is timely, given our recent advances in understanding of the functional meningeal lymphatic system (3). Of note, additional mechanistic studies are required to determine if the CLN FDC reservoir is an archive of CNS egressing virus.

KEY CELLULAR PLAYERS IN THE CLN

Cervical lymph nodes like other LNs play a central role in the development of adaptive immunity against pathogens and, particularly, the generation of antigen-specific B cell responses in specialized areas within GCs (82). Very early in the HIV epidemic, LN pathology was recognized as an important consequence of HIV infection since the beginning of the epidemic. Studies focused on lymphoid tissue architecture during HIV/SIV infection have highlighted the key role of the LN in the disease pathogenesis. The LN environment is unique for viral evolution, primarily because of the relative exclusion of HIV-specific CD8 T cells (83). In a subsequent study, SIV-specific CD8 T cells in GC and non-GC regions were quantitated (84). Therefore, further investigations are necessary to understand the biology of immune cells in HIV-infected LNs and their critical role in achieving complete viral eradication.

Follicular dendritic cells are a subset of DCs that are of mesenchymal origin and essential for GC formation and production of various types of antibodies (16). They reside in secondary lymphoid tissues such as spleen, tonsils, LNs, and follicles that appear at extranodal sites (85). GCs of secondary lymphoid tissues are composed of several types of immune cells, such as activated B cells, T_{FH} cells, and FDCs. FDCs interact with their GC counterparts. In the GC microenvironment, activated B cells communicate with FDCs by interacting with an antigen on their surface and then present this antigen to T_{FH} cells. FDCs can select for B cells to re-enter the GC or exit with the help of T_{FH} cells (15). FDCs have a unique ability to retain immune complexes on their dendritic processes. These immune complexes consist of antigen–antibody complexes and complement (86), which can retain infectious virions for several months even in the presence of neutralizing antibodies or under cART (54). FDCs interact with T_{FH} cells in GCs, and these cells serve as a reservoir of infectious virus. Surrounding GC, T cells become highly susceptible to infection with HIV X4 isolates (39). HIV production increases to twofold when viral particles are transferred from FDCs to susceptible CD4+ T cells (35). FDCs can secrete inflammatory cytokines (29) including TNF alpha and thereby contribute to enhanced transcription in the LNs (35).

The underlying mechanisms of HIV/SIV FDC reservoirs remain unclear and require further studies. FDC trapped HIV virions in human lymphoid tissues remain infectious (56). In murine FDCs, HIV virions in immune complexes remained infectious *ex vivo* for up to 9 months after being captured by FDCs (54). This characteristic of the FDCs is particularly interesting because most of the identified reservoirs of persistent virus are found in the integrated pro-viral stage of the HIV replication cycle. It is important to note that current approaches to eliminate persistent HIV have largely focused on elimination of HIV pro-viral DNA. The HIV/SIV lymphoid reservoir has been well characterized utilizing RNAscope and DNAscope methodologies (30). However, the CLN FDC HIV reservoir has only recently been partially characterized (24) and requires detailed investigation (**Table 1**).

T follicular helper are a subset of CD4 T lymphocytes (87) that play a key role in B-cell differentiation. T_{FH} cells assist B cells in the production of antigen-specific antibodies and are essential for memory B cell activation, survival, and differentiation. Even under conditions of durable control, such as in elite controller

macaques, T_{FH} cells contribute significantly to ongoing viral replication and production, and are the single CD4 subset in the LN's most highly enriched in SIV (31). During HIV infection, cellular interactions between FDCs, GC B-cells, and T_{FH} cells result in reservoir establishment. T_{FH}-associated replication competent virus may be the source of resurgent HIV after cART interruption or failure. As such, T_{FH} are increasingly recognized as another major CLN-associated reservoir of HIV infection (88–90). However, mechanisms by which these cells get infected remain unclear. T_{FH} express very little CCR5 and in macaque studies, it has been shown that T_{FH} lacking CCR5 cells can be infected *in vivo* with CCR5-tropic SIV (91, 92). Infection of the T_{FH} population by CCR-5 tropic viruses appears to be the result of infection of the pre-T_{FH} cells that express CCR-5 (93).

In cART-naïve as well as treated individuals, T_{FH} and GC B cells are elevated (94). In addition, there is a direct correlation of T_{FH} and GC B cells with the activated T-cell population in the LNs (95). In absence of cART, during chronic HIV infection, viral replication is concentrated in secondary lymphoid follicles (SLF). T_{FH} cells have been shown to be highly permissive to HIV within SLF and are the source of replication competent HIV during latency (28). HIV vaccines are not strong inducers of neutralizing antibodies. However, in one of the recently described study, rhesus macaques were immunized with HIV envelope glycoprotein trimer, and there was a substantial production of HIV neutralizing antibodies (96). The high antibody titers had a strong correlation to GC B cells and T_{FH} (96). These observations underscore the need to study more details of LNs, since previous HIV reservoir studies have frequently focused primarily on the peripheral blood.

Follicular regulatory T (TFR) cells are another subset of T cells in SLF (97, 98). TFR share some phenotypic characteristics with the T_{FH} cells. Importantly, both T_{FH} and TFR are permissive to HIV infection (99). However, TFR express greater levels of CCR5 and CD4 as compared with the T_{FH} cells. They also support higher frequency of viral replication. Expression of Ki67, a marker of proliferative capacity appears to correlate with viral replication in these cells. As such, TFR differ from T_{FH} in their susceptibility to R5 HIV infection (99). Furthermore, it has recently been shown that natural killer (NK) cells migrate into the follicles of secondary LNs. The role of NK cells in LNs is not clear. However, a particular study in African green monkeys demonstrated that entry and persistence of NK cells in LNs was IL-15 dependent, as depletion of IL-15 resulted in an increase in viral replication. These data suggest a key role for NK cells in the establishment and maintenance of this viral reservoir (100).

ERADICATION OF FDC VIRAL RESERVOIR

A significant challenge to HIV eradication is the elimination of viral reservoirs in GC T_{FH} cells. GCs are considered to represent an immune privileged site within the LN where antiviral CD8+ T cells are primarily excluded (83, 84). However, unselected CD8+ T cells engineered to express CXCR5 (C-X-C chemokine receptor type 5, a chemokine receptor required for homing to GCs) direct them to GCs (26). CXCR5 expressing cytotoxic T cells are able to selectively enter BCFs and eradicate infected T_{FH} and B cells (27). A population of SIV-specific CD8+ T cells expressed CXCR5 and expanded in LNs following pathogenic SIV infection in a cohort of vaccinated macaques (101). Animals that exhibited greater control of SIV replication had a greater expansion of these cells. The increase in CXCR5+ CD8 T cells was associated with the presence of higher frequencies of SIV-specific CD8 T cells in the GC (101). Thus, CXCR5+ CD8 T cells represent a unique subset of antiviral CD8+ T cells that expand in LNs during chronic SIV infection and may play a significant role in the control of pathogenic SIV infection (101) (**Table 1**).

An important milestone in purging the FDC reservoir was demonstrated by utilization of soluble complement receptor 2 or CD21 (102). CD21 is necessary for HIV interaction with FDCs and B-cells (40) as interaction of HIV with FDC stabilizes the virus (38). Despite the stabilized interaction, Heesters and co-workers were able to purge the FDCs of HIV virions by utilizing a soluble form of CD21 (102). Thus, intersecting CD21:C3d interactions significantly reduced recycling of virions through the endosomal compartment. In addition, viral transmission to T_{FH} was diminished in *in vitro* studies (102). In an alternate approach to purge FDC HIV reservoir, monoclonal antibodies targeting CD21 were utilized (36). Thus, blocking CD21 interactions appears to be a potential strategy for purging the FDC HIV reservoir.

FUTURE PERSPECTIVES

Profound and durable suppression of HIV by cART represents a major accomplishment in HIV/AIDS research (103, 104). However, HIV persists in patients despite long-term administration of cART (55). Withdrawing cART invariably results in viral rebound (105, 106). One of the major challenges with cART is to maintain virologic control. Two types of research strategies have been utilized in HIV cure research. Eradication of replication-competent HIV is considered as a "classic cure" and the best example is Timothy Brown, also known as the Berlin patient (107). Timothy Brown received a stem cell transplant from a donor that was homozygous for delta32 mutation in the CCR5 gene (107). On the other hand, in a "functional cure," viral rebound after cessation of cART is controlled without eradication of HIV. Such functional cure was demonstrated in SIV-infected rhesus macaques with α4β7 monoclonal antibody (108). Even after cART withdrawal, sustained virologic control was maintained with passive administration of α4β7 monoclonal antibody (108). However, mechanisms underlying this sustained virologic suppression remain to be elucidated. Other HIV cure strategies include (a) latency-reversing agents (e.g., anti-CD3, Bryostatin, IL-7, Romidepsin, TLR-7 agonists, Valproic acid), (b) immunotoxic therapy with bi-functional antibodies, and (c) precise excision of HIV genomes by CRISPR/Cas9 gene editing in mice (109–111). It needs to be determined if such cure strategies can successfully purge the CNS and the LN HIV reservoirs based on our current understanding of the functional meningeal system, CSF outflow (1), and viral acquisition by FDCs (**Figures 1A–C**) (24). Lifetime cART is associated with toxicity, residual chronic inflammation, and the accelerated onset of comorbidities associated with aging. Therefore, optimizing other cure strategies

in combination with cART will be critical to reducing cART-associated complications and the overall viral burden.

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Follicular CD4 T Helper Cells As a Major HIV Reservoir Compartment: A Molecular Perspective

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Effective antiretroviral therapy (ART) has prevented the progression to AIDS and reduced HIV-related morbidities and mortality for the majority of infected individuals. However, a lifelong administration of ART is necessary, placing an inordinate burden on individuals and public health systems. Therefore, discovering therapeutic regimens able to eradicate or functionally cure HIV infection is of great importance. ART interruption leads to viral rebound highlighting the establishment and maintenance of a latent viral reservoir compartment even under long-term treatment. Follicular helper CD4 T cells (TFH) have been reported as a major cell compartment contributing to viral persistence, consequent to their susceptibility to infection and ability to release replication-competent new virions. Here, we discuss the molecular profiles and potential mechanisms that support the role of TFH cells as one of the major HIV reservoirs.

Keywords: HIV, lymph nodes, TFH cell, cure, gene expression

INTRODUCTION

Antiretroviral therapy (ART) has impacted on the quality of life of HIV-infected subjects. However, the persistence of a long-lasting viral reservoir, where fewer than 10% of the infected cells harbor replication-competent provirus (1–4), poses a major obstacle for viral eradication. Recently, TFH cells have been reported as a potential sanctuary for HIV/SIV replication and an important compartment for viral persistence (5, 6), making them an important target for cure therapies.

GERMINAL CENTER (GC) AND CIRCULATING (c) TFH CELLS

TFH cells localize specifically in B cell follicles (BCF) in secondary lymphoid organs (5, 6) and express a CCR7^{lo} CXCR5^{hi}PD-1^{hi}ICOS^{hi} BTLA^{hi}CD69^{hi}SAP^{hi} phenotype (7). Through surface receptors and soluble factors like IL-21 and IL-4 (8), TFH cells provide help for the survival, activation, and maturation of GC B cells. A complex network of transcription factors (TFs), including BCL-6, interferon regulatory factor (IRF4), c-Maf, and BATF, promote TFH differentiation while inhibiting alternate CD4 T cell differentiation pathways (9). The downregulation of CCR7 and upregulation of CXCR5 licenses the migration of activated T cells to BCFs of secondary lymphoid organs and

promotes their interaction with B cells, further upregulating the expression of Bcl-6 and leading to the establishment of effector and memory TFH cell programs (10). The mutual regulation of TFH and B cells (11), through receptor–ligand interactions like ICOS/ICOSL, PD-1/PD-L1, and CD40/CD40L (11) and soluble mediators like IL-21 and IL-4 (12), is critical for the formation and maintenance of GC structure and provision of critical help for the development of antigen-specific B cell responses (13–15). TFH cells can populate different areas of the follicle, including the marginal zone that surrounds the GC and the "light" zone (**Figure 1A**).

The fate of memory TFH cells after a resolved infection, immunization, or response to foreign antigen is not known. Several studies have focused on the characterization of cTFH cells (17–20) and their ability to promote differentiation and/or class switching of autologous B cells (19). However, their lineage commitment and relationship to GC TFH cells is not well understood. Given the limited accessibility to lymph nodes during clinical trials, the identification and validation of blood biomarkers (21) that could provide robust estimation of GC reactivity is of great interest. In this context, the development of single cell sequencing, allowing for the TCR clonotypic analysis

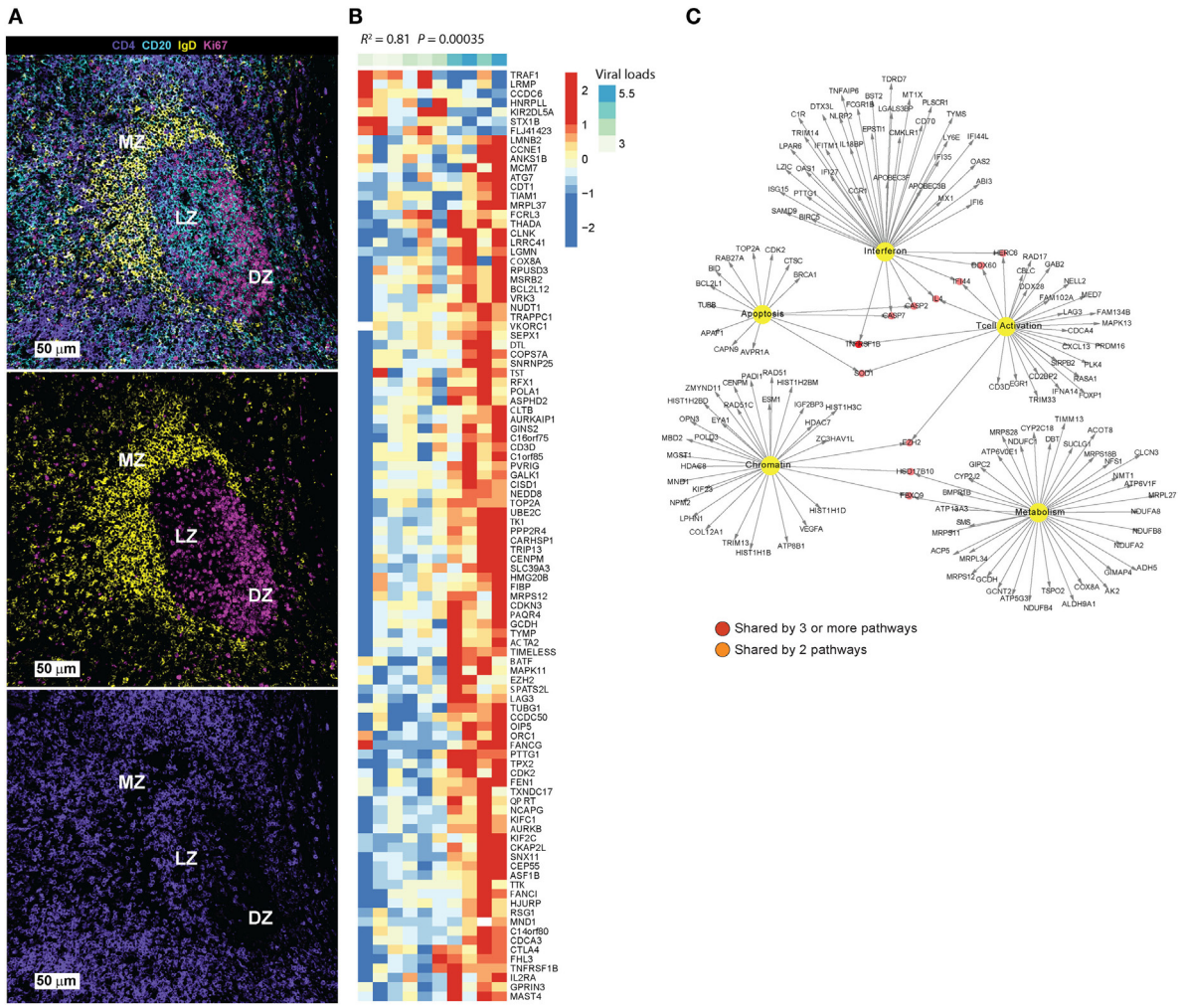

FIGURE 1 | **(A)** Main areas of a tonsillar follicle defined by IgD (yellow), CD20 (cyan), and Ki67 (magenta) are shown: marginal zone ($IgD^{hi}CD20^{dim}$), germinal center light zone ($IgD^{neg}sCD20^{hi}Ki67^{hi/lo}$) and dark zone ($IgD^{neg}PCD20^{dim}Ki67^{hi}$). The distribution of CD4 T cells (purple) is shown in the lower image. **(B)** Differential gene expression analysis was performed using Limma model by comparing sorted TFH cells ($CXCR5^{high}$) vs. Non-TFH ($CXCR5^{low}$) from eight HIV- donors in LNs. A corrected *p*-value cutoff of 0.05 (BH method) was used to select significant upregulated genes (731 genes) in $CXCR5^{high}$ vs. $CXCR5^{low}$ TFH cells. Linear regression model to correlate these genes to viral load in a cohort of HIV viremic subjects (10 viremic subjects) was performed in R. Heatmap shows the $\log_2$ normalized expression of each gene transformed to *z*-score where the average expression of each gene was subtracted and divided by its SD across samples. Genes that correlated significantly to viral load (*p*-value <0.05) were shown on the heatmap. **(C)** Pathways enrichment analysis using genes in panel **(B)** and a compiled set of pathways from MsigDB C2 collection (http://software.broadinstitute.org/gsea/msigdb/collections.jsp#C2) and pathways from Chaussabel et al. (16) was performed. A FDR cutoff of 0.05 was used to selected pathways significantly correlated to viral loads. Interferon, metabolism, T cell activation and differentiation, apoptosis, and chromatin regulators were enriched. Cytoscape was used to infer gene-interacting networks of the leading genes of these pathways. Yellow nodes represent pathways name, red nodes represent genes shared by more than three pathways, orange nodes represent genes shared by two pathways, and white nodes represent genes specific to each pathway.

and global transcriptional profiling, would be instrumental for the understanding of the lineage relationship between cTFH and follicular TFH cells.

TRANSCRIPTIONAL REGULATION OF TFH CELL DIFFERENTIATION

A complex network of signaling molecules and TFs has been described for the development and maintenance of TFH cells (9). In humans, the generation of TFH is promoted by the synergistic function of Bcl-6 and c-MAF (22). Several TFs can promote TFH differentiation in a Bcl-6-dependent (i.e., LEF1 and TCF1) (23) or -independent (i.e., ASCL2) (24) manner. On the other hand, Blimp1 represents a major TF that suppresses TFH differentiation, by modulating the expression of BCL-6 (25). KLF2 restricts the *in vivo* development of TFH, an activity mediated by the induction of S1PR1 and Blimp1 (26). How the course of HIV/SIV infection modulates this complex network of TFs is not well understood. To this end, longitudinal NHP studies will be highly informative (27).

Members of STAT family play a central role in TFH differentiation upon the engagement of receptors for γ-C cytokines which are required for TFH survival and differentiation. The cytokines IL-6 and IL-21, both positive regulators of TFH differentiation, induce BCL-6 expression through STAT-3 activation (28), while IL-27 acts likely *via* its indirect impact on IL-21 production (29). IRF4, expression of which is dependent on TCR signaling strength (30, 31), globally cooperates with STAT-3 (9) as a complex to regulate IL-21-mediated gene expression. In contrast to STAT-3, STAT-5 has a negative impact on TFH development at least by suppressing the expression of TFs like c-Maf, BCL-6, and Batf (25). IL-2 inhibits TFH differentiation by activating STAT-5 which prevents the binding of STAT-3 to the Bcl-6 promoter. Alternatively, STAT-5 deficiency greatly enhances TFH gene expression *in vitro*, in part associated to dampened Blimp1 expression (32). These observations highlight the inhibitory crosstalk that takes place between STAT-5/Blimp1 and the IL-6/IL21/STAT-3/BCL-6 pathways in TFH development (10). In addition to STAT-3 and STAT-5, several studies have indicated that STAT-1 and STAT-4 are also involved in TFH differentiation. IL-6 and IFNg induced STAT-1 was shown be required for BCL-6 induction of early TFH differentiation *in vivo* (33, 34). Additionally, IL-12-mediated STAT-4 activation can induce expression of IL-21 and BCL-6 to generate cells with features of both TFH and Th1 cells (35). Altogether, these findings indicate that the interactions among TFs that determine the fate of specialized $CD4^+$ T-cell lineages are complex, giving them flexibility and potential to respond to environmental conditions by altering the expression of critical specific TFs as needed.

GC DYNAMICS IN HIV/SIV INFECTION

The GC dynamics in HIV infection is a subject of intense research. The susceptibility of TFH cells to infection (36), the local inflammatory microenvironment (37, 38) and potential sequestration of innate and pro-inflammatory cells (39, 40), as well as their close proximity to Follicular Dendritic Cells (FDCs) that harbor infectious virus for long periods of time (41–43) represent biological factors that could contribute to TFH cell dynamics during the course of HIV/SIV infection. Acute SIV infection is characterized by modest increases in the relative frequency of TFH cells (36, 44, 45) while chronic viremia has a dramatic effect on extrafollicular and follicular architecture and TFH dynamics affecting the development of HIV/SIV specific antibody responses (46). Available viral antigen, possible preferential deletion of Env-specific TFH CD4 T cells, loss of stromal cells like fibroblastic reticular cells (47) that directly affects the dynamics of T cells (47) and their trafficking within lymph node areas (48) and altered tissue architecture due to progressive deposition of fibrotic collagen (49), a major determinant of altered LN architecture (47, 49, 50), could contribute to altered GC T-B cell interactions with direct implications for the development of broadly neutralizing antibodies. In fact, circulating GC-related factors like CXCL-13 have been proposed for monitoring the development of such antibodies (21, 51). In the advanced phase of disease (AIDS), significantly lower frequencies of TFH cells were found indicating accelerated loss of TFH cells under these conditions (52) when compared to other CD4 subsets. TFH cells express unusually high levels of the co-inhibitory receptor PD-1 further sensitizing them to "pre-apoptotic" signals (53) upon interaction with locally expressed PD-1 ligands during chronic infection (54). Whether the loss of TFH cells is due to their accelerated "exhaustion" associated with AIDS, an increased operation of pre-apoptotic pathways, or a result of an advanced loss of structure and vital signals (50) is not known and needs further investigation. The delineation of local pro- and anti-inflammatory networks will further inform on the cellular and molecular mechanisms governing the dynamics of TFH cells in chronic infection and might lead to novel strategies for virus elimination by manipulating such pathways. Thus, although early ART rapidly controls HIV/SIV replication, it only partially reduces lymphoid and systemic markers of cellular activation, resulting in increased TFH frequencies and persistent hyperplastic BCFs, which may contribute to the seeding and magnitude of viral reservoirs within these lymphoid tissue compartments (55).

TFH CELLS: A PREFERENTIAL HIV/SIV RESERVOIR

TFH cells have been demonstrated to be both productively (i.e., $vRNA^+$) and latently (i.e., vDNA) infected at higher frequencies (5, 56) than non-TFH cells. TFH cells are in the vicinity of cells harboring virus-immune complexes like FDCs and B cells (57–59) that serve as a significant reservoir and are able to effectively infect $CD4^+$ T cells *in vitro* (41, 60, 61). Their unique localization in combination with their activation status could contribute to the higher infection levels of TFH cells *in vivo*. *In vitro* studies have further supported the preferential infection (5) and production of infectious virus from TFH compared to non-TFH cells (5, 6, 43). HIV RNA was found in TFH cells even after ART initiation, although those levels were lower in long-term ART treated donors (6). Similar results were found during chronic SIV infection (44, 62). Recently, follicular regulatory T (TFR) cells, shown by gene

profiling as not originated from the same lineage as TFH cells (63, 64), share phenotypic characteristics with TFHs and are even more permissive to HIV-1 infection both *ex vivo* and *in vivo*. This can be in part mediated by the higher surface expression of CCR5 and CD4 (65) and their higher activation and *in vivo* cycling status, judged by the expression of Ki67 (65). Given the shared surface characteristics with TFH cells, TFR cells might contribute to data ascribed as specific for TFHs. However, what impacts the preferential infection of TFH and TFRs cells *in vivo* is not well understood and warrants further investigation. Generation of viral targets due to TFH cell accumulation in chronic infection (64), their unique cellular profile, the low penetrance of ART in the tissues (66), the relative exclusion of virus-specific CD8 T cells from the BCFs and particularly the GCs (67, 68), as well as the expression of local cytokines like IL-10 (69), TGFb (47) that interfere with the activation of CD4 T cells or chemokines involved in HIV infection like CXCL9 (70) could represent biological factors contributing to the preferential establishment of virus persistence in TFH cells (70). Further delineation of the cellular and molecular mechanisms underlying TFH dynamics will inform the design of novel therapies for viral elimination. However, novel therapeutics targeting the virus reactivation in TFH should be carefully evaluated given the unique biology and localization of TFH.

MOLECULAR PATHWAYS PROMOTING TFH SUSCEPTIBILITY TO HIV INFECTION AND RESERVOIR ESTABLISHMENT

In an attempt to understand better the preferential infection of TFH cells, we performed microarray analysis on TFH ($CD4^+CXCR5^{high}$) and non-TFH ($CD4^+CXCR5^{low}$) T cells sorted from lymph nodes from healthy subjects (not published data). Differential gene expression analysis of TFH ($CD4^+CXCR5^{high}$) compared to non-TFH ($CD4^+CXCR5^{low}$) revealed upregulation of major TFH markers (CXCR5, PDCD1, MAF, BCL-6, LAG3, IRF4) (data not shown).

In order to identify TFH genes and pathways associated with HIV replication and/or reservoir establishment, we performed a linear regression analysis using a compiled set of TFH signatures (see **Figures 1B,C** legend for more details) with integrated DNA (IntDNA—latent reservoir), TILDA [inducible HIV reservoir Ref. (71)], and plasma viral load (VL—active replicating virus) measurements from a cohort of HIV viremic subjects. We found a significant and positive correlation of several TFH specific signatures to IntDNA, TILDA, and VL (data not shown). This regression analysis was performed in viremic subjects, allowing us to correlate specific pathways of gene expression to active viral replication. The TFH genes associated to high viral load are shown (**Figure 1B**). Pathways enrichment analysis using these genes was performed using MsigDB C2 collection and pathways from Chaussabel et al. (16). We observed an enrichment in genes related to modulation of T cell activation, co-stimulation and MHC protein binding (Lag3, CD3D, NELL2, FAM102A), apoptosis (CASP2, CASP7, APAF1, BID, associated to positive regulation of programmed cell death, intrinsic apoptotic signaling), metabolism (NUDFB8, NDUFA2, NDYFB8, NDUFC1, all associated with NADH dehydrogenase activity and ATP synthesis coupled electron transport), chromatin organization (RAD51, RAD51C, POLD3, NPM2, associated with DNA polymerase activity and DNA repair), and interferon genes (CASP7, IFI44, DDX60, OAS1, OAS2, ISG15, associated with response to virus, regulation of viral genome replication, IFNg mediated signaling) (**Figure 1C**). The presence of genes related to T cell activation would support the hypothesis that TFH cells are more prone to infection; this is confirmed by the expression in TFH of genes related to DNA polymerase and DNA repair from the chromatin module. Epigenetic modifications as shown by the expression of permissive (Ex. H3K4me3) or repressive (Ex. H2K27me3) chromatin modifications, have been reported as modulating T cell fate (29) and can have an impact on HIV integration and expression.

The transcriptional suppressor BCL-6 has been shown to suppress the expression of antiviral genes in TFH cells (61, 72). Indeed, several genes are under direct or indirect control of BCL-6 and are positively correlated to the maintenance of HIV reservoir (IntDNA) and to the other viral outcomes (TILDA and VL) (**Figure 2A**). Conversely, silencing viral gene expression allows the survival of infected cells *via* two mechanisms: (i) the diminished viral gene expression downregulates viral production, which in turn prevents the virus-induced cytopathic effect and (ii) the reduced antigen presentation on MHC-I prevents recognition by CTLs or natural killer cells and, therefore, prevents cell-mediated cytotoxic killing (61). The low frequency of HIV-specific CD8 T cells in GC (67), their impaired cytotoxic capacity compared to their blood counterparts (70) or even their inability to sense the infected cells could represent cellular mechanisms for this outcome. Future studies are needed to understand the mechanism of viral gene suppression and to determine if inducing the expression of antiviral genes could lead to apoptosis of the infected cells and, therefore, the reduction of viral reservoir. Alternative, recent reports have indicated (73, 74) that combinatorial approaches aiming at virus reactivation and infected cell elimination by immunotherapies like bispecific antibodies could represent promising strategies in the context of HIV cure.

INTERFERON FAMILY OF GENES AND ITS EXPRESSION PROFILES IN TFH CELLS FROM VIREMIC SUBJECTS

Analysis of the type I IFN component in human TFH cells has shown a decreased expression of genes related to restriction factors (**Figure 2B**). This is corroborated by previous findings where microarray data sets (GEO #GSE50391) of tonsil TFH cells revealed that human TFH cells exhibit diminished expression of several anti-HIV restriction factors, including MX2, IFITMs, SAMHD1, and SLFN11, which are the IFN-stimulated genes (ISGs) shown to inhibit HIV infection/replication (75, 76). Additionally, BCL-6 has been reported to inhibit IRF7, an important antiviral TF (15, 77), and might thus contribute to the lack of intrinsic antiviral immunity in TFH cells. This fact can lead to sustained virus infection, replication, and integration, which combined with lower immune pressure could lead to the establishment of intact DNA in this T cell compartment.

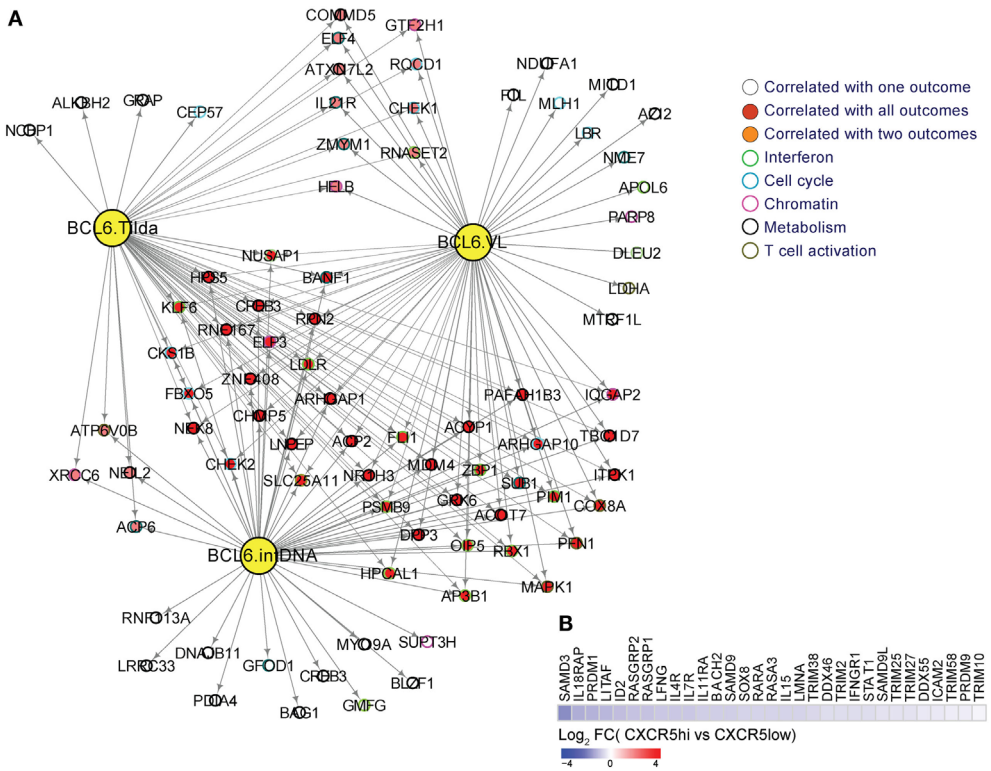

FIGURE 2 | **(A)** BCL-6 target genes are enriched in genes from interferon, metabolism, T cell activation and differentiation, cell cycle, and chromatin regulators pathways. A linear regression model to correlate BCL-6 targets to viral load, integrated DNA, and inducible RNA (TILDA) from a cohort of viremic subjects was performed using R. BCL-6 targets correlated significantly to viral load, integrated DNA and TILDA (p-value <0.05) were used to infer a gene-interacting network using Cytoscape. Yellow nodes represent BCL-6 targets correlated respectively to intDNA, TILDA, and viral loads. Red nodes represent genes correlated to viral load, integrated DNA, and TILDA, orange nodes represent genes correlated to two pathways and white nodes represent genes specific to each outcome. Circles color depict the different molecular functions enriched in these genes; **(B)** downregulation of restriction factors and genes involved in interferon type 1 signaling in $CXCR5^{high}$ vs. $CXCR5^{low}$ cells from TFH cells from HIV^{-} donors in LNs. Heatmap representing the gene log_2-fold change expression ranging from blue (down regulation) to red (upregulation).

Indeed, diminished constitutive expression of ISGs including the antiviral resistance factor MX dynamin-like GTPase 2 (MX2) and IFN-induced transmembrane 3 (IFITM3) in TFH compared with non-TFH cells might contribute to their higher susceptibility to HIV infection as previously reported. The lower antiviral resistance of TFH is consistent with a profile of higher susceptibility to retroviral infections (15). In addition, the lack of intracellular host restriction factors, such as SAMHD1, was also reported to enhance infection with high degree of viral replication (78). Additionally, BCL-6 binds to ISG loci and inhibits the expression of MX2 and IFITM3 in TFH cells. The ability of BCL-6 to control these pathways impacts directly on the TFH susceptibility to HIV infection. Amet et al. (15) demonstrated that inhibition of the BCL-6, BR-C, ttk, and bab (BTB) domain function increased the expression of ISGs and suppressed HIV infection and replication in TFH cells, revealing a regulatory role of BCL-6 in inhibiting antiviral resistance factors, thereby promoting TFH susceptibility to viral infections (15). These suggests that the modulation of BCL-6 function in TFH cells could be a potential strategy to enhance TFH cell resistance to retroviral infections and potentially decrease cellular reservoirs during HIV infection (15).

CONCLUSION

HIV infection leads to hyperplastic BCFs and increased TFH cell frequencies. TFH cells have been reported to represent an important HIV reservoir compartment harboring intact and infective proviruses. BCL-6 is the master regulator of TFH cell differentiation and plays a role in the modulation of a series of other TFs and their downstream targets. Among those genes are the ones related to IFNs, cell cycle, chromatin modifiers, metabolism, and T cell activation. All these genes play a role in the heightened susceptibility of TFH cells to infection as well as the integration of intact provirus. Further understanding of molecular pathways and genes involved in the establishment of viral infection in TFH cells could represent a strategy for efficient depletion of the HIV reservoir in this T cell compartment.

AUTHOR CONTRIBUTIONS

MA: data analyses and interpretation, FD and EM: data generation, SM: data analysis and writing, EH and JE: data generation and writing, RS and CP: writing, SR: review outline, guidance on data analysis, and writing.

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Simian Immunodeficiency Virus (SIV)-Specific Chimeric Antigen Receptor-T Cells Engineered to Target B Cell Follicles and Suppress SIV Replication

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There is a need to develop improved methods to treat and potentially cure HIV infection. During chronic HIV infection, replication is concentrated within T follicular helper cells (Tfh) located within B cell follicles, where low levels of virus-specific CTL permit ongoing viral replication. We previously showed that elevated levels of simian immunodeficiency virus (SIV)-specific CTL in B cell follicles are linked to both decreased levels of viral replication in follicles and decreased plasma viral loads. These findings provide the rationale to develop a strategy for targeting follicular viral-producing (Tfh) cells using antiviral chimeric antigen receptor (CAR) T cells co-expressing the follicular homing chemokine receptor CXCR5. We hypothesize that antiviral CAR/CXCR5-expressing T cells, when infused into an SIV-infected animal or an HIV-infected individual, will home to B cell follicles, suppress viral replication, and lead to long-term durable remission of SIV and HIV. To begin to test this hypothesis, we engineered gammaretroviral transduction vectors for co-expression of a bispecific anti-SIV CAR and rhesus macaque CXCR5. Viral suppression by CAR/CXCR5-transduced T cells was measured *in vitro*, and CXCR5-mediated migration was evaluated using both an *in vitro* transwell migration assay, as well as a novel *ex vivo* tissue migration assay. The functionality of the CAR/CXCR5 T cells was demonstrated through their potent suppression of SIV_{mac239} and SIV_{E660} replication in *in vitro* and migration to the ligand CXCL13 *in vitro*, and concentration in B cell follicles in tissues *ex vivo*. These novel antiviral immunotherapy products have the potential to provide long-term durable remission (functional cure) of HIV and SIV infections.

Keywords: HIV, simian immunodeficiency virus, chimeric antigen receptor, CAR-T cells, CXCR5, B cell follicles, $CD8^+$ T cells, HIV cure strategies

INTRODUCTION

Over 2 million individuals become infected with HIV each year, and nearly 37 million people are currently infected with HIV (1). Current antiretroviral therapy (ART), while effective at reducing viral loads, does not eliminate the virus, thus requiring HIV-infected individuals to remain on ART for life. ART is expensive, inconvenient, demands strict adherence, and, in some cases, leads to

drug resistance. In addition, HIV-infected individuals remain at increased risk of cardiovascular disease (2), neurological disease (3), and malignancies (4), and have decreased life expectancies (5). Given these issues and risks, there is great global interest in developing strategies to fully eradicate infectious HIV from the body ("sterilizing cure"), or to achieve durable viral remission in the absence of ART ("functional cure") (6).

During chronic HIV and simian immunodeficiency virus (SIV) infections prior to the development of AIDS, virus replication is most concentrated within B cell follicles (7–12), primarily within T follicular helper cells (Tfh) (10, 13, 14). Replication is further sustained by infectious virions adhering to the surface of follicular dendritic cells (FDC) *via* antibody and complement complexes in germinal centers (15–19). Although virus-specific $CD8^+$ T cells are critical for controlling HIV and SIV infections, they fail to fully suppress viral replication (20). Several mechanisms are thought to contribute to this failure including: the emergence of CTL escape variants (21–28), viral induced MHC class I down-modulation (29, 30), viral latency (31), CTL exhaustion (32–34), and potential Treg inhibition of CTL (35–39). A particularly compelling factor, which we address in this study, is that levels of virus-specific $CD8^+$ T cells are low within B cell follicles, thereby permitting ongoing viral replication (8, 9, 40–42).

Migration of cells into the B cell follicle is mediated through the chemokine receptor, CXCR5 (43–45), and its ligand, the chemokine CXCL13 (46, 47), which is expressed by B cells (48–50) and FDCs in follicles (47, 51). We hypothesize that increasing levels of virus-specific CTL in B cell follicles will lead to significantly better control of viral replication in B cell follicles and might lead to sustained remission of HIV infection (42). Several lines of evidence support this hypothesis. In lymphocytic choriomeningitis virus (LCMV)-infected mouse models, adoptive transfer of CXCR5-expressing, LCMV-specific $CD8^+$ T cells controlled LCMV infection of Tfh cells and reduced viral loads significantly better than $CXCR5^-$ $CD8^+$ T cells (52, 53). We previously showed that levels of SIV-specific CTL in lymphoid compartments predicted levels of viral replication in lymphoid compartments (8) and that levels of SIV-specific CTL in follicles tended to predict plasma viral loads (36). Furthermore, it was reported recently that levels of virus-specific $CXCR5^+$ cells inversely correlated with viral load in HIV-infected individuals (52). In addition, in a recent SIV CTL vaccine study, it was found that vaccine induced protection from pathogenic SIV challenge was associated with increased levels of $CXCR5^+$ virus-specific $CD8^+$ T cells (54). Thus, increasing virus-specific $CD8^+$ T cells in B cell follicles is predicted to lead to better control of viral replication in lymphoid follicles and decreased viral loads.

In the field of cancer immunotherapy, dramatic successes have been achieved by genetically engineering autologous patient T cells to express a chimeric antigen receptor (CAR). CAR-T cells have shown great promise in treating certain B cell leukemias and lymphomas, and are being actively pursued to treat additional cancers including solid tumors (55–57). Several features make CAR technology particularly appealing in HIV functional cure efforts (58–61). CAR activity is MHC-independent, and thus not compromised by HIV-1 nef-mediated down-modulation of MHC-I in infected cells that facilitates their evasion from conventional cytotoxic T cells (62). The target for an anti-HIV CAR is the viral Env glycoprotein, which is expressed exclusively on infected cells. Env is absolutely essential for virus infectivity and spread, and the targeting motif of the CAR can be designed to recognize strictly conserved Env elements that are refractory to mutational escape. Interestingly, the very first clinical tests of CAR technology were directed against HIV-1 infection, using first-generation CAR constructs employing CD4 as the targeting motif; while minimal virus suppression was achieved, the gammaretroviral-engineered CAR-T were found to be safe, and had stable levels of engraftment with a decay half-life exceeding 16 years (63–66).

Achieving durable HIV/SIV remission in the absence of ART demands long-term persistence of functional CAR-T cells, with minimal chance for virus mutational escape and immune response against the CAR. To this end, we have designed bispecific CARs containing CD4 (domains 1 and 2) linked to a second moiety that binds to a distinct highly conserved site on the HIV-1 Env glycoprotein. The second moiety both enhances CAR potency and prevents the CD4 from acting as an entry receptor in CAR-expressing $CD8^+$ T cells (67, 68). In a favored CAR construct (68), the second moiety is the carbohydrate recognition domain of mannose-binding lectin (MBL), which binds to the dense oligomannose patch that is highly conserved on clinically relevant HIV-1 variants. Indeed, compared to a monospecific CD4 CAR, the CD4–MBL CAR displays superior suppressive activity against genetically diverse HIV-1 primary isolates. Immunogenicity concerns are minimized with the CD4–MBL CAR, since both Env-binding components are derived entirely from human protein sequences; moreover, the MBL moiety lacks the equivalent of variable regions that are likely to elicit immune responses during the long-term persistence required to durably maintain HIV suppression.

In the present study, we address another major concern for achieving effective HIV control, namely the likely requirement to enhance CAR-T cell trafficking to B cell follicles. To this end, we engineered rhesus macaque T cells to co-express CXCR5 along with an all-rhesus variant of the CD4–MBL CAR. Results from *in vitro* and *ex vivo* assay systems suggest promising potential of this approach as a means to direct CAR-T cells to B cell follicles, where HIV replication is concentrated.

MATERIALS AND METHODS

Plasmid Constructs and Retroviral Vectors Encoding CARs

All CAR targeting motifs were synthesized by GenScript, codon-optimized for expression in rhesus macaque cells, and subcloned into the plasmid pMSGV1 gammaretrovirus vector backbone (69). The active antiviral CAR employed in this study was a rhesus variant of the human bispecific CAR designated CD4–MBL (68). As a non-reactive negative control, we used the previously described 139 CAR, which does not react with cells in this system. The targeting domains were linked to extracellular hinge, transmembrane and cytoplasmic co-stimulatory domain of rhesus CD8 followed by the activation domain of rhesus CD3 zeta, as previously described (67, 68).

T cells were transduced to express either the rhCD4–MBL CAR, rhCXCR5, or the rhCD4–MBL CAR plus rhCXCR5. For co-expression, bicistronic plasmid constructs (produced by GenScript) were designed in which the rhCD4–MBL gene was linked to the downstream rhCXCR5 gene. CXCR5 expression was driven by either the ECMV internal ribosome entry site (IRES) or the self-cleaving P2A peptide from porcine teschovirus-1 with a GSG linker added at the N-terminus of the P2A peptide sequence (70). The corresponding gammaretroviruses were generated for expression of these genes in rhesus macaque T cells. In most experiments, these plasmids were co-transfected with the plasmid pBS-CMV-gagpol (71) (a gift from Dr. Patrick Salmon, Addgene plasmid #35614), a plasmid encoding RD114 envelope glycoprotein (72), and the plasmid pMD.G encoding VSV-G envelope (73) (a gift from Dr. Scott McIvor) at ratios of 3:1:1:0.4, respectively. Retroviral vector supernatants were collected 48 h after transfection, and were titrated by transducing HEK293T cells. Retrovirus was snap frozen and stored at −80°C. In the SIV suppression studies, gammaretrovirus vector production was carried out as previously described (67).

Transduction of Rhesus T Cells

Primary rhesus macaque PBMC, or $CD8^+$ T cells enriched by negative selection (Miltenyi), were activated for 2 to 3 days in six-well plates with plate-bound anti-CD3 (FN18) and soluble anti-CD28.2 (both from NHP Reagent Resource) in either RPMI supplemented with 10% heat inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 300 IU/ml IL-2, for early experiments, or in X-Vivo 15 completed with 10% heat inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, and 50 IU/ml IL-2 for later experiments. RetroNectin (TaKaRa)-mediated transduction was carried out on the activated T cells. Retroviral vector supernatants, diluted in serum-free media, were added (eventual MOI of 0.5) to RetroNectin-coated six-well plates and centrifuged for 2 h at 2,000 × *g* to facilitate binding of the retrovirus. After removal of the unbound retrovirus, activated PBMC or $CD8^+$ T cells (1.5×10^6 cells/well) were added to the wells and centrifuged at 1,000 × *g* for 10 min. Mock-transduced cells were subjected to exactly the same procedures without the addition of retrovirus to the RetroNectin-coated wells. Cells were cultivated in the media listed above for 5–6 days prior to analysis by flow cytometry.

Flow Cytometry

Cells were analyzed using an LSR Fortessa flow cytometer (BD Bioscience). The following antibodies were used: CD4 (M-T477, reactive with endogenous rhCD4 and the rhCD4–MBL CAR), CD3 (SP34-2), CD8 (RPA-T8) (all from BD Bioscience), CXCR5 (MU5UBEE) (eBioscience), MBL2 (3E7) (Invitrogen). Viability was assessed with the Live/Dead Fixable Near IR Dead Cell Stain Kit (Invitrogen). A minimum of 70,000 events were acquired for each sample. Data analysis utilized FlowJo v10 (FlowJo, LLC).

In Vitro Transwell Migration Assay

Rhesus macaque PBMCs were transduced with the CAR or CAR/CXCR5 vectors, or mock-transduced. Samples were run in duplicate. For each sample, one million cells in 100 µl X-Vivo-15 media containing 0.1% BSA were placed in the upper chamber of a 24-well plate, with a 5.0-µm transwell membrane (Costar). To the lower chamber containing 600 µl X-Vivo 15 and 0.1% BSA, either CXCL12 at 1 µg/ml or CXCL13 at 2.5 µg/ml (both from ProSpec) were added. No chemokine was added to control wells. After incubation for 4 h at 37°C, cells were collected from the lower chamber, fixed with 1% paraformaldehyde, and counted on a Cytoflex flow cytometer (Beckman). All samples were normalized with the addition of AccuCheck Counting Beads (Invitrogen). Specific cell migration was determined by first subtracting the number of cells that migrated to media alone from the number of cells that migrated to the chemokine and then dividing by the number of cells added to the upper chamber.

Ex Vivo B Cell Follicle Migration Assay

Chimeric antigen receptor- and CAR/CXCR5-transduced rhesus $CD8^+$ T cells were used in conjunction with fresh lymph node tissue sections from allogeneic rhesus macaques. A gelatin sponge (7 mm Gel foam by Pfizer) was cut to fit and placed into a six-well plate containing 3–4 ml of RPMI with 20% heat inactivated FBS. The sponge was hydrated for 1 h at 37°C. Fresh rhesus macaque lymph nodes, collected at the Wisconsin National Primate Research Center, were shipped in chilled RPMI containing 100 µg/ml heparin overnight on ice blocks. Lymph nodes were cut into 0.5 cm × 0.5 cm pieces and embedded in 40°C PBS-buffered 4% low-melt agarose and cut into 300-µm thick slices using a Compresstome, as we have previously described (74). Tissue sections and associated agarose were laid flat on the hydrated sponge without being submerged. Transduced $CD8^+$ T cells were stained with a 5-µM solution of Cell Trace Violet Dye (CTV) (Molecular Probes). The dye was added at a 1:1 ratio to 1×10^7 cells/ml suspended in PBS/10% FBS, and cells were incubated for 15 min at 37°C, followed by two washes with complete RPMI supplemented with 10% heat inactivated FBS 100 U/ml penicillin, and 100 µg/ml streptomycin. For each fresh tissue section, one million CTV-stained transduced $CD8^+$ T cells were re-suspended in 20–30 µl complete RPMI and were slowly pipetted onto the surface of the tissue. Tissue sections were incubated at 37°C for 6 h followed fixation with 4% PBS-buffered paraformaldehyde for 2 h at RT. After fixation, sections were washed with chilled PBS containing 100 µg/ml heparin (PBS-H). Antigen retrieval was carried out by boiling tissues 3× in 0.01 M urea for 30 s. Tissues were permeabilized and blocked with PBS-H containing 0.3% Triton x-100 and 2% normal goat serum for 1 h, then incubated overnight with mouse-anti-human CD20 (0.19 µg/ml, clone L26, Novocastra) to label B cells and rat-anti-human CD3 (2 µg/ml, CD3-12, Bio-Rad) to label T cells. After washing with PBS-H, secondary antibody staining was carried out by incubating tissues overnight with goat-anti-mouse-IgG/Alexa 488 (0.75 µg/ml Jackson ImmunoResearch Laboratories) and goat-anti-rat-IgG/Cy5 (0.3 µg/ml, Jackson ImmunoResearch Laboratories). All incubations were done at 4°C on a rocking platform. Sections were imaged using a Leica confocal microscope. 512 × 512 pixel *z*-series were collected using a step size of 2 µm and with collection initiated at least 50 µm deep into each section. B cell follicles were identified morphologically as clusters of brightly stained closely aggregated $CD20^+$ cells. Areas

that showed loosely aggregated B cells that were ambiguous as to whether the area was a follicle were not included. Cell counts were done with individual *z*-scans. The total number of CTV-stained cells was counted inside follicles and the adjacent area outside of the follicles. For each sample, 2–3 tissue sections and a minimum of three follicles (range 3–8) were evaluated.

SIV Suppression Assay

To generate SIV-infected target cells, rhesus macaque PBMCs were re-suspended at 5×10^5/ml in complete medium, transferred to a T25 flask, and incubated at 37°C in 5% CO_2 for 2–3 days. The PBMCs were washed, adjusted to 3×10^6/ml in total of 4 ml volume in complete media containing 30 IU/ml IL-2, and incubated with 200–600 $TCID_{50}$/ml of virus for 24 h at 37°C in 5% CO_2. Infected cells were washed three times using 20 ml of medium per wash and then re-suspended in complete medium at a density of 1.5×10^6 cells per ml in 96-well round bottom plates. To generate effector cells, T cells (derived from activated PBMCs) were transduced with the indicated gammaretroviral vectors. In triplicate, 100 µl of SIV-infected targets were mixed with 100 µl of serially diluted effectors. Cocultures were incubated at 37°C in 5% CO_2 for a total of 16 days. On the indicated days, supernatants were collected, and p27 content was determined by ELISA (ABL, Inc.).

Statistical Analysis

All statistical analyses assumed two-sided tests with $P < 0.05$ considered significant. Paired *t*-tests with pooled variance were used to evaluate co-expression levels of the CAR and CXCR5 *via* IRES versus P2A constructs An unpaired *t*-test with pooled variance was used to evaluate groups in the CXCL12 *in vitro* migration assays while an unpaired Welch's *t*-test of unequal variance was used to evaluate groups in the CXCL13 *in vitro* migration assays. Paired *t*-tests with pooled variance were used in all statistical analyses in the *ex vivo* migration assay. The F:EF ratios were log transformed before analysis. Statistical analyses were conducted using GraphPad Prism (Version 6.01; GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS

The goal of this study was to engineer rhesus macaque T cells to co-express a potent anti-SIV CAR along with CXCR5, in order to promote CAR-T cell trafficking to B cell follicles. To this end, we designed constructs for expression of the CARs, without or with co-expression of CXCR5. CAR-transduced T cells were analyzed using both an *in vitro* transwell assay of chemokine-directed cell migration and a novel *ex vivo* B cell follicle migration assay. In addition, we tested the ability of T cells expressing CAR and CXCR5 to suppress viral replication *in vitro*.

CAR and CXCR5 Expression in Transduced Primary Rhesus Macaque T Cells

For this study, we developed gammaretroviral vectors encoding the rhCD4–MBL CAR and rhCXCR5, and vectors encoding bicistronic constructs to express both proteins. We developed two variations of bicistronic vectors, one with CXCR5 co-expression driven by an internal ribosome entry site (IRES) and the other *via* a P2A self-cleavage site (70). For simplicity, the constructs encoding the rhCD4–MBL CAR alone or the bicistronic rhCD4–MBL CAR plus rhCXCR5 are, respectively, referred to as CAR or CAR/CXCR5; for the latter, the use of either the IRES or P2A modalities is indicated. The constructs are shown schematically in **Figure 1A**.

In **Figures 1B–F**, T cells derived from activated rhesus PBMCs were transduced with gammaretroviral vectors encoding the CAR or CXCR5 genes alone, or the bicistronic CAR/CXCR5 constructs (IRES or P2A). Cell viabilities posttransduction were 87–90% (data not shown). Antibodies directed against MBL or CXCR5 were used to detect surface expression of the CAR and CXCR5, respectively. Transduction with the CAR (**Figure 1B**) or CXCR5 (**Figure 1C**) vectors gave the expected surface expression of the corresponding individual proteins. For vectors encoding the bicistronic CAR/CXCR5, the P2A-based construct yielded a clear population of cells expressing both CAR and CXCR5, with only a small fraction of cells expressing only one of the proteins; by contrast, the IRES-based construct appeared less effective at co-expressing CXCR5 relative to CAR, since the fraction of cells expressing only the CAR was comparable to that expressing both proteins, with a minimal fraction expressing only CXCR5 only (**Figures 1D,E**). These results are consistent with the efficient P2A system producing equivalent amounts of the two post-cleavage components of a bicistronic construct, as contrasted with the relatively inefficient expression of the downstream component in the IRES system (75, 76). Moreover, as indicated in **Figure 1G**, the P2A-based construct produced cells with nearly twofold higher surface expression levels of CXCR5 than obtained with the IRES-based construct (median 1.8-fold higher; range 1.4- to 2.2-fold).

The percentages of T cells that expressed the CAR and CXCR5 with each construct are shown in **Figure 1H**. Transduction with the vectors encoding CAR-only or CXCR5-only yielded a median of 44.4% (range 40–47.6%) and 81.1% (range 51.8–84.2%) of cells expressing each protein, respectively. Cells transduced with the IRES-based bicistronic CAR/CXCR5 vector showed higher number of cells expressing the CAR compared to CXCR5, with a median cell expression of 37.2% (range 5.6–50.2%) for the CAR and 19.4% (range 4.7–19.7%) for CXCR5. In contrast, cells transduced with the P2A-based CAR/CXCR5 vector showed similar expression of the two proteins, with a median of 40.2% (range 37.3–52.2%) for the CAR and 38.9% (range 27.5–50.2%) for CXCR5. Similar transduction efficiencies were found with enriched rhesus CD8 T cells transduced with these vectors (data not shown). The percentage of cells that co-expressed CAR and CXCR5 is shown in **Figure 1I**. Cells transduced with the IRES-based construct showed a median co-expression efficiency of 18.1% (range 4.3–18.9%), whereas cells transduced with the P2A-based construct resulted in a significantly higher co-expression efficiency of 33.3% (range of 25.9–38.3). Thus, the data in **Figure 1** establish the suitability of the P2A-based bicistronic system for efficient co-expression of CAR and the B cell follicle-homing chemokine receptor CXCR5, and its superiority over the IRES-based system.

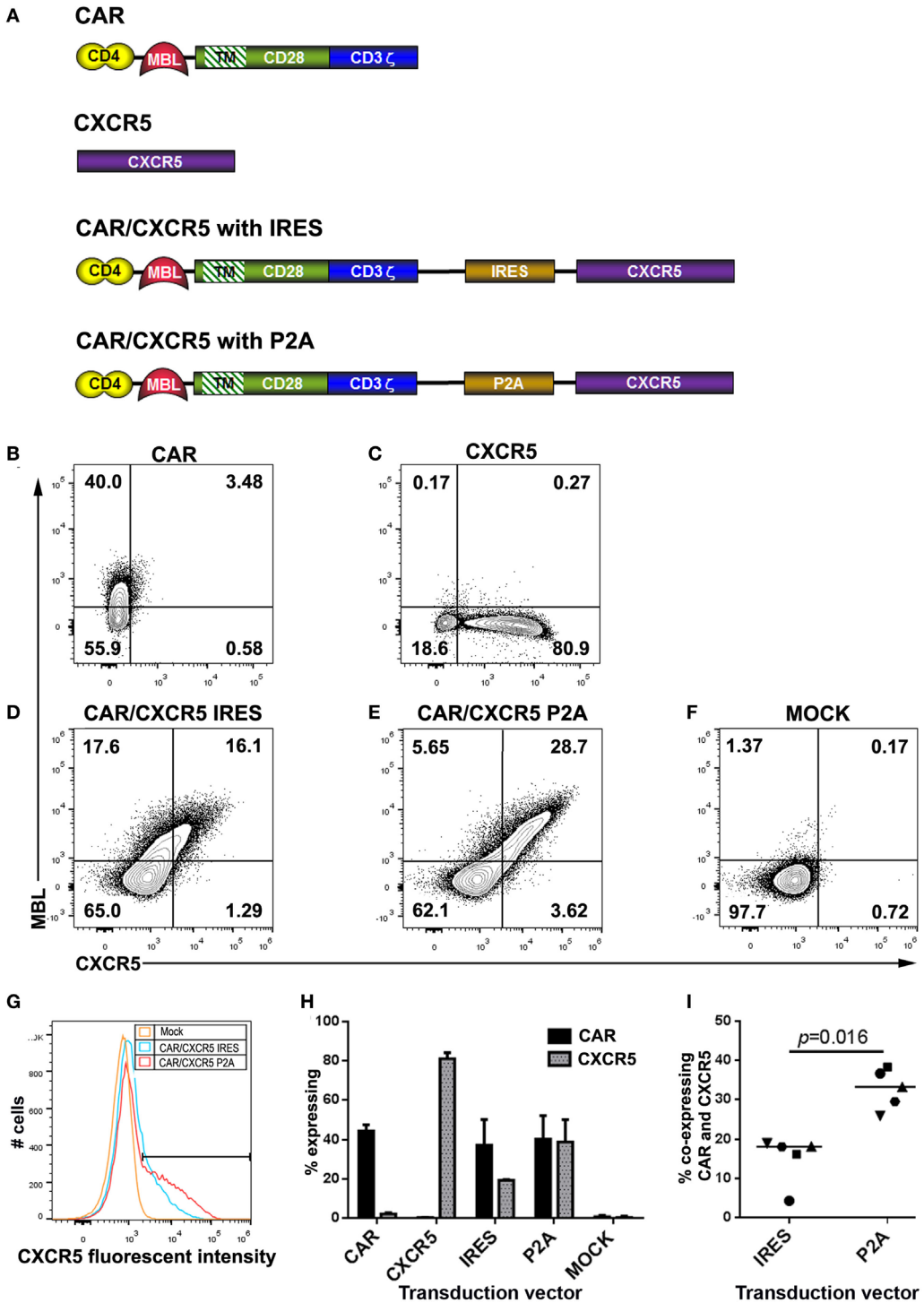

FIGURE 1 | Construct design, and expression in rhesus macaque T cells. **(A)** Schematic figures showing constructs encoding the chimeric antigen receptor (CAR) (rhCD4–MBL CAR), rhCXCR5, and the bicistronic CAR/CXCR5 encoding both proteins, with CXCR5 expression mediated by IRES or P2A. In all cases, the targeting domains are linked to domains from rhesus CD28 including a short extracellular hinge, transmembrane TM, and cytoplasmic signaling, followed by the CD3 activation domain. Cells were transduced with gammaretroviral vectors encoding **(B)** CAR, **(C)** CXCR5, **(D)** CAR/CXCR5 (IRES), **(E)** CAR/CXCR5 (P2A), or **(F)** mock-transfected, and analyzed by flow cytometry. Cells were pre-gated sequentially on lymphocytes, singlets, live cells, and $CD3^+$ cells (T cells) and evaluated for CAR and CXCR5 expression, using antibodies against mannose binding lectin (MBL) and CXCR5, respectively. **(G)** Histogram depicting fluoroscent intensites of CXCR5 expression from samples shown in panels **(D–F)**. **(H)** Median percentage of T cells that expressed the CAR and CXCR5 in activated PBMCs transduced with CAR ($n = 3$), CXCR5 ($n = 3$), CAR/CXCR5 (IRES) ($n = 5$), CAR/CXCR5 (P2A) ($n = 5$) and mock-transduced ($n = 5$). **(I)** The percentage of T cells that co-expressed the CAR and CXCR5 in activated PBMCs transduced with either CAR/CXCR5 (IRES) or CAR/CXCR5 (P2A).

CXCR5 Co-Expression Promotes CAR-T Cell Migration Selectively to CXCL13 *In Vitro*

We next tested the ability of CXCR5 co-expression to promote migration of CAR-T cells toward CXCL13, the chemokine ligand for CXCR5. To this end, we utilized an *in vitro* transwell migration assay. Using this assay, we found that both CAR-transduced and CAR/CXCR5-transduced PBMCs similarly migrated toward a positive control chemokine CXCL12 (SDF-1α) that is strongly chemotactic for lymphocytes (77) demonstrating the ability of both CAR and CAR/CXCR5-transduced cells to migrate to a chemotactic stimulus (**Figure 2A**). In contrast, significantly more CAR/CXCR5-transduced than CAR-transduced PBMCs migrated toward CXCL13 (**Figure 2B**). Furthermore, increasing specific migration to CXCL13 was seen with an increase in the percentage of cells expressing CXCR5 (**Figure 2C**). For these studies, a median of 54% (range 12–64%) of CAR/CXCR5-transduced cells expressed CXCR5. By contrast, a median of only 2% (range 1–5%) of the CAR-transduced cells expressed CXCR5 and they showed minimal migration to the stimulus. These results demonstrate that co-expression of CXCR5 promotes selective migration of the CAR-T cells toward CXCL13 *in vitro*.

CAR/CXCR5-Transduced CD8$^+$ T Cells Selectively Migrate into B Cell Follicles *Ex Vivo*

As an additional means to evaluate the ability of CXCR5 to promote selective migration of CAR-T cells, we developed a novel *ex vivo* B cell follicle migration assay. This method was adapted from previously described *ex vivo* live tissue migration assays that tracked T cells in mouse thymus tissue using two-photon microscopy (78, 79). For these studies, we evaluated the migration of CTV-labeled CAR- and CAR/CXCR5-transduced primary rhesus macaque CD8$^+$ T cells in fresh lymph node tissue sections. **Figure 3A** shows representative images of sections incubated with CTV-labeled CAR and CAR/CXCR5-transduced cells. Similar levels of total CTV$^+$ cells were detected in lymph node sections incubated with CAR versus CAR/CXCR5-transduced cells (**Figure 3B**). While total numbers of cells were similar, significant differences were observed in the levels of CAR- compared to CAR/CXCR5-transduced cells in follicular and extrafollicular compartments. Significantly lower levels of CTV$^+$ cells were found in follicular compared to extrafollicular areas in sections incubated with CAR-transduced cells (**Figure 3C**). In contrast, significantly higher levels of CTV$^+$ cells were found in follicular compared to extrafollicular areas in sections incubated with CAR/CXCR5-transduced cells (**Figure 3D**). As a result, significantly large increases in the follicular to extrafollicular ratios (F:EF) of CTV-labeled cells were detected in the tissue sections incubated with CAR/CXCR5- compared to CAR-transduced T cells. Sections incubated with CAR/CXCR5-transduced cells showed a median F:EF ratio of 2.8 (range of 1.5–6.9), whereas sections incubated with CAR-transduced T cells showed a median ratio of 0.4 (range 0.3–0.7) (**Figure 3E**). An increased follicular to extrafollicular ratio was seen with an increase in the percentage of cells expressing CXCR5 (**Figure 3F**). A median of 46% (range 23–71%) of CAR/CXCR5-transduced cells expressed CXCR5 and they showed relatively high F:EF ratios. By contrast, a median of only 1.6% (range 0.2–4.1%) of the CAR-transduced cells expressed CXCR5 and they showed correspondingly low F:EF ratios. Thus, in this novel *ex vivo* B cell follicle migration assay, CAR/CXCR5- but not CAR-transduced CD8$^+$ T cells preferentially migrated to B cell follicles.

CXCR5 Co-Expression Does Not Impair CAR-T Cell-Mediated Suppression of SIV Replication *In Vitro*

The all-rhesus CD4–MBL CAR (rhCD4–MBL) displayed potent suppression of multiple SIV strains (Hajduczki et al., manuscript in preparation). For this study, we tested whether co-expression of CXCR5 affected the potency of SIV suppression by T cells expressing the rhesus CD4–MBL CAR. PBMCs transduced with the CAR or CAR/CXCR5 vectors, were cocultured with rhesus PBMC targets infected with two different pathogenic SIV

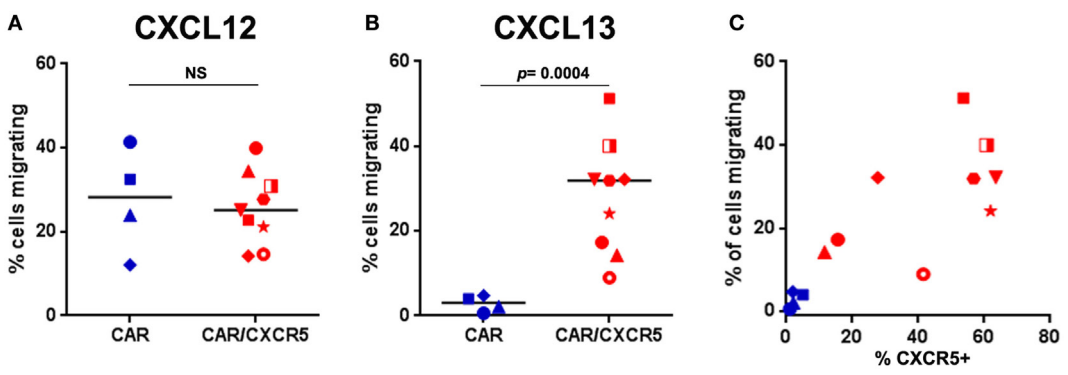

FIGURE 2 | Co-expression of CXCR5 promotes selective migration of chimeric antigen receptor (CAR)-T cells toward CXCL13 *in vitro*. The percentage of CAR- or CAR/CXCR5-transduced PBMC that migrated toward **(A)** CXCL12 (SDF-1) or **(B)** CXCL13 was measured in transwell plates. **(C)** The relationship between the percentage of cells expressing CXCR5 and the percentage of cells that migrated. In all panels, each data point symbol represents the mean value of duplicate samples obtained with cells from individual animals, with colors indicating transduction with CAR (blue) or CAR/CXCR5 (red).

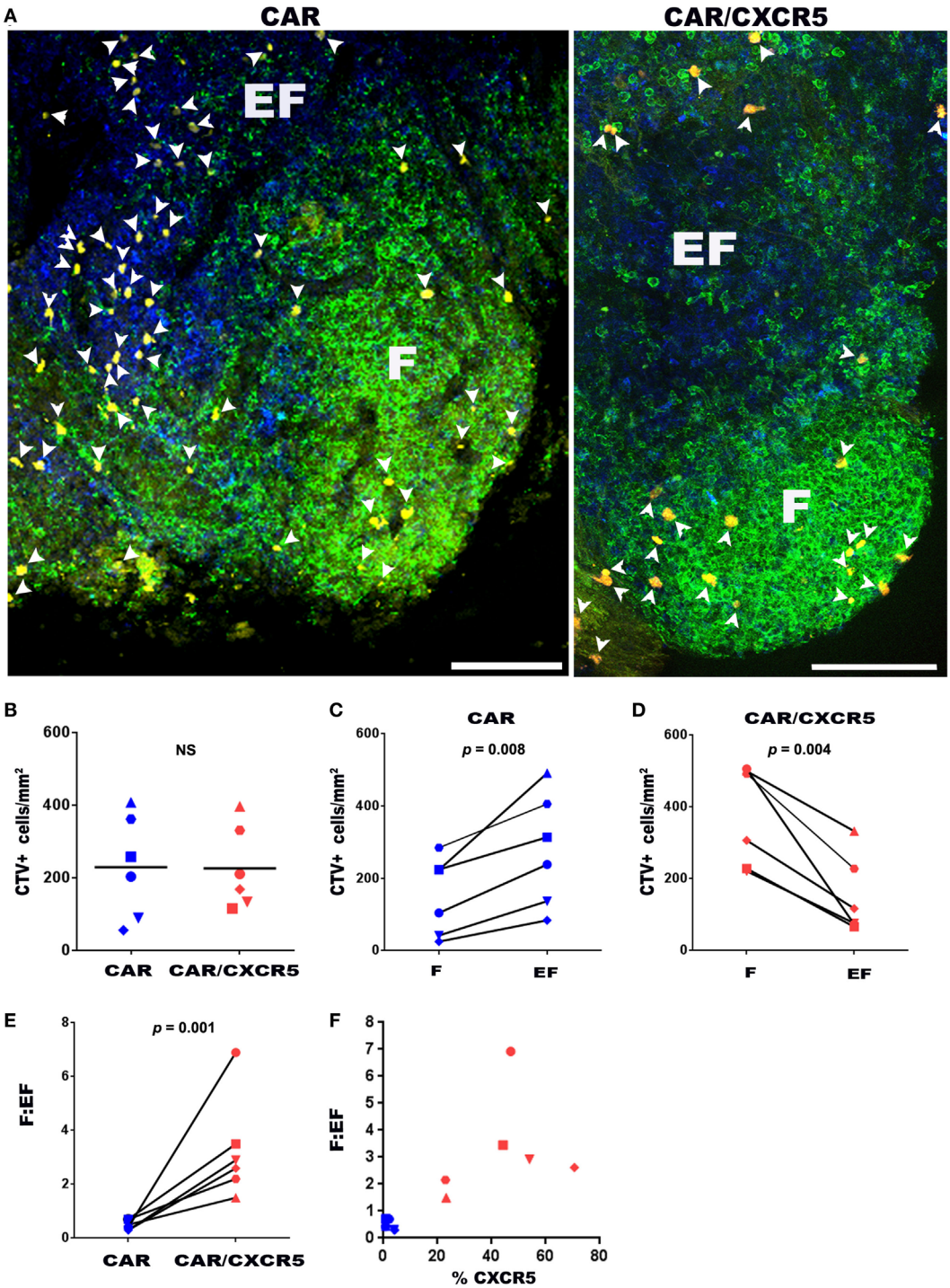

FIGURE 3 | *CXCR5* co-expression enhances CD8⁺ CAR-T cell migration to B cell follicles *ex vivo*. **(A)** Chimeric antigen receptor (CAR) or CAR/CXCR5-transduced rhesus macaque CD8⁺ T cells were stained with cell trace violet dye (CTV) (pseudo-colored yellow), then pipetted on to fresh rhesus macaque lymph node sections and incubated for 6 h at 37°C. Sections were then fixed and stained with anti-CD20 antibodies (green) to delineate B cell follicles (F) and anti-CD3 antibodies (blue) to delineate the T cell zone and extrafollicular areas (EF). Arrowheads indicate CTV⁺ cells. Confocal images were collected with a 20× objective. Scale bars equal 100 µm. **(B)** Similar total levels of CFSE-labeled CD8⁺ T cells were detected in tissues incubated with CAR- and CAR/CXCR5- transduced cells. **(C)** CAR-transduced cells showed higher levels in the extrafollicular regions than in the follicles. **(D)** By contrast, CAR/CXCR5-transduced cells showed increased levels within B cell follicles. **(E)** CAR/CXCR5-transduced cells showed higher F:EF ratios compared to CAR-transduced cells. **(F)** The relationship between the percentage of transduced cells that expressed CXCR5 and F:EF ratios. Each symbol represents individual animals from which CD8⁺ T cells were derived.

isolates, SIV_{mac239} and SIV_{E660}. The negative controls employed included adding no effector T cells, and adding effector T cells that were transduced with the 139 CAR that recognizes an irrelevant epitope [a glioma-specific variant of the epidermal growth factor receptor (80)]. Robust spreading of viral infection by both SIV strains was evident in the presence of the negative control effector cells (no effector T cells and 139 CAR-transduced T cells). In contrast, CAR-transduced and CAR/CXCR5-transduced effectors suppressed infection by both strains with equivalent high potency over the 12-day infection, at E:T ratios of 1:1 or 0.2:1 (**Figure 4**). These data demonstrate that the antiviral activity of CAR-T cells is not altered by co-expression of the CXCR5 follicular trafficking chemokine receptor on the effector cell surface.

DISCUSSION

In most HIV-infected individuals and SIV-infected rhesus macaques, virus-specific CTL fail to accumulate to high levels in B cell follicles (8, 9, 40–42), where virus replication is most concentrated prior to the development of AIDS (7–12). The paucity of virus-specific CTL in follicles permit ongoing replication (8, 36, 52, 54). We hypothesize that increasing levels of virus-specific CTL in follicles will lead to better control of viral replication and may lead to long-term durable remission in the absence of ART, i.e., a "functional cure." In this study, we developed tools to test this hypothesis in the SIV-infected rhesus macaque model of HIV infection. To this end, we developed gammaretroviral vectors for co-expression of a potent bispecific anti-SIV CAR (rhCD4–MBL) and the B cell follicle-homing chemokine receptor CXCR5.

Our initial bicistronic constructs encoding both the CAR and CXCR5 utilized an internal ribosome entry site (IRES) to achieve co-expression. We found inconsistent and often low levels of cells that co-expressed both the CAR and CXCR5. This was likely due to inefficient initiation of translation at the IRES. This finding was not altogether surprising as it is well known that IRES-dependent gene expression from a bicistronic construct is not always efficient in all cell systems (75, 76). To achieve more consistent levels of CAR and CXCR5 co-expression, we tested an alternative construct with the porcine teschovirus-1 P2A self-cleavage site between the CAR and CXCR5 genes. The P2A-sequence allows the cell to produce both proteins without re-initiation of translation due to a "stop and go" translational effect mediated by the ribosome, thereby resulting in similar levels of expression of the two proteins (70, 81). In contrast with the IRES-based CAR/CXCR5 construct, T cells transduced with the P2A-based construct consistently produced efficient co-expression of both proteins on the T cell surface. Moreover, the P2A yielded nearly twofold higher levels of CXCR5 at the cell surface. These results highlight the superiority of the P2A compared to the IRES modality for advancing the CAR/CXCR5 system as an immunotherapy product.

Using the P2A system, we demonstrated CXCR5 functionality in promoting targeted migration of CAR-T cells. In an *in vitro* migration assay, CXCR5 co-expression drove selective migration of rhesus CAR-T cells toward CXCL13, the chemokine ligand for CXCR5 responsible for follicular homing. Moreover, using in a novel *ex vivo* B cell follicle migration assay, we demonstrated that CXCR5 co-expression promoted accumulation of rhesus CAR-T cells in B cell follicles of rhesus lymphoid tissue. This finding is supported by the recent report showing that rhesus $CD8^+$ T cells engineered to express human CXCR5 and infused into rhesus macaques accumulated within B cell follicles *in vivo* (82). The T cells used in that study, however, did not contain a

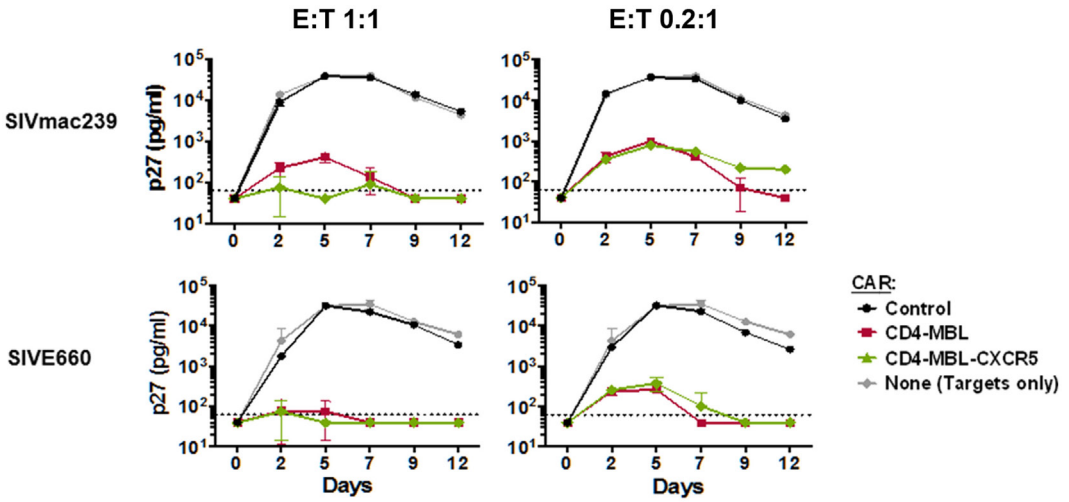

FIGURE 4 | Chimeric antigen receptor (CAR)/CXCR5-transduced T cells suppress simian immunodeficiency virus (SIV) *in vitro*. PBMC target cells were infected with the indicated SIV_{mac239} and SIV_{E660} isolates for 24 h. The cells were then washed and mixed with the effector cells transduced as indicated, at effector-to-target ratios (E:T) of 1:1 (left panels) or 0.2:1 (right panels). Culture supernatants were collected on the indicated days, and the presence of virus was determined by p27 ELISA. The effector cells were transduced either with the CD4–mannose-binding lectin (MBL)–CAR alone or CD4–MBL–CAR plus CXCR5. As negative controls, no effector cells, or cells transduced with the negative control 139 CAR were used.

viral-targeting CAR or other antiviral moiety required for suppressing virus replication.

We previously demonstrated that human CD4–MBL CAR-T cells are capable of potently suppressing *in vitro* replication of genetically diverse HIV-1 isolates (68). The rhesus variant of this CAR displays potent suppressive activity against multiple SIV strains (Hajduczki et al., in preparation). Here, we show that co-expression of rhesus CXCR5 causes no impairment of the SIV-suppressive activity of this CAR.

As mentioned, we hypothesize that treatment with autologous CAR/CXCR5-transduced T cells can be a valuable component for achieving sustained remission of HIV. Future studies to evaluate the *in vivo* efficacy of CAR/CXCR5 immunotherapy must address multiple complexities under active study in the cancer field (56, 83, 84), plus others distinct for HIV (60, 85, 86). Robust proliferation and persistence of the adoptively transferred cells is especially critical for the long-term (life-long?) viral suppression required for an HIV functional cure. Diverse aspects are being investigated, including choice of optimal cell type (T cells early stages of differentiation, hematopoietic stem cells, etc.), mode of *ex vivo* cell expansion, requirements for CAR expression on both $CD4^+$ and $CD8^+$ T cells, alternative methods for CAR gene introduction (viral vector transduction, targeted gene insertion), influence of alternative co-stimulatory domains (CD28, 4-1BB, etc.), and strategies to limit CAR-T cell exhaustion.

Additional challenges confront CAR-based immunotherapy against HIV. A particular concern involves the potential for the CAR-T cells to become infected, which would likely compromise their function and persistence. The bispecific CD4-based CARs such as CD4–MBL (67, 68) are advantageous in that the second moiety prevents the CD4 from acting as an HIV entry receptor on CAR-expressing $CD8^+$ T cells; however, an additional mode of protection is required for CAR-expressing $CD4^+$ T cells, which are susceptible to infection *via* the endogenous CD4 molecules. Another issue is the requirement for antigenic stimulation to maintain the CAR-T cells. Within the B cell follicle, infected Tfh cells may provide the necessary stimulatory activity. If CAR-T cells are administered after effective HIV suppression with ART, the required antigenic stimulation presumably would occur upon drug cessation. For maintenance of CAR-mediated suppression in the absence of ART, the spontaneous activation of latently infected cells may provide the necessary antigenic stimulation. Well-designed studies in suitable animal models will help pave the way toward efficacious CAR-T cell therapy as a component of an HIV functional cure.

ETHICS STATEMENT

Indian-derived rhesus macaque monkeys (*Macacca mulatta*) described in this study were housed at the Wisconsin National Primate Reasearch Center in accordance with the regulations of the American Association of Accreditation of Laboratory Animal Care and the standards of the Association for Assessment and Accreditation of Laboratory Animal Care International. All protocols and procedures were approved by the relevant Institutional Animal Care and Use Committee at the University of Wisconsin-Madison. All animals were housed indoors in an SOP-driven, AAALAC-accredited facility. Husbandry and care met the guidance of the Animal Welfare Regulations, OLAW reporting and the standards set forth in The Guide for the Care and Use of Laboratory Animals.

AUTHOR CONTRIBUTIONS

KH assisted with vector construction, performed transduction experiments, performed the *in vitro* migration assays, and assisted with drafting this manuscript; AH performed the viral suppression assays and assisted with drafting of this manuscript, MP optimized protocols, oversaw and performed virus production and transduction experiments in the Skinner lab, assisted with subcloning genes and sequencing vectors, and drafted this manuscript; GM developed and performed the *ex vivo* migration assays; DV-I constructed and characterized CAR constructs; ER provided study oversight, assisted with flow cytometry, and oversaw acquisition and isolation of primate cells; EC provided study oversight, obtained funding, and assisted with drafting this manuscript; EB oversaw all aspects of CAR design and functional characterization viral suppression assays, obtained funding, and contributed to drafting this manuscript; PS conceived of the studies, obtained funding, provided study oversight, and drafted this manuscript.

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REVIEW
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Potential Epigenetic Regulation in the Germinal Center Reaction of Lymphoid Tissues in HIV/SIV Infection

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The production of high-affinity and broadly neutralizing antibodies plays a key role in the defense against pathogens. These antibody responses require effective germinal center (GC) reaction within anatomical niches of GCs, where follicular helper T (Tfh) cells provide cognate help to B cells for T cell-dependent antibody responses. Emerging evidences indicate that GC reaction in normal state and perhaps establishment of latent Tfh cell reservoir in HIV/SIV infection are tightly regulated by epigenetic histone modifications, which are responsible for activating or silencing chromatin. A better understanding of the mechanisms behind GC responses at cellular and molecular levels thus provides necessary knowledge for vaccination and immunotherapy. In this review, we discussed the epigenetic regulation of GC responses, especially for GC B and Tfh cell under normal state or HIV/SIV infection.

Keywords: Epigenectic regulation, germinal center reactions, follicular CD4 T helper cells, B cells, HIV

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INTRODUCTION

B-cell lineage commitment develops in primary lymphoid tissues such as fetal liver and bone marrow, and enters circulation (1). In secondary lymphoid tissues [such as lymph nodes, spleen, and gut-associated lymphoid tissues (GALT)], antigen-activated B cells experience clonal expansion, somatic hypermutation (SHM), and selection, and ultimately differentiate into antigen-specific memory subsets and plasma cells, which require T cell-dependent interactions for full responses (2, 3). Of these, germinal center (GC) reaction is the critical checkpoint in the development of T-dependent B-cell responses against foreign pathogens. Emerging studies have shown GC responses are strictly regulated by epigenetic modifications, which cooperate with timely expression of transcriptional factors for follicular B/T helper cell differentiation, thereby modulating antibody responses to foreign- and self-antigens (4). Therefore, understanding the intrinsic mechanisms involved in GC responses, and their dysregulation in HIV infection provides potential for the development of improved vaccines and immunotherapy.

GC FORMATION AND REACTION IN INTERACTION BETWEEN GC B AND FOLLICULAR HELPER T (Tfh) CELLS

Germinal centers are unique highly organized structures that formed within organized lymphoid tissues of both peripheral and mucosal (GALT) lymphoid tissues in response to T cell-dependent antigen. In GCs, Ag-activated B-cell clones proliferate and undergo SHM and selection, eventually produce antibodies with high-affinity and antigen specificity (5–7). For example, early GCs can first be histologically observed in mice at day 4 after immunization, in which B cells expand and

differentiate into B cell blasts within the network of follicular dendritic cells (FDC) in the center of the follicle (5, 8). The dark zone (DZ) and light zone (LZ) in GCs could be microscopically distinguishable in lymphoid tissues. The DZ B cells (called centroblasts) highly proliferate, with opportunity to produce random immunoglobulin gene hypermutation and diversify Ig repertoire against foreign antigens. These DZ B cells leave DZ, and then migrate to the LZ, form LZ B cells (known as centrocytes), which are subject to clonal selection and terminal differentiation into memory B cells and plasma cells by signals from Tfh cells and FDCs. GCs are major sites for humoral immune responses, including B-cell development, differentiation and maturation, production of high-affinity antibodies that recognize and/or neutralize infectious pathogens.

The GC reaction is responsible for T-dependent humoral immune responses and is defined as the sequential process of B-cell differentiation, activation, maturation, resulting in antibody affinity maturation, and terminal differentiation, all that occurring within the GCs of lymphoid tissues. GC B cells undergo random SHM, Ig gene rearrangement, and clonal selection and eventually differentiate into long-lived memory B cells and high-affinity antibody-secreting plasma cells (8–12). By B cell receptor signaling *via* antigen binding, naïve B cells are initially activated and then migrate to the interfollicular (IF) region, where they interact with antigen-specific T cells and are thoroughly activated (13–15). These GC B founders express intermediate levels of BCL6 prior to follicular entry and GC seeding, and subsequent transit to the BCL6high state in B-cell commitment to the GC lineage, lagging behind Tfh migration into the follicle interior (16). The transcriptional repressor BCL6 is indispensable for GC B cell differentiation, repressing expression of the transcriptional factors IRF4/Blimp1 and formation of short-term antibody-secreting cell (ASC) (8, 17). However, only a proportion of these antigen-activated B cells are able to enter the GC zones and participate in the GC reaction (8). A subset of activated B cells in the IF zones at the peripheral follicles could differentiate into ASCs, which produce low-affinity antibodies to pathogens, albeit with a rapid antibody responses (18). Another pool of antigen-specific GC B cells with the highest relative affinity gains access to the lymphoid follicles, aggregated to form GCs (19–22). Within anatomical niches of mature GCs, GC B cells in the DZ (densely packed blasts, centroblasts) rapidly proliferate, undergo random SHM catalyzed by activation-induced cytidine deaminase (AID), and rearrange and diversify their IgV genes, resulting in mutant GC B cell clones with a broader repertoire of antibody specificity (23–25). Upon transition into the LZ (sparsely populated B cells, centrocytes), GC B cells with the highest affinity B cell receptors are positively selected by GC Tfh cells. Signaling from GC Tfh cells, such as CD40, IL-4, IL-9, IL-21, and ICOS, plays a pivotal role in the GC reaction during intermittent cognate engagement between GC B and Tfh cells (26–28). Rapid interactions between GC B and Tfh cells in DZ/LZ occur, as indicated by fluctuating CXCR4 and/or CXCR5 expression, which facilitate several reiterative rounds of B cell mutation and selection, resulting in terminal differentiation into highly specific memory B cells and plasma cells (5, 7, 11, 29). In the GC reaction, increasing evidence indicates that Ig SHM and selection of antigen-experienced B cells are needed for development of broadly neutralizing antibodies at checkpoints during B cell activation (30).

EPIGENETIC HISTONE REGULATION AND ITS POTENTIAL IN B-CELL DIFFERENTIATION AND ANTIBODY RESPONSES

Epigenetic alteration at posttranslational modification (PTM) is able to regulate gene expression or repression, and control cellular function without genomic sequences changes (4). Epigenetic histone modification, either by adding or removing histone methylation, acetylation, phosphorylation, or ubiquitination at histone posttranslational levels, alters chromatin structure and represses (such as chromosomal condensation) or promotes target gene transcriptional pathways affecting cell development, differentiation, and cell fates, and thereby modulates cell functions in both programmed development, or in response to disease states (31). Under the control of epigenetic regulation, cell commitment to a specific differentiated lineage involves the activation of specific genes while maintaining the other gene silence at the genomic loci (32). Among various chromatin-modifying epigenetic factors, polycomb G (PcG) proteins act in multimeric complexes known as polycomb repressive complexes (PRCs, including PRC1, PRC2, and PhoRC), which are specifically involved in histone PTMs. PRC2, composed of three subunits [enhancer of zeste homolog 2 (EZH2)/EZH1, SUZ12, and EED], binds to specific targets of chromatin, and then the enzymatic subunit EZH2 catalyzes the di- and tri-methylation of Lys 27 residues on histone H3 to generate H3K27me2/3 (33), which mediates changes in chromatin structure, transcriptional repression, somatic processes during embryonic development, lineage commitment, and even tumorigenesis (34–41). H3K27me3 could recruit PRC1 (BMI1 subunit) (42, 43), and thus stabilize polycomb G-mediated repression (39, 44, 45). EZH2 is a central core component of the PcG family, as it serves as histone-lysine *N*-methyltransferase to catalyze H3-K27 methylation (13). Conversely, aberrant EZH2 overexpression and subsequent SHMs are associated with cancer occurrence (13, 46, 47). Although EZH2 is directly responsible for the trimethylation of H3-K27, EZH2 overexpression does not directly increase H3K27me3, but instead results in PRC4-mediated H1K26 trimethylation, upregulation of demethylase (JMJD3/UTX), and phosphorylation of Ezh2 (P-Ezh2-Ser21) (48–51). Loss of H3K27me3 despite high EZH2 and demethylase levels is thus believed to be due to transcriptional suppression of H3K27me3-target genes by increased demethylase or other unknown mechanisms. The degree of lysine methylation within histones (mono-, di-, and tri-) is one modification with distinctive nuclear features and transcriptional states of target genes, and a major determinant for genome organization. Both lysine methyltransferases (KMTs) and lysine demethylases (KDMs) have specificity for specific lysine residues and degrees of methylation within the histone tails. Lysine (K) motifs within the histone tails are primary sites to recruit chromatin-modifying enzymes such as methyltransferase, leading to specific gene repression or

activation (52). For examples, H4K20 and H3K27 monomethyation (H4K20/27me1) is associated with active promoters, while H4K20 and H3K27 trimethylation (H4K20m/27me3) is affiliated with gene repression and compacted genomic regions. However, H3K4me3 is generally responsible for active chromatin (53, 54). H3K27me2 shows a similar distribution and role to H3K27me3 (55, 56). In addition, histone demethylation/acetylation, respectively, catalyzed by demethylase UTX/JMJD3 (H3K27me2/3 substrate), LSD1 (H3K4me2 substrate), JMJD2 (H3K9me3 substrate), JARID (H3K4me3 substrate), or acetyltransferase, is also associated with active transcription, antagonizing the repression of gene expression induced by H3K27me2/3 (57, 58).

In the context of antibody responses, B-cell development and the GC reaction is precisely fine-tuned by histone modifiers (59). Specifically, epigenetic modification controls B-cell differentiation and maturation, thereby regulating Ab responses (4, 13, 60–64). Upon activation by antigens, GC B cells upregulate and highly express EZH2, which segregates primarily in either the LZ or/and DZ (60, 65), and plays a pivotal role in B cell differentiation, GC formation, normal immunoglobulin VDJ recombination, inhibition of terminal B-cell differentiation, and lymphomagenesis *via* histone trimethylation (H3K27me3) (13, 61, 63, 66). High expression of EZH2, cooperating with Bcl6, is required to maintain the GC B cell phenotype but its relevance diminishes concomitant with GC B cells exiting GCs and terminal differentiation (upregulated IRF4 and BLIMP1), suggesting an important role for this protein in maintaining B cell division (8, 61, 67). EZH2 depletion or mutation perturbs B-cell differentiation and GC reaction with reduction in high-affinity antibodies, while overexpression of EZH2 promotes lymphomagenesis (63, 66). These findings suggest that EZH2 is essential for normal B-cell differentiation, activation, as well as maturation. Additionally, expression of EZH2 is also precisely regulated in various physiological and pathogenic processes (13, 68). Factors, including c-Rel, E2F1/2, Elk-1, and HIF-1α directly bind to the EZH2 promoter, leading to EZH2 expression (69–72). For example, c-Rel supports GC B cell proliferation and maintains the GC through upregulation of EZH2. Another factor, Myc could also indirectly induce EZH2 expression through miRNA or retinoblastoma protein-E2F (pRB-E2F) (73). Myc also enables GC B cell division and transformation, as Myc+ GC B cells are highly proliferative cell subsets (12, 74, 75), compared with p53-mediated suppression of EZH2 expression (76). Combined, multiple *B cell-intrinsic epigenetic alterations* may be involved in instructing B cells to undergo B cell development, GC formation, SHM, and Ab affinity maturation in the GC reaction, including differentiation to memory B cells or long-lived plasma cells (8, 12, 63, 66) (**Figure 1**).

EPIGENETIC REGULATION IN Tfh CELL RESERVOIRS IN HIV/SIV INFECTION

CD4 T cells preferentially develop into Tfh cells following repetitive T cell receptor interactions and activation, and the proinflammatory cytokines produced during persistent viral infections (77–79). Notably, epigenetic regulation is also involved in T cell differentiation and memory formation (80–82). These epigenetic alterations include PTMs. For example, EZH2 restricts

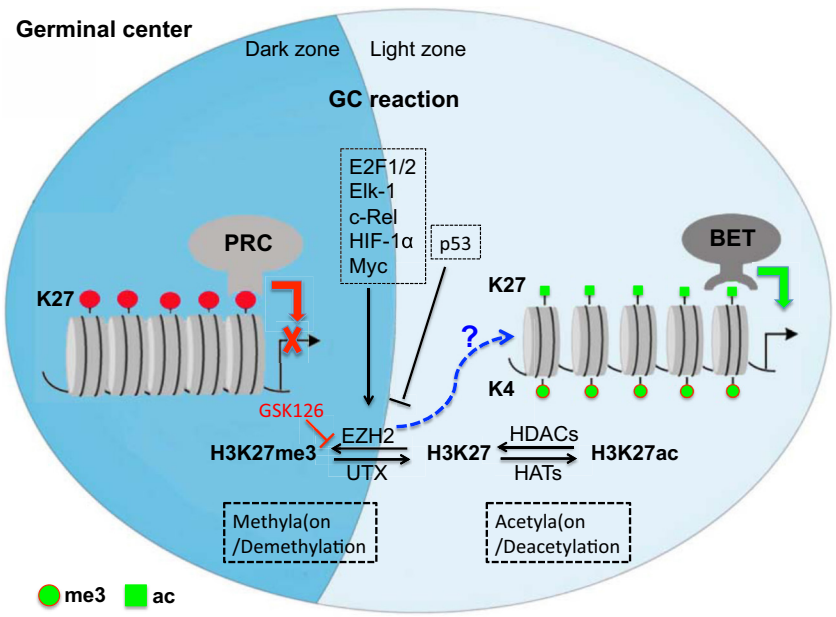

FIGURE 1 | Repressive and active gene regulation in germinal center (GC) reaction by epigenetic histone modification at posttranslation level in GCs. Epigenetic regulation, histone methylation/demethylation, or/and acetylation/deacetylation, is involved in B cell development, GC formation, somatic hypermutation, and Ab affinity maturation. Note that methyltransferase EZH2 is highly expressed in GC B cells (see our preliminary study), but its unique target mark-H3K27me3 is undetectable in developing neonates, suggesting that EZH2 may regulate the GC reaction *via* alternative mechanisms or balanced by histone demethylation/acetylation. PRC, polycomb repressive complex; EZH2, enhancer of zeste homolog 2; UTX, X chromosome-encoded histone demethylase; HATs, histone acetyltransferases; HDACs, histone deacetylases; BET, bromodomain and extra-terminal motif protein.

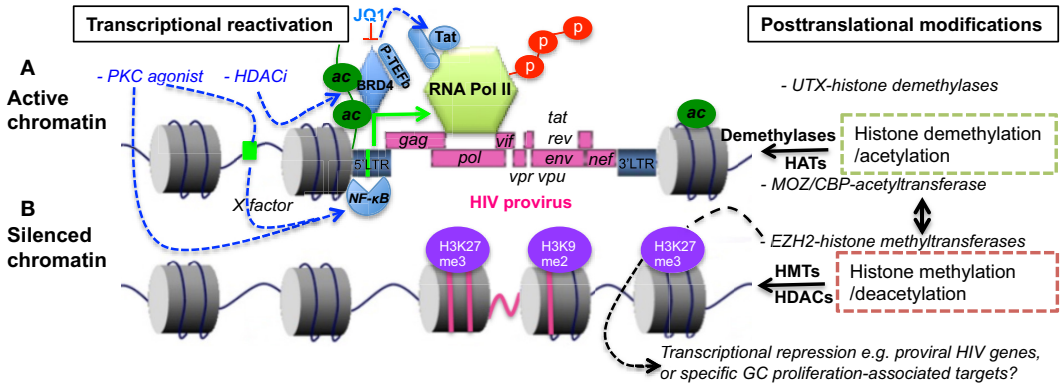

FIGURE 2 | Epigenetic regulation in transcriptional reactivation **(A)** or repression **(B)** of proviral transcription. Histone acetylation or/and specific demethylation leads to a less compact chromatin and active transcription. In comparison, histone deacetylation or/and specific methylation causes condensed and transcriptionally silenced chromatin. BRD4 binds to chromatin *via* acetylated histone, blocking histone deacetylation and recruiting P-TEFb that initiates phosphorylation of polymerase II. BET bromodomain inhibitor JQ1 disassociates BRD4/P-TEFb, resulting in recruitment of P-TEFb/Tat complexes to LTR promoter, and specific reactivation of HIV latency. PKC agonists or HDACi, which are regulators of gene expression that enzymatically remove the acetyl group from histone, facilitate transcriptional reactivation of provirus. *BET*, bromodomain and extra-terminal motif; HATs, histone acetyltransferases, e.g., MOZ or CBP; HDACs, histone deacetylases; HMTs, histone methyltransferases; *BRD4*, BET protein 4; P-TEFb, positive transcription elongation factor b; EZH2, enhancer of zeste homolog 2; UTX, X chromosome-encoded histone demethylase; CBP, CREB-binding protein; MOZ, monocytic leukemia zinc finger protein; PKC, proteinase kinase C; HDACi, histone deacetylase inhibitor.

the differentiation of Th1 and Th2 cells *via* H3K27me3-mediated gene repression (83). Conversely, upregulation of UTX, an H3K27 demethylase supports Tfh cell differentiation and eliminates persistent viral infections (84). As indicated in **Figure 2**, epigenetic histone modification in virus-infected cells is implicated in the immune evasion and latency in HIV infection and AIDS (85–90). The reactivation of HIV latency could be regulated by epigenetic modification through effects on the chromatin state of the viral promoter in the LTR sequence (90–93). The BET (bromodomain and extraterminal domain) family, including BRD2, BRD3, BRD4, and BRDT, are important epigenetic regulators facilitating the gene transcription in chromatin (94). BRD4, a chromatin adaptor protein, forms a tight complex with chromatin through two tandem bromodomains (BD1 and BD2), acetylate lysine residues in histone 3 and 4 at both enhancer and general promoter regions of chromatin, recruiting positive transcription elongation factor-b (P-TEFb) (95). The latter facilitates cellular transcription by phosphorylating RNA polymerase II at the serine residue in the C-terminal domain (96). However, recent studies indicate that BET bromodomain inhibitor (e.g., JQ1) dissociates BRD4 from BRD4/P-TEFb complex, resulting in P-TEFb/HIV Tat recruitment to the LTR promoter and reactivation of HIV-infected cells (97, 98). Antiretroviral drugs, in combination with epigenetic regulatory agents, are promising to effectively reactivate HIV latency *via* histone deacetylase inhibitors (HDACi), histone methyltransferase inhibitors, or DNA methyltransferase inhibitors.

As described above, GC Tfh cells provide help for optimal B-cell differentiation, and antibody affinity maturation (10, 99). Interactions of GC B cells with GC Tfh cells are critical for antibody production. However, persistent SIV infection leads to aberrant GC Tfh cell expansion, ultimate depletion, abnormal B-cell responses, and viral reservoir establishment as a major source of the HIV reservoir within sanctuary sites in lymphoid tissues (79, 100, 101), consistent with the facts that organized lymphoid tissues represent the major tissue reservoir for HIV replication and latency (102–104), even during prolonged ART (78, 105–109). Although studies in adults indicate that HIV infection leads to abnormal B-cell and Tfh cell responses (110–115), yet, studies on the regulation of B-cell responses, especially at the cellular and molecular levels within GCs, needed to be further investigated in HIV/SIV infection.

AUTHOR CONTRIBUTIONS

XW wrote manuscript and HX revised the manuscript.

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Complement-Opsonized HIV-1 Alters Cross Talk Between Dendritic Cells and Natural Killer (NK) Cells to Inhibit NK Killing and to Upregulate PD-1, CXCR3, and CCR4 on T Cells

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Dendritic cells (DCs), natural killer (NK) cells, and T cells play critical roles during primary HIV-1 exposure at the mucosa, where the viral particles become coated with complement fragments and mucosa-associated antibodies. The microenvironment together with subsequent interactions between these cells and HIV at the mucosal site of infection will determine the quality of immune response that ensues adaptive activation. Here, we investigated how complement and immunoglobulin opsonization influences the responses triggered in DCs and NK cells, how this affects their cross talk, and what T cell phenotypes are induced to expand following the interaction. Our results showed that DCs exposed to complement-opsonized HIV (C-HIV) were less mature and had a poor ability to trigger IFN-driven NK cell activation. In addition, when the DCs were exposed to C-HIV, the cytotolytic potentials of both NK cells and CD8 T cells were markedly suppressed. The expression of PD-1 as well as co-expression of negative immune checkpoints TIM-3 and LAG-3 on PD-1 positive cells were increased on both CD4 as well as CD8 T cells upon interaction with and priming by NK–DC cross talk cultures exposed to C-HIV. In addition, stimulation by NK–DC cross talk cultures exposed to C-HIV led to the upregulation of CD38, CXCR3, and CCR4 on T cells. Together, the immune modulation induced during the presence of complement on viral surfaces is likely to favor HIV establishment, dissemination, and viral pathogenesis.

Keywords: dendritic cells, natural killer cells, complement, HIV, cross talk, checkpoint inhibitors, CXCR3, CCR4

INTRODUCTION

The microenvironment and the interactions between immune cells and pathogens at the site of infection determine the quality of the immune responses activated. At the mucosal site of primary HIV infection, dendritic cells (DCs) represent one of the first cell types that interact with the virus particles (1, 2). DCs are part of both the innate and the adaptive immune responses and have the

ability to sense danger, such as presence of HIV, and send out signals that alert the body to fight infection and also to induce specific T cell-mediated adaptive immune responses directed against the virus (3). However, in addition to playing an essential part in host defense, DCs also play a dexterous role by enhancing viral spread to newly activated CD4 T cells in the submucosa and lymph nodes, which is a key step in the establishment of systemic HIV infection (4). The initial events and responses generated during acute retroviral exposure across the mucosal surfaces are key to establishment of viral set point and rate of HIV disease progression, and DCs are important determinants of these responses (5).

Body fluids, such as semen, vaginal secretions, and breast milk, in contact with HIV particles will cover the virus with soluble factors, including complement components and antibodies. Complement-mediated lysis of HIV is largely inefficient due to presence of regulators of complement activation across the viral membrane, resulting in virus opsonization by inactivated complement fragments, such as iC3b (6), which protect HIV from lysis and enhance the infectivity when it comes to both direct infection of DCs and transfection from DCs to T cells (7–9). The inactivated complement fragment iC3b has been shown to interact with complement receptor 3 (CR3) expressed on DCs, resulting in the promotion of HIV phagocytosis (10) and modulation of antigen presentation (11). We demonstrated that enhancement of infection in DCs is largely due to complement-mediated suppression of inflammation and antiviral responses *via* CR3 (12). Furthermore, we also found that the ability of DCs to attract other innate immune cells, especially natural killer (NK) cells to the site of infection was impaired when the DCs were exposed to complement-opsonized HIV (C-HIV) as a result of suppressed production of chemoattractants, including CCL3 and CXCL10 (13). In addition, it has also been suggested that CR3 engagement of DCs decreases their capacity to stimulate T cells (14).

The importance of functional NK cell responses is exemplified by the ability of these cells to control SIV replication in the lymph nodes they relocate to in SIV-infected animals (15). The diminished recruitment of NK cells to the site of infection appears to accentuate the establishment of HIV infection, seeing that NK cells have been shown to directly restrict viral spread by killing infected cells and indirectly by secreting antiviral factors (16, 17). In addition, NK cells can also produce inflammatory cytokines, such as IFN-γ, which promote further activation of innate and adaptive immune responses (18). The ability of NK cells to kill infected cells is key to impediment of HIV-infection (16, 17). The necessity of NK cells for HIV protection and control is further illustrated by the fact that loss of NK cell functions is associated with poor disease prognosis (19) and the correlation between protection against infection and the level of NK cell activity in HIV-exposed uninfected individuals (20). In HIV-infected individuals, there is a dysfunctional population of NK cells with reduced cytokine production and cytolytic activity (21, 22). NK cell dysfunction also appears to influence the immune activation potential of DCs, which affects the ensuing T-cell responses (22).

In order to necessitate protective antiviral immune responses, DCs must receive optimal activation and maturation signals. The cross talk between DCs and NK cells can have either positive or negative effects on the respective cell's functionality. When interaction between these cells occurs in a setting where there is a high proportion of NK cells per DC, this can result in high level of lysis of DCs (16, 17). In settings with a low NK cell to DC ratio, the NK–DC cell interactions enhance the expression of activation markers, e.g., MHC class II, CD80, CD86, and increase the synthesis of IL-12 by DCs (23). Thus, support from NK cells is imperative for proper DC maturation (24, 25) and the DC maturation depends on cell–cell interaction between DCs and NK cells, and may possibly involve the association of NKp30 receptor and production of TNF and IFN-γ by NK cells (26). While the DCs contribute by releasing IL-18, which triggers HMGB1 secretion by NK cells that further enhances the DC maturation process.

The NK–DC cross talk will also influence subsequent development of T cell responses, with the NK cell IFN-γ production affecting both the CD4 and CD8 T cell responses (27). HIV susceptibility is influenced both by the availability as well as the phenotypes of target cells present across the mucosa (28). Hence, T cell migration as well as phenotypes that are induced as a result of initial NK cell and DC responses during HIV transmission is likely to have an important impact on the outcome of infection.

There are numerous studies on the direct effects of free HIV on single cultures of DCs, NK cells, and T cells, and here, we aimed to investigate how the virus, and presence of complement on its surface, affects the interactions between them. The effects C-HIV exerts on NK cells directly and on the NK–DC cross talk have to our knowledge never been investigated previously. We found that complement opsonization of HIV altered DC responses in a way that suppressed NK activation and their killing ability. In addition, NK–DC cross talk in the presence of C-HIV generated T cells with a higher expression of immune checkpoint inhibitors such as PD-1, and chemokine receptors CXCR3 and CCR4. The observed immune modulation is likely to aid HIV in establishing infection in the host and contributes to HIV pathogenesis.

MATERIALS AND METHODS

Virus Generation and Opsonization

HIV-1BaL (lot no. 4238) was produced from SUP-T 1/CCR5 cells and purified as described previously (29). The virus (30 ng/μl) was incubated for 1 h with either an equal volume of RPMI 1640 (Sigma-Aldrich, Stockholm, Sweden) to generate free HIV (F-HIV), single-donor human serum, to generate C-HIV, or single-donor human serum supplemented with 2 μg/ml HIV-specific IgG and 20 μg/ml γ-globulins, to generate complement- and antibody-opsonized HIV (CI-HIV).

Cell Purification and Culture

Monocyte-derived DCs were prepared and cultured as described previously (30). In brief, PBMCs were separated from whole blood from healthy volunteers (Ethical approval No. EPN 173-07), $N = 30$. DC progenitors were enriched from the PBMCs by plastic adhesion to tissue culture plates for 1 h at 37°C. The non-adherent cells were harvested and cryopreserved for

subsequent purification of NK cells, or were used directly for purification of T cells. Adherent cells were cultured in RPMI1640 with L-glutamine supplemented with 10 mM HEPES, 20 μg/ml gentamicin (Fisher Scientific, Leicestershire, UK), 100 IU/ml recombinant human GM-CSF, 300 U/ml recombinant human IL-4 (Prepotech, UK), and 1% human plasma for 5 days. The DC purity and maturation status was assessed by flow cytometry staining for CD14 and CD83. All DC preparations used were more than 98% pure and had less than 5% CD14 and CD83 expression. Pan-T cells were purified from fresh non-adherent cells by negative magnetic bead purification using a commercial human Pan-T cell Isolation kit (Miltenyi Biotec, Lund, Sweden) according to the manufacturer's protocols. Memory T cells were depleted using CD45RO microbeads (Miltenyi Biotec). NK cells from frozen non-adherent cells were purified the same day as they were added to the NK–DC cross talk assays using a commercial human NK cell Isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. NK cells and DCs from the NK–DC cross talk assays were separated by positive selection of CD1c+ DCs using microbeads separation (Miltenyi Biotec).

NK–DC Cross Talk Assay

Autologous DCs and NK cells from the same donor were exposed to mock, i.e., exposed to the same media as added to the HIV preparation, 100 ng/1 × 10^5 cells F-HIV, C-HIV, or CI-HIV for 24 h, either in single cultures or in NK–DC cross talk cultures. For the NK–DC cross talk cultures, NK cells were added to DC cultures 3 h post HIV exposure at a 1:1 ratio followed by additional 21 h incubation. An overview of the cross talk assays and the design of this study can be found in **Figure 1**.

Cytometric Bead Array (CBA) and ELISA

The protein levels of IFN-γ, IL-6, IL-12, CXCL10, IL-5, CX3CL1, CXCL9, CCL3, and CCL4 in cell supernatants were assessed using a commercial CBA (BD Biosciences, Stockholm, Sweden) performed on a BD FACSCanto II flow cytometer

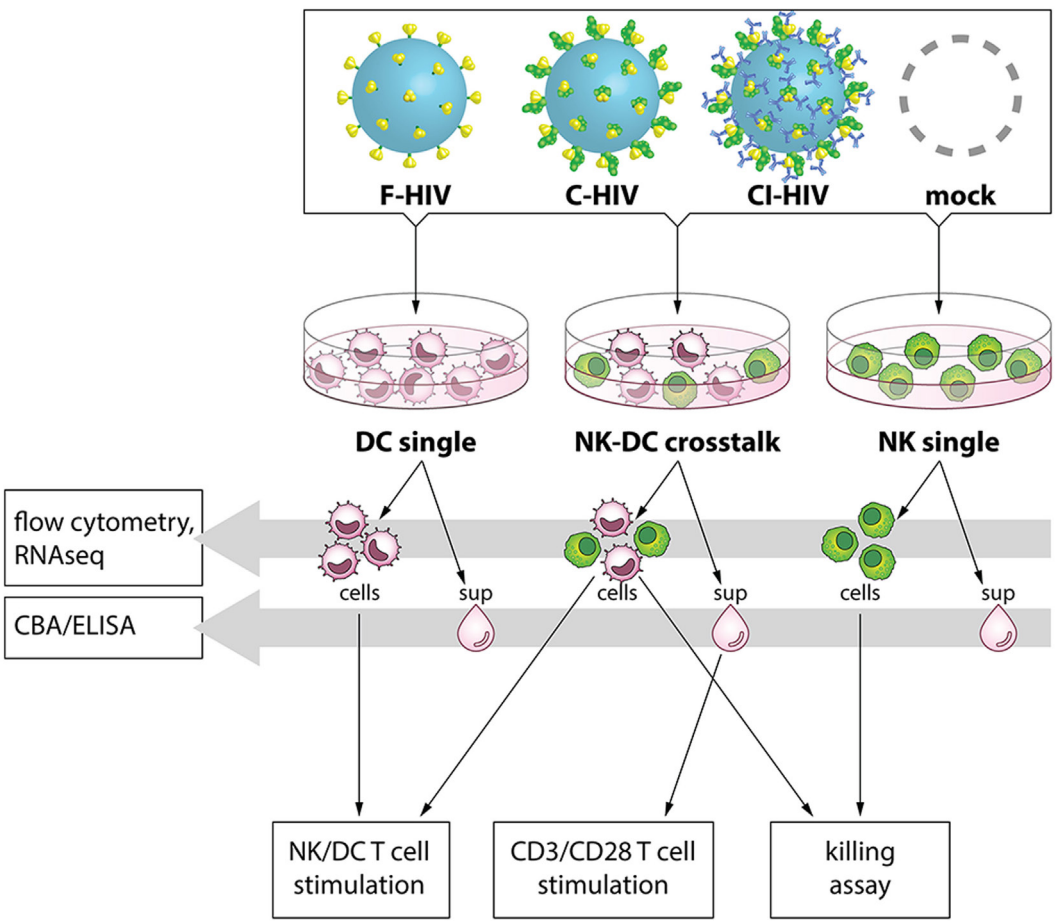

FIGURE 1 | Experimental study design. Dendritic cells (DCs) and natural killer (NK) cells were kept in single cultures, or in cross talk cultures consisting of NK cells and DCs from the same donor at a 1:1 ratio and exposed to 1 μg/ml F-HIV, complement-opsonized HIV (C-HIV), complement- and antibody opsonized HIV (CI-HIV), or mock treated for 24 h. Flow cytometry was performed to assess expression of activation markers on the cells, and the pathways activated were assessed by RNAseq. The concentration of cytokines in the supernatants was measured using a cytometric bead array (CBA) or ELISA. Allogeneic T cells were stimulated using cells from the cultures, or by CD3/CD28 ligation together with culture supernatants. The proliferation and the phenotype of the T cells was subsequently evaluated.

(BD Biosciences) and analyzed using a FCAP Array version 3 software (BD Biosciences) according to the manufacturer's protocols. Furthermore, the protein levels of IL-15 (Sino Biological, NordicBiosite, Stockholm, Sweden) and IL-23 (Mabtech, Stockholm, Sweden) were measured using commercial ELISA kits according to the manufacturer's protocols.

RNA Sequencing and Data Handling

Whole transcriptome amplification of RNA purified from DCs and NK cells from single and NK–DC cross talk cultures was done using NuGEN's Ovation RNA-Seq V2 kit following the protocol provided by the company (San Carlos, CA, USA). cDNA was amplified from total RNA using a single primer isothermal amplification and purified using a MinElute Reaction Cleanup Kit (Qiagen; Valencia, CA, USA). The cDNA samples were fragmented, barcoded with adaptors, and amplified using an Ultralow System V2 kit. Distribution of the size of the library was determined using an Agilent Bioanalyzer 2100. Libraries from three different donors were sequenced on the Illumina NextSeq500 platform (San Diego, CA, USA). The fastq files were uploaded and the quality checked using fastQC (31). Trimmomatic (32) was used to remove adaptors and low-quality bases and the reads were then mapped to human reference genome hg19 using STAR. FeatureCounts was used to calculate counts for each gene (33). The data were normalized and R/DeSeq2 used to determine differentially expressed genes (34). Analysis of pathways was done by Ingenuity Pathway Analysis (Qiagen), R analysis, and custom gene lists.

NK Cell Killing Assay

K562 cells (ATCC, Manassas, VA, USA) were cultured in RPMI 1640 supplemented with 10% FBS, 20 µg/ml gentamicin, and 10 mM HEPES (Fisher Scientific, Leicestershire, UK) and used as target cells for killing by NK cells from single or purified from NK–DC cross talk cultures exposed to mock, F-HIV, C-HIV, or CI-HIV. PHA (1 µg/ml)-activated NK cells were used as positive controls. The frequency of CFSE-labeled K562 cells undergoing cell death following incubation with NK cells at a 1:8 ratio for 6 h was determined using a BD FACSCanto™ II flow cytometer and analyzed using FlowJo™ Software (Treestar, OH, USA).

T Cell Activation and Proliferation Assay

Allogeneic pan-T cells were added to the NK–DC cross talk cultures or to DC single cultures 24 h after stimulation with mock, F-HIV, C-HIV, or CI-HIV and cultured at a DC:T cell ratio of 1:10 in 96-well plates in 5% PHS supplemented with 10 µM azidothymidine (AZT). In addition, pan-T cells were activated with CD3/CD28 T Cell Activator according to the manufacturer's protocol (ImmunoCult™, STEMCELL technology) in the presence of supernatant harvested at 24 h from the NK–DC cross talk or DC single cultures and cultured in 96-well plates in 5% PHS supplemented with 10 µM AZT. After 24 h exposure, the T cells were washed and re-cultured with IL-2 and 10 µM AZT. PHA stimulation (1 µg/ml) of the Pan-T cells was used as a control for T-cell activation. T-cell phenotypes were assessed by flow cytometry on day 3 (FACSCalibur, BD Immunocytometry Systems, San Jose, CA, USA). The antibodies used were AmCyan mouse anti-human CD3 (Clone SK7), FITC anti-human CD45RA (Clone HI100), PE anti-human TIM-3 (CD366) (Clone 7D3) (all from BD Biosciences). PerCP/Cy5.5 anti-human CD8, APC anti-human CD4 Brilliant Violet 421 anti-human CD4, Pacific Blue anti-human CD197 (CCR7), Alexa Fluor 647 anti-human Granzyme B, PE anti-mouse CD183 (CXCR3), Pacific Blue anti-human perforin, Alexa Fluor 647 anti-human CD194 (CCR4), PE/Cy7 anti-human CD38, FITC anti-human CD223 (LAG-3), Zombie NIR Fixable Viability Kit, PE anti-human CD366 (TIM-3), Brilliant Violet 421 anti-human CD279 (PD-1) (all from NordicBiosite), and PE-eFluor 610 anti-human CD279 (PD-1) from eBioscience. T-cell proliferation was assessed by adding 4µCi of ^{3}H-Thymidine (Amersham Pharmacia Biotech) to the assay on day 4 and measuring the incorporation after 20 h.

Phenotypic Analysis of NK Cells and DCs

Phenotypic analysis of NK cells and DCs was performed by flow cytometry. The cells were collected and resuspended in RPMI1640 supplemented with EDTA (Fisher Scientific) in order to disrupt any cell aggregates and were subsequently stained with antibodies specific for CD1c, CD25, CD40, CD56, CD69, CD80, CD86, and HLADR and their corresponding isotype controls (BD Biosciences, Stockholm, Sweden). The cells were analyzed with a FACS flow cytometer (BD Immunocytometry Systems, San Jose, CA, USA) and FlowJo software (TreeStar, AsMHCnd, OR, USA).

Statistics

The RNA data were normalized and R/DeSeq2 used to determine differentially expressed genes. Analysis of pathways was done by Ingenuity Pathway Analysis (Qiagen), and R analysis, and custom gene lists. All other results were analyzed using Graph Pad Prism 5 (GraphPad Software, La Jolla, CA, USA), with repeated measures ANOVA followed by Bonferroni posttest. $p < 0.05$ was considered statistically significant. In all figures, *N* denotes the number of times an experiment was repeated, each time with cells derived from a different donor.

Data Availability

The RNAseq datasets generated for this study can be found at Sequence Read Archive, accession SRP131436. The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

RESULTS

Complement Opsonization of HIV Reduced DC-Induced NK Activation During NK–DC Cross Talk

Dendritic cells were preexposed to mock, F-HIV, C-HIV, or CI-HIV for 3 h. After this, DCs were maintained either as a single culture, or NK cells were added to facilitate cross talk for 21 h. RNAseq was performed on the DCs isolated from the cultures, to examine the effect of different virus exposures on the DCs and their cross talk with NK cells. Analysis of the RNAseq data using IPA revealed that in DCs, complement opsonization of HIV

decreased the activation of upstream regulators associated with inflammatory and antiviral responses, such as TNF, IL-1β, NFκβ, and IFNγ, while several regulators of growth such as RABL5 and ERBB2 were increased (**Figure 2A**). This profile was similar for DCs derived from both single and cross talk cultures, i.e., the presence of NK cells did not have a dramatic effect on DC activation (**Figure 2A**). The majority of regulators fell into three main functional groups–danger (factors shown to be involved in inflammatory, antiviral, or stress responses), transcription (factors associated with GO annotation terms "transcription factor activity," "sequence specific DNA binding," and "chromatin binding"), and survival (factors regulating cell growth, cycle, and survival). The upstream regulators involved in danger signaling pathways showed a high positive clustering for F-HIV, whereas the upstream regulators for cell survival had a higher positive clustering for C-HIV and CI-HIV groups (**Figure 2B**). The list of the top regulators generated by IPA and to which group they were assigned can be found in Table S1 in Supplementary Material. The RNAseq results were filtered for genes reported to be involved in DC maturation that were significantly affected ($p < 0.05$) for at least one culture condition and normalized to the mock-treated cross talk sample (**Figure 2C**). Several factors involved in the

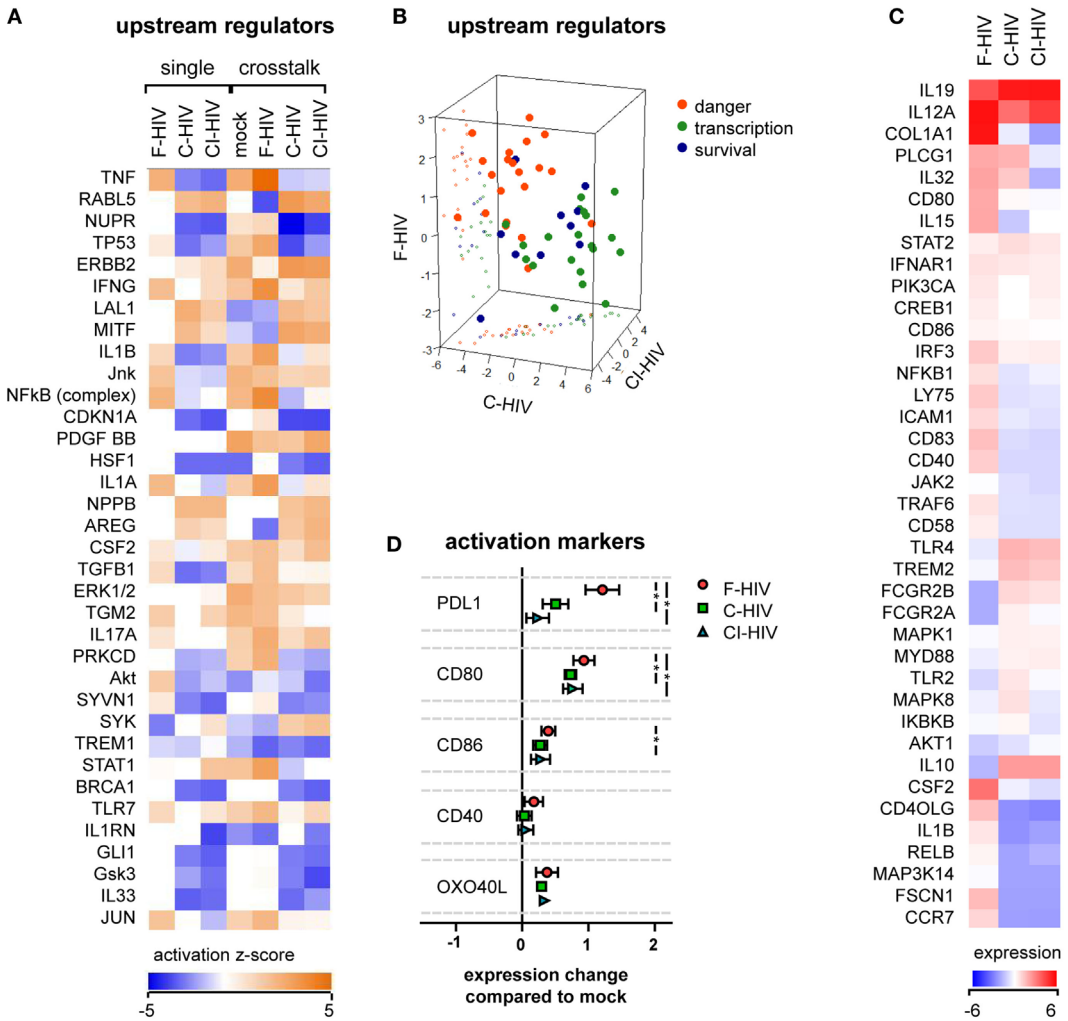

FIGURE 2 | HIV effects on dendritic cell (DC) activation in the absence or presence of natural killer (NK) cells. DCs were exposed to 1 μg/ml F-HIV, complement-opsonized HIV complement- and antibody opsonized HIV (CI-HIV), CI-HIV, or mock treated either in DC single culture, or in a cross talk culture consisting of NK cells and DCs from the same donor at a 1:1 ratio for 24 h. The experiment was replicated three times using cells derived from three different donors. **(A)** RNAseq was performed on the DCs from the single culture or DCs isolated from the NK–DC cross talk, and the genes that were significantly differentially expressed were analyzed using IPA (Qiagen) to determine the most affected upstream regulators. **(B)** The top upstream regulators were divided into functional groups, and a 3D scatterplot was used to visualize clustering. **(C)** A heatmap was generated for genes that were significantly differentially expressed in at least one condition and that were defined by IPA to be involved in DC maturation. The heatmap shows fold change in the virus exposed cross talk samples compared to the mock treated cross talk samples. **(D)** The protein expression of surface activation markers on the DCs from the cross talk cultures was assessed using flow cytometry, and the values normalized to the expression in the mock-treated cross talk culture. One-way ANOVA followed by a Bonferroni posttest was used to test for statistically significant differences in protein expression. Boxplots show mean ± SEM (*$p < 0.05$).

maturation of DCs, such as CD40LG, IL1B, RELB, and CCR7, were upregulated in F-HIV-exposed cells, while expression of these factors in cells exposed to C-HIV or CI-HIV were downregulated (**Figure 2C**). When assessing the expression on protein level, the presence of HIV during the cross talk between DCs and NK cell increased the expression levels of factors involved in the DC maturation (**Figure 2D**). The highest impact was seen for the expression of PD-L1, a receptor for PD-1 involved in immune suppression, where F-HIV induced a significantly higher upregulation than C-HIV or CI-HIV (**Figure 2D**). In addition, the costimulatory molecule CD80 was upregulated on DCs exposed to F-HIV (**Figure 2D**).

Activation of NK Cells Was Altered by Cross talk With HIV-Exposed DCs

The transcriptome profiles of NK cells from the cross talk cultures exposed to mock, F-HIV, C-HIV, or CI-HIV showed high effects on many factors involved in NK cell activation compared to the NK cell groups cultured without DCs (**Figure 3A**). The cross talk between the NK cell and DCs in itself had a stronger influence on NK cell activation than the HIV exposure. The top upstream regulators in the cross talk cultures according to IPA analysis fell under three main categories: IFN (factors induced by IFN or involved in IFN signaling), inflammation, and survival. The upstream regulators involved in inflammation and

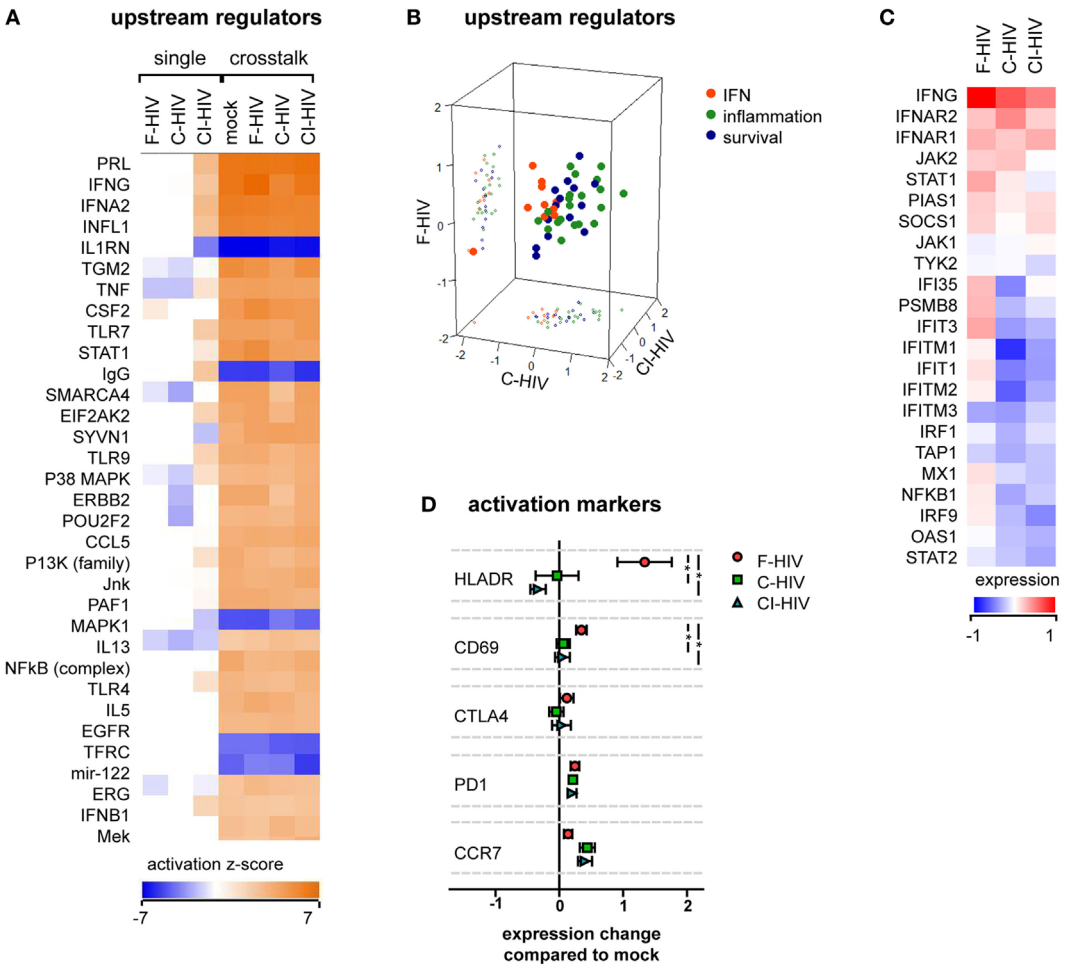

FIGURE 3 | HIV effects on natural killer (NK) cell activation in the absence or presence of dendritic cells (DCs). NK cells were exposed to 1 μg/ml F-HIV, complement-opsonized HIV (C-HIV), CI-HIV either in single NK cell culture, or in a cross talk culture consisting of NK cells and DCs from the same donor at a 1:1 ratio. The experiment was replicated three times using cells derived from three different donors. **(A)** RNAseq was performed on the NK cells from the single culture or NK cells isolated from the NK–DC cross talk, and the genes that were significantly differentially expressed were analyzed using IPA (Qiagen) to determine the most affected upstream regulators. **(B)** The top upstream regulators were divided into functional groups, and a 3D scatterplot was used to visualize clustering. **(C)** A heatmap was generated for genes that were significantly differentially expressed in at least one condition and that were defined by IPA to be involved in IFN signaling. The heatmap shows fold change in the virus exposed cross talk samples compared to the mock-treated cross talk samples. **(D)** The protein expression of activation markers on the NK cells from the cross talk cultures was assessed using flow cytometry, and normalized to the expression in the mock-treated cross talk culture. One-way ANOVA followed by a Bonferroni posttest was used to test for statistically significant differences in protein expression. Boxplots show mean ± SEM (*$p < 0.05$).

survival responses were upregulated in all HIV-exposed cross talk groups compared to mock, whereas regulators in the IFN group tended to be upregulated in cultures exposed to F-HIV only (**Figure 3B**). The list of the top regulators generated by IPA and the groups to which they were assigned in can be found in Table S2 in Supplementary Material. The RNAseq results were filtered for genes reported by IPA to be involved in IFN signaling and normalized to the mock treated cross talk NK cell sample (**Figure 3C**). Several factors involved in IFN signaling were higher in NK cells from cross talk cultures exposed to F-HIV compared to cultures exposed to C-HIV or CI-HIV (**Figure 3C**). When assessing the expression of NK cell activation markers on protein level, HLADR and CD69 were upregulated on the F-HIV-treated NK cells from the NK–DC cross talk compared to cells exposed to complement-opsonized virus. The homing receptor CCR7 was slightly more upregulated (not statistically significant) on NK cells derived cultures exposed to C-HIV and CI-HIV compared to F-HIV (**Figure 3D**). Next, we assessed the expression of PD-1 on NK cells in the NK–DC cocultures as this negative immune checkpoint molecule is expressed not only on T cells but also on NK cells (35–37). The expression of PD-1 on NK cells activated by the DCs in the cross talk assay was induced in the presence of any of the HIV groups (**Figure 3D**).

The Cellular Cross Talk Altered the Profiles of Cytokine and Chemokine Production in Both DCs and NK

Release of inflammatory and chemotactic factors into the supernatants of the single DC and NK cultures as well as from cross talk cultures was assessed using a CBA or ELISA. TNF, IL-1β, IL-15, IL-12, and CX3CL1 were produced by NK cells in single culture, but the production was downregulated during cross talk with DCs (**Figure 4A**). IL-5, CXCL10, IL-10, and CXCL8 were produced by DCs in single culture, and were upregulated by HIV exposure (**Figure 4A**). These cytokines were to some extent downregulated during cross talk with NK cells (**Figure 4A**). The release of IL-1β and IL-15 was downregulated by C-HIV compared to free HIV (**Figure 4A**). Production of IFN-γ, CXCL9, IL-6, and CCL3 were highest during cross talk compared to single cultures of both NK and DCs (**Figure 4A**). Secretion of the chemotactic cytokines CXCL9 (MIG) and CCL3 were higher in the NK–DC cross talk cultures exposed to C-HIV or CI-HIV compared to F-HIV (**Figures 4A,B**). In contrast, cytokines that have been shown to activate NK cells, such as IFN-γ, CX3CL1 (Fraktalkine), and IL-15, were higher in the cross talk cultures exposed to F-HIV than in the cultures exposed to C-HIV or CI-HIV (**Figures 4A,B**). IL-6 secretion was highest in the cross talk cultures exposed to C-HIV or CI-HIV (**Figures 4A,B**). In the cross talk cultures, IFN-γ production was higher in F-HIV and lower in C-HIV and CI-HIV compared to mock (**Figures 4A,B**).

HIV Decreased the Cytotoxicity in NK Cells Activated by Cross Talk Between NK Cells and DCs

We assessed the functionality of NK cells exposed to F-HIV, C-HIV, CI-HIV, or mock either from single cultures or from NK–DC cross talk cultures. The NK cell's ability to kill target cells (CFSE-stained K562) was assessed using flow cytometry (**Figures 5A,B**). The NK–DC cross talk greatly increased NK cell killing compared to the single cultured NK cells, independent on the treatment (**Figures 5A,B**). The HIV-exposed DCs activated less cytotoxicity in the NK cells compared to mock (**Figures 5A,B**). C-HIV and CI-HIV exposure led to a significantly lower killing ability than that F-HIV exposure. The amount of target cells killed by NK cells from cross talk cultures exposed to complement-opsonized virus decreased by approximately 50% (**Figures 5A,B**). The DCs were protected from the NK cell cytotoxicity as the killing of these cells was quite low (below 2%), with a very low but significant decrease for DCs exposed to C-HIV (**Figure 5C**).

DCs Exposed to HIV and Conditioned by NK Cells Suppressed T Cell Proliferation and Promoted the Differentiation of Central Memory T Cells

Next, we assessed the quality and type of T cell responses activated by DCs conditioned by NK (NK–DC cross talk) in the presence of mock, F-HIV, C-HIV, or CI-HIV. Naïve allogeneic T cells were isolated and added to the NK–DC cross talk cultures. T cell proliferation was assessed by ^{3}H-thymidine incorporation assay. DCs exposed to both the free and C-HIV significantly suppressed the proliferation of T cells (**Figure 6A**). The profiles were similar for T cells exposed to DCs cultured both in single culture and DCs conditioned by NK cells (**Figure 6A** and data not shown). In the naïve T cells primed by CD3/CD28 beads in the presence of supernatant from NK–DC cross talk cultures, the CD4 and CD8 subsets differentiated into central memory T cells (T_{CM}, CD45RA-CCR7+), with higher levels of T_{CM} T cells in the virus exposed groups, and with C-HIV and CI-HIV leading to a higher increase than F-HIV (**Figures 6B,C**; Figures S1A,B in Supplementary Material). Gating strategy for the T_{CM} flow cytometry analysis can be seen in **Figure 6D**. In order to assess whether the T cells primed by DCs conditioned by NK cells had acquired effector functions, the T cell expression of perforin was evaluated by flow cytometry and all HIV conditions induced perforin and granzyme B expression in approximately 80% of the CD8 T cells (Figure S2 in Supplementary Material). The amount of perforin per cell was higher in CD8 T cells activated by NK-conditioned DCs exposed to F-HIV compared to C-HIV, CI-HIV, or mock (**Figure 6B**), indicating that C-HIV and CI-HIV could suppress the cytolytic responses in T cells as well as in NK cells.

Cells From NK–DC Cross Talk Cultures Exposed to C-HIV Induced CXCR3+ CCR4+ CD4 T Cells

Naïve allogeneic T cells that were added to NK–DC cultures were stained for an array of phenotypic markers and assessed by flow cytometry (**Figure 7A**). Expression levels of CXCR3 and CCR4 in CD4 T cells were significantly upregulated when the cross talk cultures had been exposed to C-HIV or CI-HIV but not to F-HIV (**Figures 7A,B**). In addition, C-HIV and CI-HIV significantly increased the level of CXCR3+ CCR4+ CD4 T cells, whereas F-HIV decreased the level compared to mock (**Figure 7B**). The

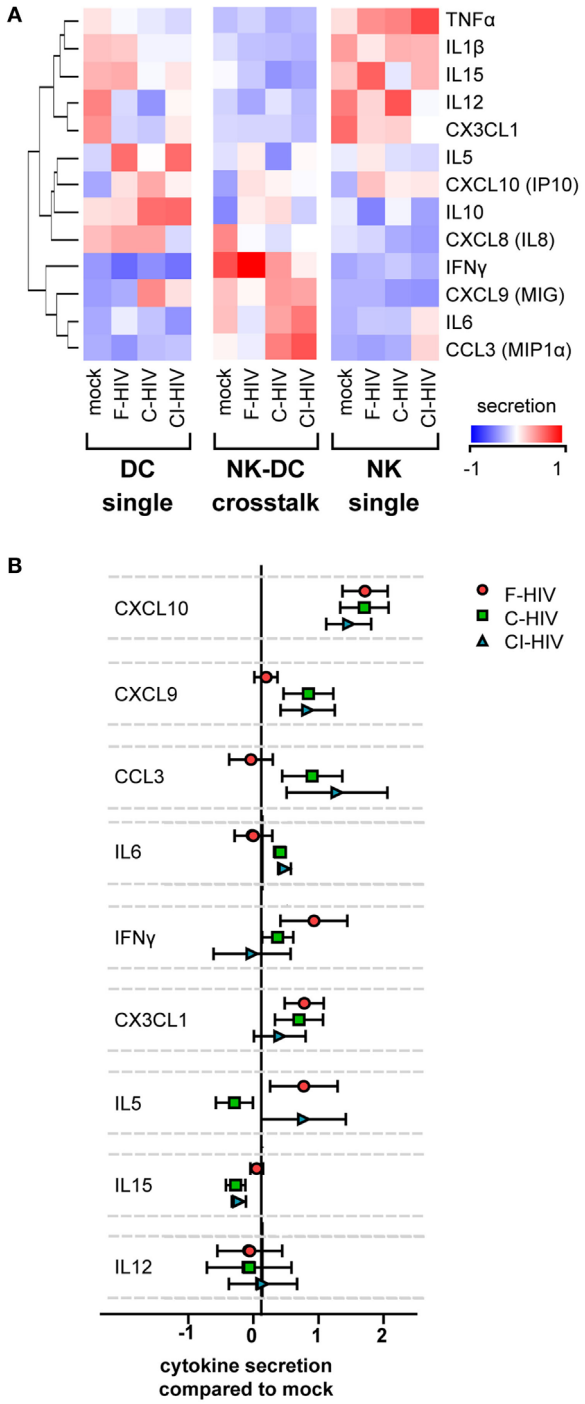

FIGURE 4 | Cytokine secretion profiles in the natural killer (NK)–dendritic cell (DC) cross talk assay. DCs and NK cells were kept in single cultures, or in cross talk cultures consisting of NK cells and DCs from the same donor at a 1:1 ratio and exposed to 1 μg/ml F-HIV, complement-opsonized HIV (C-HIV), CI-HIV, or mock treated for 24 h. The experiment was replicated four times using cells derived from four different donors. **(A)** The supernatants were harvested, and the concentration of cytokines was determined using a cytometric bead array or ELISA. The values were normalized (centered) and a heat map was created. **(B)** Cytokine concentration in the NK–DC cross talk cultures were normalized to the mock treated samples. Boxplots show mean ± SEM.

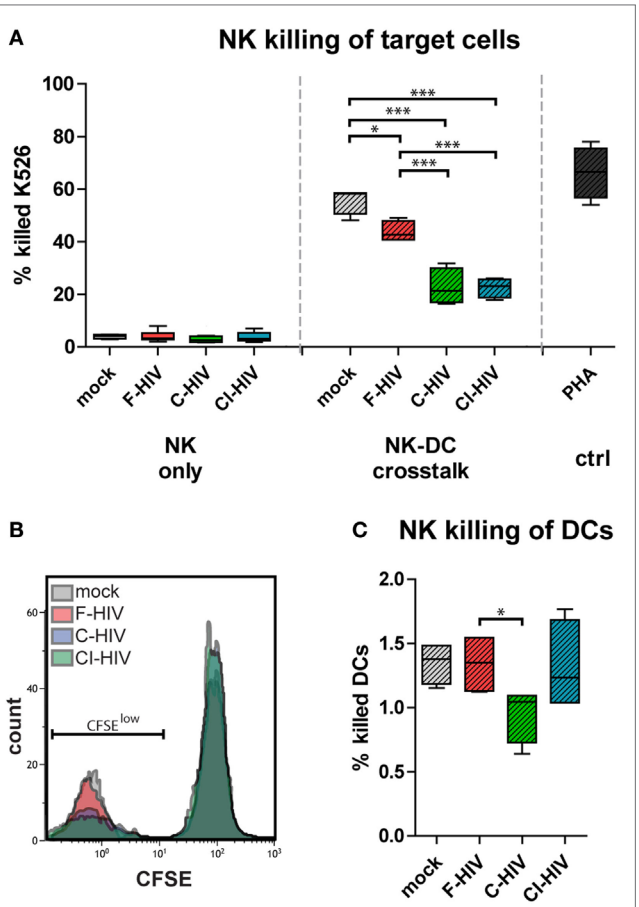

FIGURE 5 | HIV decreased the cytotoxicity in natural killer (NK) cells activated by cross talk between NK cells and dendritic cells (DCs). NK cells were maintained in single NK cell cultures, or in cross talk cultures consisting of NK cells and DCs from the same donor at a 1:1 ratio and exposed to 1 μg/ml F-HIV, complement-opsonized HIV (C-HIV), CI-HIV, PHA, or mock treated for 24 h. The experiment was replicated four times using cells derived from four different donors. **(A)** NK cells from single or purified from the cross talk assays were added to target cells (CFSE-stained K562 cells) at a 1:8 ratio for 6 h and the amount of killed cells were assessed using flow cytometry. **(B)** Representative histograms for the different experimental conditions. **(C)** A similar assay was performed, using CFSE-stained DCs as target cells. Boxplots show mean with Tukey error bars. One-way ANOVA followed by a Bonferroni posttest was used to test for statistical significance (*$p < 0.05$, $N = 4$).

upregulation of CXCR3 and CCR4 by NK–DC cross talk cultures exposed to C-HIV or CI-HIV was also seen for CD8 T cells, although receptor expression was induced in a lower number of cells (**Figure 7C**). The upregulation of CXCR3 and CCR4 expression on T cells by C-HIV was only achieved in the by NK–DC cross talk cultures, but not when the T cells were merely activated by DCs exposed to HIV (**Figure 7B**). This indicates that the effect is likely dependent on contact-mediated interaction with NK cells or both DCs and NK cells. The flow cytometry gating was first performed on singlet viable CD3+ T cells followed by gating on CD4 or CD8 positive cells and finally by assessing the expression levels of CCR4 and CXCR3 on these cells. The gating strategy for flow cytometry analysis is presented in **Figure 7D**.

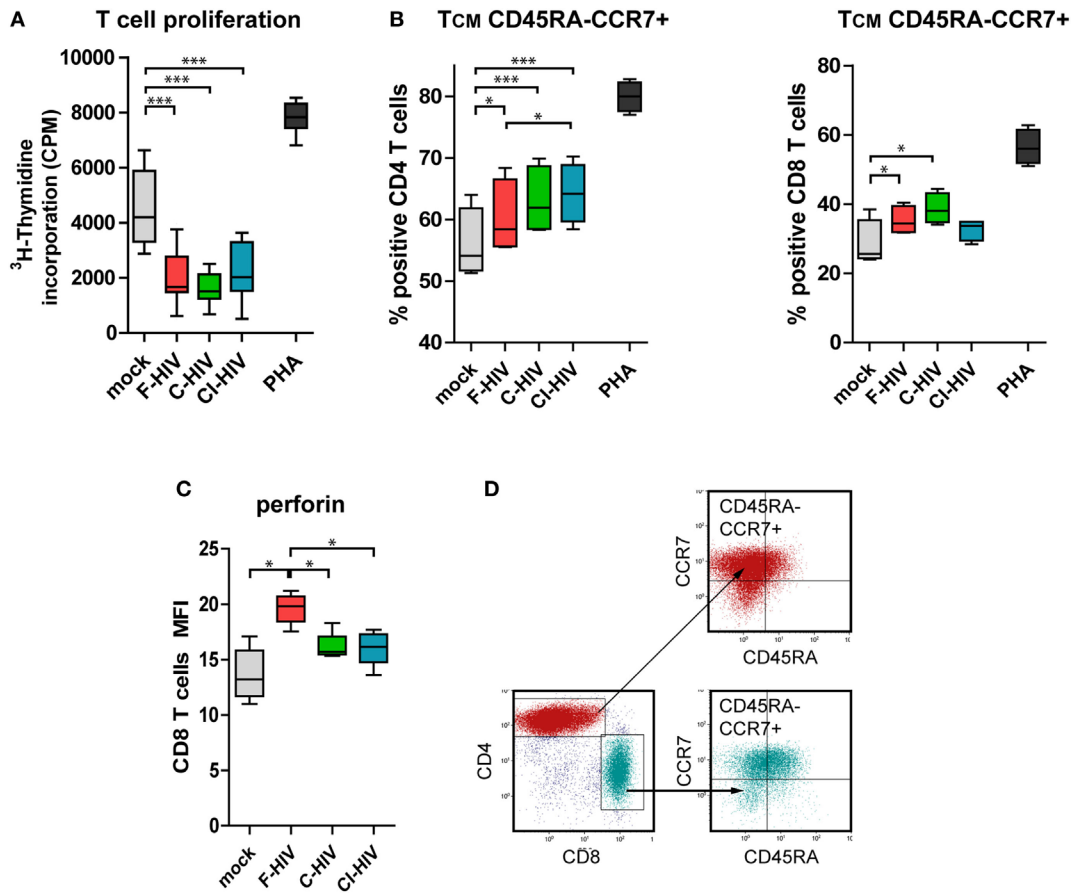

FIGURE 6 | T cell proliferation and memory phenotype activated by natural killer (NK) cell–dendritic cell (DC) stimulation assay. DCs and NK cells from the same donor were exposed to 1 μg/ml F-HIV, complement-opsonized HIV (C-HIV), complement- and antibody opsonized HIV (CI-HIV), PHA, or mock-treated for 24 h. The experiment was replicated four times using cells derived from four different donors. Allogeneic T cells were stimulated by cells from these cultures at a 1:10 DC:T cell ratio. **(A)** T cell proliferation was assessed using a ^{3}H-Thymidine incorporation assay. **(B)** Supernatants from the NK–DC cross talk cultures were added to allogeneic T cells stimulated by CD3/CD28 ligation and the percentage of cells with central memory (T_{CM}) phenotype in the CD4 and CD8 T cell populations was determined using flow cytometry. **(C)** The amount of perforin in the CD8 T cells from the NK–DC stimulation assay was evaluated. **(D)** Gating strategy for flow cytometry analysis of a representative sample can be seen in. Boxplots show mean with Tukey error bars. One-way ANOVA followed by a Bonferroni posttest was used to test for statistical significance (*$p < 0.05$, $N = 4$).

DCs Exposed to C-HIV Upregulated Negative Costimulatory Molecule Expression on CD4 T Cells

Supernatants were harvested from to NK–DC cultures exposed to different forms of HIV or mock treated and added to allogenic T cells activated by CD3 and CD28 ligation. The T cell phenotype was assessed by flow cytometry. CD3 and CD28 ligation together with supernatants from cultures exposed to C-HIV and CI-HIV induced CD4 T cell upregulation of the activation marker CD38 (**Figures 8A,B**), whereas supernatants from cultures exposed to F-HIV did not. All virus conditions led to the upregulation of the co-inhibitory molecules PD-1, TIM-3, and LAG-3 on CD4 T cells, with the highest upregulation seen for C-HIV and CI-HIV (**Figure 8A**; Figure S3 in Supplementary Material). The populations of CD4 T cells that were positive for PD-1 or PD-1 in combination with TIM-3 and LAG-3 were significantly higher when activated with CD3 and CD28 ligation and exposed to supernatants from C-HIV and CI-HIV than from F-HIV (**Figure 8B**; Figure S3 in Supplementary Material). The expression of the inhibitory molecules on CD8 T cells was similar to that for CD4 T cells described above, but less pronounced. In addition, the T cells activated by CD3 and CD28 ligation and exposed to C-HIV or CI-HIV supernatants induced a larger population of CD8 T cells that coexpressed PD-1, TIM-3, and LAG-3 (**Figures 8D,E**). In addition, the CD3 and CD28 ligation activated CD8 T cells upregulated CD38 upon all virus exposures, whereas CD4 T cells only upregulated CD38 in the presence of supernatants derived from cultures exposed to C-HIV and CI-HIV (**Figures 8B,E**). Noteworthy, PHA stimulation led a substantial increase in amount of PD-1 positive cells (Figures S2 and S3 in Supplementary Material), whereas the percentage of cells coexpressing PD-1, TIM-3, and LAG-3 was decreased. PHA did not induce a high upregulation of CD38; which could be due to the expression kinetics of this marker (38, 39). The gating strategy

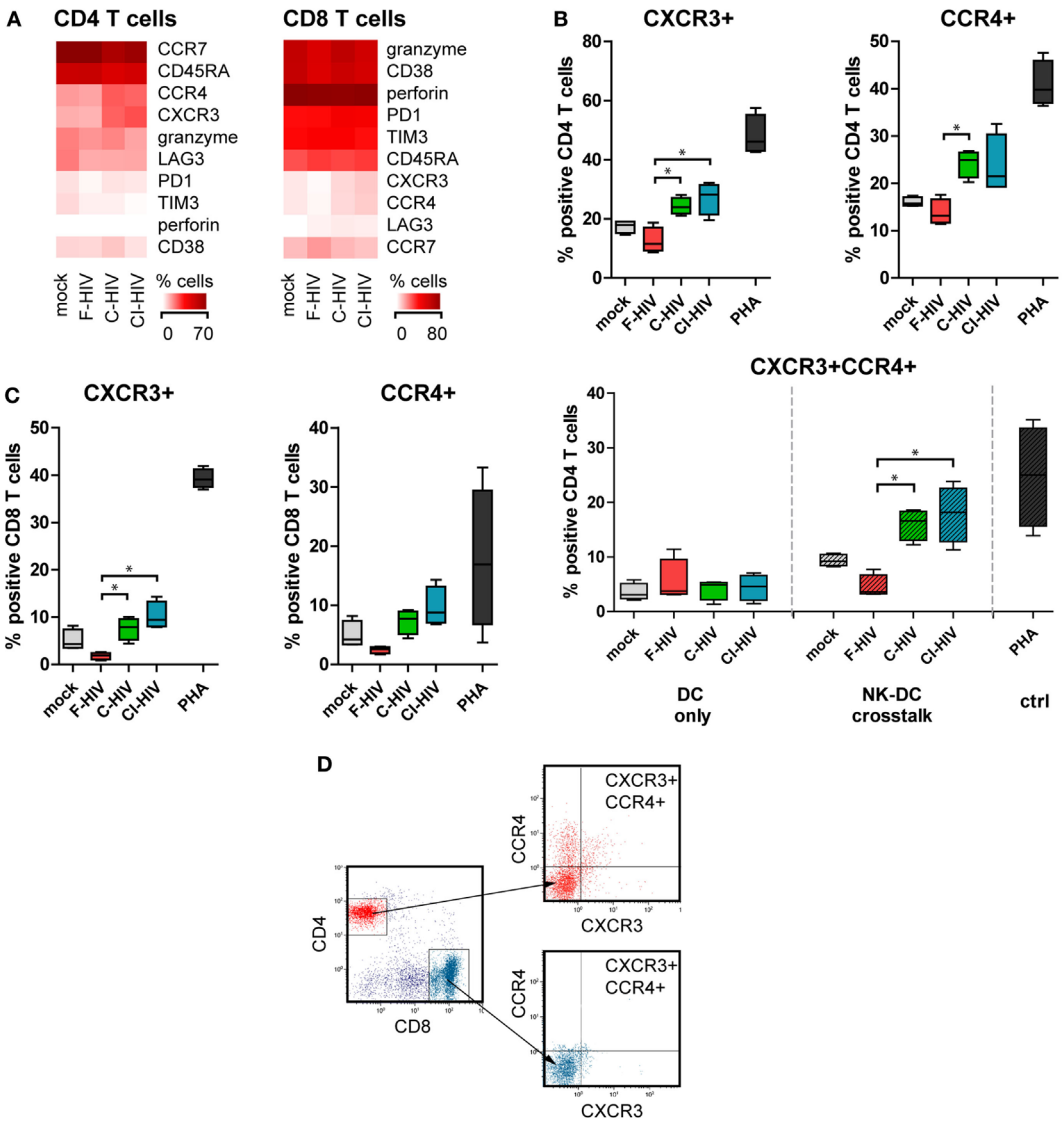

FIGURE 7 | T cell phenotype activated by natural killer (NK)–dendritic cell (DC) stimulation assay. DCs and NK cells from the same donor were cocultured and exposed to 1 μg/ml F-HIV, complement-opsonized HIV (C-HIV), complement- and antibody opsonized HIV (CI-HIV), PHA, or mock treated for 24 h. The experiment was replicated four times using cells derived from four different donors. Allogeneic T cells were then added to the cultures at a 1:10 DC:T cell ratio. **(A)** The T cell phenotype induced was assessed by flow cytometry. Heat maps of the percentage of CD4 and CD8 cells positive for phenotypic markers were created. **(B)** The number of CD4 T cells positive for CXCR3 or CCR4 were evaluated in culture with DCs conditioned with NK cells, and the number of CD4 T cells positive for both CXCR3 and CCR4 were evaluated in culture with DCs conditioned with NK cells or DCs alone. **(C)** The number of CD8 T cells positive for CXCR3 and CCR4 was evaluated in culture with DCs conditioned with NK cells. **(D)** Gating strategy for flow cytometry analysis of a representative sample can be seen. Boxplots show mean with Tukey error bars. One-way ANOVA followed by a Bonferroni posttest was used to test for statistical significance (*$p < 0.05$, $N = 4$).

for CD38, PD-1, TIM-3, and LAG-3 on CD4 and CD8 T cells is presented in **Figures 8C,F**.

DISCUSSION

Dendritic cells, NK cells, and T cells at the mucosal linings all have important roles during HIV exposure and interactions between these critical immune cells are likely to determine whether the exposure leads to establishment of HIV infection as well as shaping the immune responses against the virus. In this study, we investigated how free HIV (F-HIV), and HIV opsonized with complement only (C-HIV), or with both complement and antibodies (CI-HIV) affected DCs and NK cells alone and during cross talk, and how this cellular cross talk in turn affected their ability to activate naïve T cells and the T cell phenotypes generated.

Our transcriptome profiling and flow cytometry experiments revealed that C-HIV induced lower activation of DC maturation pathways and factors compared to free HIV and also less NK cell

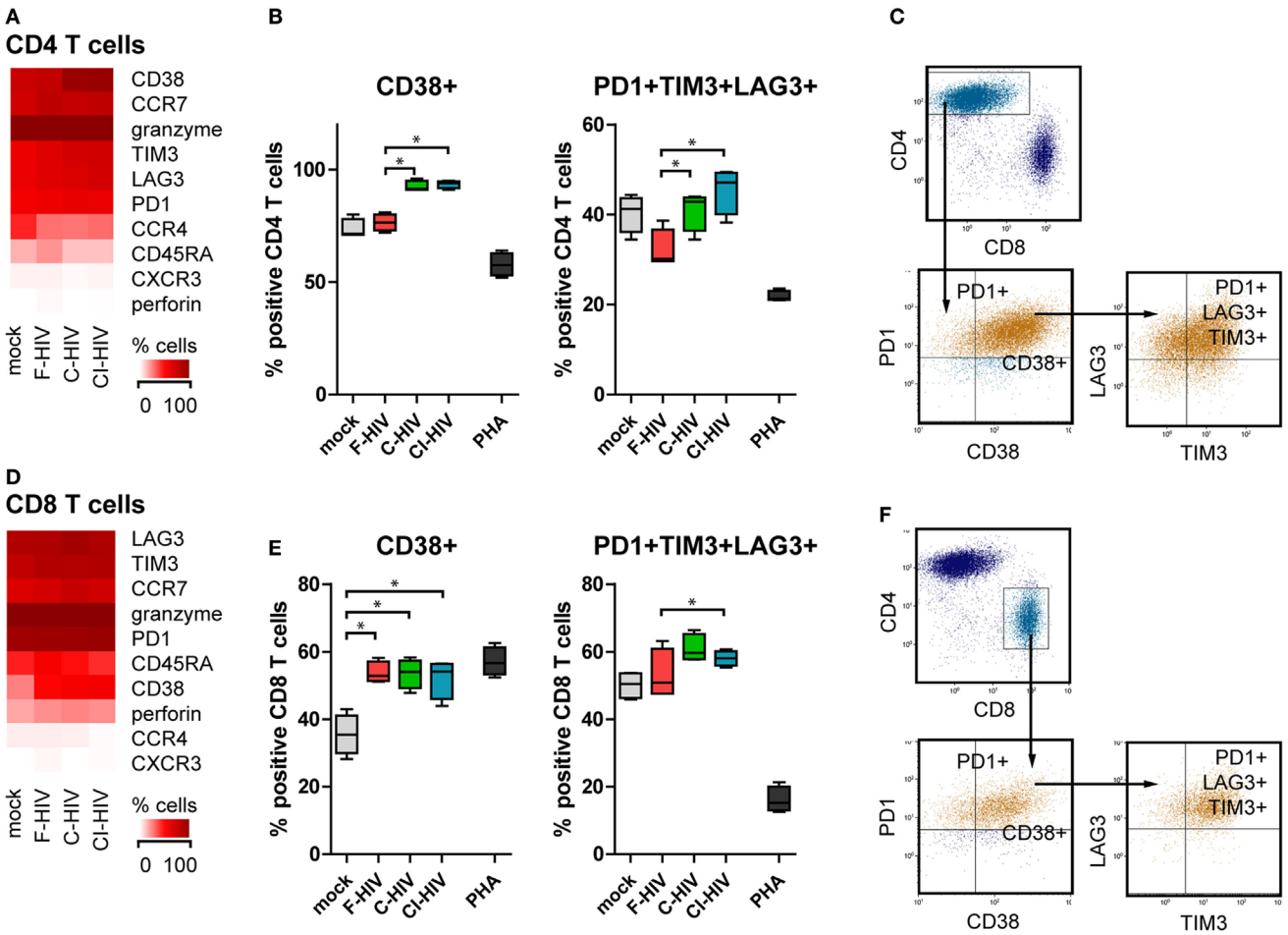

FIGURE 8 | Expression of immune checkpoint factors on the CD4 and CD8 T cells activated by CD3 and CD28 ligation and supernatants from the natural killer (NK)–dendritic cell (DC) cross talk assay. DCs and NK cells from the same donor were exposed to 1 μg/ml F-HIV, complement-opsonized HIV (C-HIV), CI-HIV, PHA, or mock treated for 24 h. The experiment was replicated four times using cells derived from four different donors. Allogeneic T cells were stimulated by CD3/CD28 ligation in the presence of supernatants from these cultures. **(A,D)** The CD4 T cell **(A)** and CD8 T cell **(D)** phenotype generated was assessed using flow cytometry. Heat maps of the percentage of CD4 cells or CD8 T cells positive for phenotypic markers was created. The number of CD4 T cells **(B)** or CD8 T cells **(E)** positive for CD38, and the number of cells coexpressing PD-1, TIM-3, and LAG-3 were evaluated. **(C,F)** Gating strategy for flow cytometry analysis of a representative sample. Boxplots show mean with Tukey error bars. One-way ANOVA followed by a Bonferroni posttest was used to test for statistical significance (*$p < 0.05$, $N = 4$).

activation during NK–DC cross talk. We have previously shown that C-HIV inhibits inflammatory and antiviral responses in DCs in a CR3-dependent manner (12), and in this study, the suppression of factors associated with regulating responses to danger was clearly visible both for DCs cultured alone and for DCs derived from NK–DC cross talk experiments. Danger signaling is tightly linked to DC maturation (40), as well as their ability to activate NK cells (41). Even if the NK cells in single culture were activated by HIV, it was apparent that the responses induced by HIV in the DCs defined the NK responses since the transcriptomes of the DCs were considerably less affected by the cross talk than the transcriptomes of the NK cells. In addition, the presence of C-HIV did not suppress NK cell activation in the absence of DCs. These findings support the concept of the DC as the determining factor during HIV transmission (5). Of note, *in vivo* studies have also described that during acute infection, DCs produce reduced amounts of IL-12, IL-15, and IL-18, leading to lower IFN-γ production by NK cells, which consequently results in poor DC maturation (21, 42).

Natural killer cells have the ability to recognize and kill virus infected target cells by the release of perforin and granzymes, or engagement of target cell death receptors such as Fas by Fas-L (43). NK-mediated killing creates a substantial immune pressure on HIV (44), and it has been suggested that the ability of NK cells to eliminate infected cells from the primary site of HIV infection can avert systemic infection (2, 42). In this study, DCs exposed to HIV suppressed the pathways involved in the activation of the NK cell ability to kill target cells, which is in accordance with *in vitro* studies by others (25). The effect DCs exposed to C-HIV had on the NK cell killing was striking as it severely suppressed

this important NK effector function, with less than half the number of target cells killed. This dexterous effect by C-HIV on the NK cell's cytotoxic function should impair the ability of the host to control the HIV infection.

Dendritic cell and NK cell communications occur in the presence of HIV both locally in tissues as well as in the lymphoid system (45, 46). The ability of NK cells to lyse immature DCs in order to avoid defective T cell priming is known as "DC-editing," and this has been shown to enhance the expansion of antigen specific cytotoxic T cells (47), which are important effector cells involved in controlling the HIV infection. However, the capacity of NK cells to carry out DC-editing is reduced by HIV *in vivo* (21, 48). NK cell lysis of immature DCs has been shown to involve the NKp30 and DNAM-1 receptors whereas the upregulation of MHC class I on activated DCs can protect them from NK killing (26). In our system, DCs exposed to C-HIV or CI-HIV inhibited NK killing of both target cells and of the DCs themselves. Of note, RNAseq analysis revealed that several upstream regulators that promote survival were upregulated in the DCs, i.e., the survival of DCs could be due to both suppressed NK cytotoxicity and a consequence of higher resistance to killing in the DCs. The precise mechanisms involved in the suppression of NK killing by DCs exposed to C-HIV remain to be elucidated. Interestingly, in our system the upregulation of perforin levels in CD8 T cells exposed to NK–DC cultures was also inhibited by C-HIV and CI-HIV, i.e., it is possible that the DC-mediated inhibition of cytotoxic responses when exposed to C-HIV occurred through pathways common to both NK cells and CD8 T cells.

The effects HIV exerted on the NK-conditioned DCs' ability to prime and activate naïve T cells were assessed in HIV-exposed NK–DC cell cross talk cultures. All three forms of HIV directed naïve T cells to differentiate to central memory (CCR7+ CD45RA−) T cells. The DCs exposed to C-HIV or CI-HIV induced a slightly higher amount of CD4 and CD8 T cells with central memory phenotype, which indicates that the presence of complement did not interfere with the T cell differentiation from naïve to memory T cells, rather increase the pool of central memory T cells. Of note, central memory CD4 T cells are permissive to HIV infection and can form latent reservoirs (49) with replication-competent virus (50), and their induction and quantity is, therefore, highly relevant to HIV pathogenesis. Noteworthy, exposure to complement-opsonized virus was associated with lower levels of effector memory T cells than exposure to free virus, which could explain the higher perforin expression seen for F-HIV as perforin expression is known to be higher in effector memory T cells (51).

The effect of DC exposure to HIV on the T cell proliferation was massive with the proliferation of T cells stimulated by HIV-exposed cultures reduced to approximately 50% compared to T cells exposed to mock treated cultures. There was no significant difference in viral T cell suppression between the free and complement-opsonized virus. The reduction in DC's capacity to induce T cell proliferation after HIV exposure has been described previously, by our group and by others (52, 53), but the effect of complement has not been investigated previously.

Negative immune checkpoint molecules, such as PD-1, LAG-3, TIM-3, and PD-L1, were expressed to a higher extent on the T cells and DCs cultured in the presence of C-HIV than on cells exposed to free virus. In addition, stimulation of T cells by supernatants from NK–DC cross talk cultures exposed to C-HIV or CI-HIV led to a higher amount of both CD4 and CD8 T cells coexpressing PD-1, LAG-3, and TIM-3. The expression of PD-1 is hallmark of an exhausted T-cell phenotype with limited ability to respond to stimuli (54, 55). Exhausted T cells are characterized by a loss of proliferative capacity and cytotoxic activity (56–58). At an initial stage, the PD-1 pathway only dampens the CD8 T cell responsiveness, but can eventually lead to a hierarchical loss of proliferation, cytolytic activity, defects in cytokine production, and eventually deletion (57). A synergistic effect between PD-1 and other negative checkpoint factors has been described (59), and T cells coexpressing PD-1, TIM-3, and LAG-3 are often even more exhausted than cells expressing only PD-1 (57). While PD-1 blockade can reverse exhaustion in cells expressing PD-1 only, this is not true when TIM-3 and LAG-3 are coexpressed on the PD-1 positive cells, which indicates the existence of multiple redundant T cell suppression pathways (57). Consequently, the higher expression of both PD-1 itself, as well as the coexpression of TIM-3 and LAG-3, on T cells stimulated by cultures exposed to complement-opsonized virus is likely to have detrimental effects on immune function, thereby contributing to HIV pathogenesis.

Another factor that is an indicator of disease progression and dysfunctional T cells in untreated HIV infection is CD38. Expression of this activation marker is high on activated T cells, low on naïve T cells, and undetectable on resting memory T cells (60). Low number of CD4 and CD8 T cells expressing CD38 was associated with reduced permissiveness to HIV replication in cervical explants (61), indicating that CD38 expression either supports or is a product of HIV infection in the mucosa. We found CD38 expression to be upregulated on CD8 T cells primed and activated by CD3 and CD28 ligation following exposure to supernatants from all HIV-exposed NK–DC cross talk cultures, whereas CD4 T cells upregulated CD38 upon exposure to supernatant from C-HIV or CI-HIV cultures only. Recent observations suggest that CD38 plays an active role in HIV infection and in chronic HIV infection *in vivo*, where increased expression of CD38 on CD8 T cells appears to be associated with immune activation and HIV disease progression (60). In addition, HLADR+ CD38+ CD4 T cells have been shown to produce high levels of HIV due to higher levels CCR5 and CXCR4 (39), indicating that CD4 T cells primed by DCs exposed to C-HIV could support a higher HIV replication.

The ability to migrate to the site of infection and inflammation or into the lymph is highly relevant for most immune cells and proper migration involves an array of chemokine receptors. CD4 T cells activated by NK–DC cross talk cultures exposed to C-HIV had higher expression levels of CCR4 and CXCR3, than CD4 T cells stimulated by NK–DC exposed to free HIV. The same was also true for the CD8 T cells, although the number of CCR4 and CXCR3 positive cells was much lower than in the CD4 population. CCR4 and CXCR3 positive T cells have been shown to traffic to and infiltrate inflamed tissue (62, 63). CXCR3 expression is considered to be a signature of gut-homing potential in CD4 T cells (63). In lung, CTL migration to infection sites has been shown to be CXCR3 dependent (64), and this tissue positioning

is considered to be one of the rate-limiting steps in CTL-mediated protection (65). CXCR3+ and CCR4+ CD4 T cells are highly permissive to HIV infection and replication (66). In chronic SIV infection, there are increased levels of CXCR3 positive CD4 T cells but not of CCR4 positive CD4 T cells and this is also true in the lymph nodes, where the CXCR3 positive T follicular helper cells levels are associated with high viral loads (67).

CXCR3 expression has been shown to be induced by IFN-γ inducible ligands CXCL9, CXCL10, and CXCL11 (62). In our system, CXCL9 could play a role in the NK mediated induction of CXCR3 expression, as concentrations of this cytokine were highest in the NK–DC cross talk cultures exposed to C-HIV and CI-HIV. Both DCs and NK cells have the ability to produce CXCL9 (68). The CXCL9 mRNA expression levels was much higher in the DCs compared to the NK cells from the cross talk cultures, indicating that it is likely that the DCs account for the majority of CXCL9 production in our system. The levels of CXCL10 were upregulated in the NK–DC cross talk system by all HIV conditions. Interestingly, the upregulation of CXCR3 and CCR4 by C-HIV only occurred when both DC and NK cells were present during the T cell stimulation. Direct CD4 T cell–NK cell interactions stimulate upregulation CXCR3 on the CD4 T cells by means of IL-21 (69). However, our RNAseq did not detect any IL-21 mRNA expression, indicating that there are other mechanisms at that require direct interactions with both the DCs and NK cells during T cell conditioning, which remain to be investigated.

CCR4+ CCR5+ CD4 T cells are highly permissive to HIV infection and CCR4 is expressed by T helper type 2 and type 17 cells and also by regulatory CD4 T cells (66). Similar to CXCR3, CCR4 is important for the migration of T cells to sites of inflammation or infection (70). In addition, it has been suggested that CCR4 promotes retention of T cells in different tissues (71). HIV is highly dependent on availability of permissive cells for transmission and spread (28). The induction of a CD4 phenotype that is permissive to infection and has gut-homing capabilities, therefore, likely contribute to HIV infection and spread. An overview of the effects of C-HIV on DCs, NK cells, and T cell phenotype can be found in **Figure 9**.

In summary, presence of complement fragments on HIV's surface modulated responses in DCs and their cross talk with NK cells to inhibit killing and to promote the upregulation of factors associated with immune suppression (PD-1, TIM-3, LAG-3) and susceptibility to infection (T_{CM}, CD38, CXCR3, CCR4) on CD4 T cells. Complement opsonization, therefore, likely contributes to HIV transmission and pathogenesis.

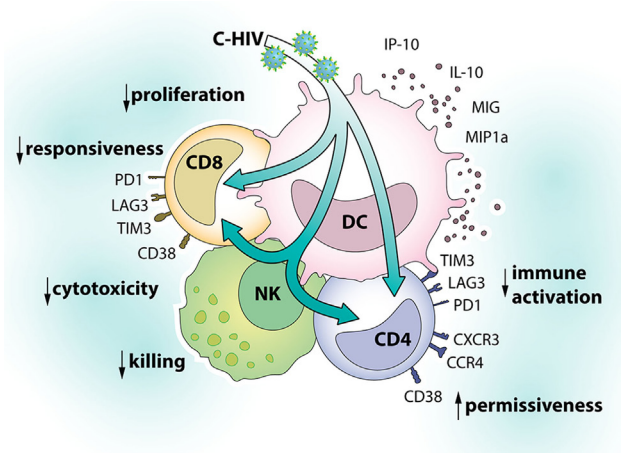

FIGURE 9 | Effects of complement-opsonized HIV (C-HIV) on dendritic cells (DCs), natural killer (NK) cells, and T cell phenotype. Complement fragments on HIV's surface modulate responses in DCs and their cross talk with NK cells to inhibit killing and promote the upregulation of factors associated with immune suppression (PD-1, TIM-3, LAG-3) and susceptibility to infection (CD38, CXCR3, CCR4) on T cells.

AUTHOR CONTRIBUTIONS

Conception/design: RE and ML. Data collection: RE, MK, HH, YT, and MW. Data analysis/interpretation: RE and MK. Drafting article: RE, ML, and ES. Critical revision of the article: CS, JH, SN, and ES. Final approval of the version to be published: RE, MK, CS, HH, YT, MW, JH, SN, ES, and ML.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00899/full#supplementary-material.

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Interferon-α Subtypes As an Adjunct Therapeutic Approach for Human Immunodeficiency Virus Functional Cure

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Human immunodeficiency virus (HIV) establishes life-long latency in infected individuals. Although highly active antiretroviral therapy (HAART) has had a significant impact on the course of HIV infection leading to a better long-term outcome, the pool of latent reservoir remains substantial even under HAART. Numerous approaches have been under development with the goal of eradicating the latent HIV reservoir though with limited success. Approaches that combine immune-mediated control of HIV to activate both the innate and the adaptive immune system under suppressive therapy along with "shock and kill" drugs may lead to a better control of the reactivated virus. Interferon-α (IFN-α) is an innate cytokine that has been shown to activate intracellular defenses capable of restricting and controlling HIV. IFN-α, however, harbors numerous functional subtypes that have been reported to display different binding affinities and potency. Recent studies have suggested that certain subtypes such as IFN-α8 and IFN-α14 have potent anti-HIV activity with little or no immune activation, whereas other subtypes such as IFN-α4, IFN-α5, and IFN-α14 activate NK cells. Could these subtypes be used in combination with other strategies to reduce the latent viral reservoir? Here, we review the role of IFN-α subtypes in HIV infection and discuss the possibility that certain subtypes could be potential adjuncts to a "shock and kill" or therapeutic vaccination strategy leading to better control of the latent reservoir and subsequent functional cure.

Keywords: human immunodeficiency virus, functional cure, interferon-α, interferon-α subtypes, human immunodeficiency virus latency

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INTRODUCTION

Human immunodeficiency virus (HIV) infections are characterized by severe immunodeficiency and onset of opportunistic infections. Currently, there are over 36 million people worldwide who are living with HIV. Onset of highly active antiretroviral therapy (HAART) has led to better viral control and long-term outcome in HIV-infected patients. As access to therapy becomes more readily available around the world, the number of new infections and transmission are expected to dramatically decrease, raising the hope that the HIV epidemic can be controlled and managed. Encouraging studies (1) showing the efficacy of neutralizing antibodies to control viral rebound and the development of long-lasting drugs are likely to have a major impact on the epidemiology of the disease. As major efforts to control the HIV epidemic gets underway, focus has shifted to finding cure for patients who are already infected with HIV.

Human immunodeficiency virus is a retrovirus that integrates into the host genome. As such, an HIV-infected individual is infected for life. The primary target cell for HIV is the CD4 T cell, with HIV establishing latency in these cells, and this latent reservoir continues to persist during HAART. Except in the case of Timothy Brown who is the only known case of HIV to have been completely cured, complete eradication of HIV reservoir has proven to be challenging not only due to the integration of HIV into the host genome but also due to the large size of the latent persistent reservoir. As such focus has recently shifted to the development of functional cure strategies, where the objective is to obtain complete remission in the absence of antiretroviral drugs.

Evidence for functional cure came rather serendipitously when an infant born to an HIV-infected mother was treated continuously for over 2 years within hours after birth. The child remained free of HIV for about 2 years after withdrawal of therapy raising the prospect that early HAART could potentially achieve full remission in HIV-infected subjects. However, the excitement was short lived as HIV rebounded suggesting the latent reservoir was not eradicated with early therapy and reactivated in the absence of long-term HAART. A number of novel approaches such as "shock and kill" using latency reversing agents (LRA) although somewhat successful in reactivating latent HIV (1), their impact on the viral reservoir has been rather limited, suggesting that LRA would need to be combined with other approaches such as vaccination against HIV that can simultaneously activate the immune system to recognize viral antigens expressed on the surface of latently infected cells following reactivation with LRA. A number of studies are currently underway to explore this strategy.

Other strategies have focused on activating intracellular defense mechanisms using interferon α (IFN-α) in combination with LRA or other immune mediators with some promising data from non-human primate models. Here, we review the progress that has been made to date in understanding the role IFN-α plays in HIV infection and explore the potential for harnessing IFN-α and its subtype as a strategy toward functional cure.

TYPE I IFN AND HIV INFECTION

Since its initial discovery in 1957 as factors that inhibit viral replication (2), the role of innate IFN in viral infections has been extensively studied. The primary source of IFN-α is the plasmacytoid DC (pDC), whereas IFN-β is produced by most cell types (3, 4). pDC plays a major role in regulating the immune system and are the earliest cells recruited to the sites of virus entry. In response to viral pathogen-associated molecular patterns, pDCs have been shown to produce ~1,000-fold more IFN-α/β than other cell types (5).

Plasmacytoid DC express a variety of pathogen recognition receptors (PRRs) such a Toll-like receptor (TLR) 3, TLR7, TLR8, and TLR9 that can sense viral nucleic acids leading to the secretion of IFN-α (6–8). Recent studies have demonstrated that the cytoplasmic DNA sensor cGAS plays an important role in the secretion of IFN-α during both HIV and SIV infections (9). Lahaye et al. (10) showed that DC's sense viral cDNA in the cytoplasm that was mediated by cGAS and blocking cGAS or reverse transcription inhibited these responses (11). Likewise, Herzner et al. (12) showed that single-stranded HIV-1 DNA activates cGAS and HIV-1 reverse transcripts was the predominant viral DNA found in the cytoplasm during early infection. George et al. (3) showed that treatment with reverse transcriptase inhibitors immediately after infection completely blocked plasma IFN-α in SIV-infected rhesus macaques. Taken together these studies show that numerous innate sensing PRR contribute to the induction of IFN-α responses during HIV infection.

Although the production of IFN-α during HIV infection has been clearly demonstrated, the exact role these IFN play during infection has been less clear. Blockade of IFN-αR with anti-IFN-αR antibody was associated with higher HIV replication, whereas HIV replicated at lower levels in pDC-depleted cultures treated with IFN-α (13). IFN-α was found to limit HIV-1 replication by decreasing the formation of late reverse transcriptase products in infected cells (14), and treatment of newly infected CD4 T cells with IFN-α for short period time was associated with significant inactivation of HIV during the early stages of replication (15). IFN-α was shown to slow HIV disease progression in randomized, placebo-controlled trials (16), and Asmuth et al. (17) reported that the treatment with pegylated IFN-α2a had a statistically significant anti-HIV effect. Others have shown that IFN-α treatment inhibited HIV and SIV replication in CD4 T-cell lines (18), monocytes (19), and macrophages (20). IFN-α has been reported to affect late stages of HIV-1 replication in chronically infected cells, by inhibiting virus assembly and release and reducing the infectivity of virions (21). Other studies have shown the IFN-α induced IFN-stimulated genes (ISGs) that effectively suppressed HIV replication (22–24).

Interferon α has been shown to induce numerous ISG that are capable of restricting HIV replication namely, apolipoprotein B mRNA-editing (APOBEC3) family of cytidine deaminases, TRIM5α, tetherin (BST-2), SAMHD1, MX2, etc. (25–27). Studies have reported high levels of ISG expression in CD4 T cells very early during infection (28), and increased levels of APOBEC3G was found to correlate with lower levels of infection in macrophages during SIV infection (29). Others have reported that ISG were significantly upregulated during SIV infection (30–33). In addition to the induction of ISG, IFN-α has been shown to prime adaptive immune responses by cross-presenting viral antigens to $CD8^+$ T cells (34–36). Interestingly, Boasso et al. demonstrated that IFN-α-induced indoleamine 2,3-dioxygenase (IDO) from pDC inhibited $CD4^+$ T-cell proliferation during HIV infection (37), and blockade of gp120/CD4 interactions was found to inhibit HIV-mediated induction of IDO and IFN-α (38, 39).

In contrast to the protective effects of IFN-α during HIV infection, increased production of IFN-α was accompanied by an increase in HIV loads (40). Mandl et al. (41) argued that the generalized immune activation and progressive CD4 T cell depletion observed in pathogenic SIV infection was likely due to an aberrant activation of the innate immune system and increased IFN-α production in contrast to natural hosts such as sooty mangabeys. Martinson et al. (42) reported that TLR stimulation and IFN-α secretion by pDC contribute to immune activation during HIV infection. Others have shown that rapid progression

of HIV was associated with continuous production of IFN-α, likely through enhanced T cell differentiation and activation (43). Parrish et al (44) demonstrated that transmitted founder viruses replicate and spread more efficiently in CD4 T cells in the presence of IFN-α. Fraietta et al. (45) showed that IFNα/β upregulated the expression of Bak, a pro-apoptotic protein that correlated with increased T cell apoptosis, low $CD4^+$ T cell counts and high viral loads in HIV-infected patients. Patients who progressed to disease were found to have lower levels of pDC but displayed higher levels of IFN-α and MxA compared to healthy individuals (46). Other studies have reported that IFN-α promoted chronic immune activation, apoptosis, and immune dysfunction during HIV-1 infection (47–51). Likewise IFN-α was found to regulate $CD4^+$ T-cell apoptosis induced by noninfectious HIV-1 by upregulating the expression of TNF-related apoptosis-inducing ligand (TRAIL) (38). Cha et al. (52) reported that IFN-α significantly enhanced activation-induced proliferation but not homeostatic proliferation, suggesting that the IFN-α likely promotes the loss of CD4 T cells by accelerating cell turnover and activation-induced cell death. On the other hand, Dondi et al. found that IFN-α displays contrasting proliferation-inducing and proapoptotic properties (53). Chronic IFN-α signaling has been implicated in other persistent viral infections such as LCMV (54, 55).

TYPE I IFN SUBTYPES AND HIV INFECTION

Since its initial discovery, numerous isoforms of type I IFN have been identified. These isoforms, encoded by single exon genes include IFN-α (which harbors 13 different subtypes namely, IFN-α1, α2, α4, α5, α6, α7, α8, α10, α13, α14, α16, α17, and α21), IFN-β, IFN-ε, IFN-κ, and IFN-ω (56). All the type I IFN subtypes signal through a common receptor complex consisting of IFN-αR1 and IFN-αR2 subunits. In humans, IFN-α subtypes share ~70–99% amino acid sequence identity with each other and a ~35% identity with IFN-β (57).

The evolutionary advantage of having multiple isoforms of the same gene that bind to a common receptor complex is not clear. However, there is evidence that the different subtypes display variable binding affinities for the common receptors (58–60), which in turn appears to influence their efficacy and potency (summarized in **Table 1**). Subtypes such as IFNα-10 binds to the IFN-αR1/2 receptor complex at affinities that is 10- to 100-fold greater than IFNα-1 (61). Interestingly, IFNα-10 was found to be highly effective against Semliki forest virus and Vesicular stomatitis virus, whereas IFN-α1 was the least effective among the nine different subtypes tested (61). Cull et al. (62) examined the expression of IFN-α1, α2, α4, α5, α6, and α9 and IFN-β in murine cytomegalovirus-induced myocarditis and observed that IFN-α6 reduced viral replication and inflammation in contrast to IFN-α2 and α5 that increased replication.

Sperber et al showed that IFNα-2 induced chemotaxis genes and was most effective against HIV-1 (72) whereas IFN-α8 induced ISG's that were protective against HCV replication (65). Foster et al (64) showed that IFN-α8 has very high antiviral potency compared to some of the other subtypes. On the other hand, Scagnolari et al. (69) reported that IFN-α5, 6, 8, and 10 had high potency against human metapneumovirus, whereas IFN-α2, 17, and 21 were the least potent. Others have shown significant differences in the *in vitro* antiviral and antiproliferative effects of various subtypes (73–75). Hibbert and Foster (76) examined the effect of various subtypes on human B cells and showed that IFN-α8 induced proliferation at very low concentrations compared to other subtypes with IFN-α1 being largely inactive. Likewise, Hilkens et al. (77) examined the signaling though Janus kinase/STAT and transcriptional responses to selected IFN-α subtypes in human T cells and dendritic cells and reported differences in the potency of various subtypes to induce ISG.

Numerous studies have examined the expression of IFN subtypes during both HIV and SIV infections. Zaritsky et al. (78) evaluated the expression of both total IFNα mRNA and the pattern of IFN-α subtype mRNA expression in macaques during acute SIV infection and found that all 13 subtypes were expressed in the spleen with IFN-α4, 17, and 21 being the least abundant as compared to high levels of IFN-α2, 8, and 13. In contrast, only subtypes IFN-α2, 6, and 13 were expressed in the brain, whereas subtypes IFN-α6 and 13 were upregulated in the lung suggesting to tissue-specific differences in the expression of various subtypes. Lehman et al. (46) reported that IFN-α2 and IFN-α6 were significantly upregulated in HIV-infected patients. On the other hand, Li et al. (79) showed that IFN-α2 and 16 were upregulated during chronic HIV infection. George et al. (3) examined the expression of both type I and III IFN subtypes in peripheral blood, jejunal mucosa, and lymph nodes (LNs) of SIV-infected rhesus macaques and reported that all subtypes (IFN-α01/13, 02, 06, 08, 14, 16, 23, 24, 25, 27, 28, and 29, IFN-β, IFN-ω, and IFN-λ1) were significantly elevated in the LNs at day 10 postinfection compared to a restricted expression in PBMC (IFN-α01/13 and IFN-λ1) and jejunal mucosa (IFN-α1, 6, 8, 14, and 23, IFN-ω, and IFN-λ). Harper et al. (80) evaluated the expression of different IFN-α subtypes and their potency in HIV-1-exposed pDC using the lamina propria aggregate *ex vivo* culture model and reported that HIV infection induced numerous IFN-α subtypes with IFN-α6, IFN-α8, and IFN-α14 being the most potent at inhibiting HIV infection. Earlier studies (72) have shown that IFN-α2 was effective at suppressing HIV-1 replication although more recent studies (70) have demonstrated that IFN-α14 displayed significantly higher antiviral activity than IFN-α2 against HIV infection in humanized mouse models.

IFN-α SUBTYPES AND POTENTIAL FOR FUNCTIONAL CURE

Given the potential for IFN-α to induce immune activation during HIV infection, there is a potential concern regarding its use in functional cure strategies although there is anecdotal evidence that IFN-α could suppress viral replication during antiretroviral therapy.

Treatment of HIV-infected subjects under HAART with pegylated-IFN-α2a was associated with the suppression of HIV RNA loads (81). Likewise, Sun et al. (82) demonstrated that the treatment of HIV/HCV co-infected patients with IFN-α/ribavirin during HAART led to a moderate but significant and sustained decline in cell-associated HIV DNA. Recent reports using IFN-α

TABLE 1 | Antiviral activity of IFN-α subtypes.

IFN subtype(s)	Viral infection	Effect	Reference
IFN-α1, 4, and 9	MCMV	IFN-α1 transgene showed better antiviral activity than IFN-α4 or IFN-α9	Yeow et al. (63)
IFN-α1, 2, 4, 5, 6, and 9 and IFN-β	MCMV	IFN-α6 transgene reduced MCMV replication, whereas IFN-α5 increased viral replication	Cull et al. (62)
IFN-α1, 2, 5, 8, 10, 14, 17, and 21 and IFN-β	MEV	IFN-α 5, 8, 10, 14, and 17 were highly effective, whereas IFN-α2 had a moderate effect and IFN-α1 was least effective	Foster et al. (64)
IFN-α1, 2, 5, and 8 and 10	HCV	IFN-α8 was effective in suppressing HCV replication, whereas IFN-α1 is least effective	Koyama et al. (65)
IFN-α2, 6, 8, and 14 and IFN-β	HIV	Plasmids encoding IFN-α2, 6, 8, and 14 and IFN-β showed IFN-α14 and IFN-β were more protective than other subtypes in humanized mice	Abraham et al. (66)
IFNα4 and IFNα5	HBV	Both proteins and plasmid encoding IFN-α4 and 5 showed anti-HBV activity	Song et al. (67)
IFN-α1, 2b, and 4b	Influenza A virus	IFN-α2b showed strong antiviral activity as compared to IFN-α1 or 4b	Moll et al. (68)
IFN-α1, 2, 5, 6, 7, 8, 10, 14, 17, and 21	hMPV	IFN-α5, 6, 8, and 10 had higher antiviral activity	Scagnolari et al. (69)
IFN-α2 and 14	HIV (humanized mice)	IFN-α14 suppressed HIV replication, induced tetherin, MX2, APOBEC3G, and increased numbers of TRAIL + NK cells compared to IFN-α2	Lavender et al. (70)
IFN-α1, 2, 4, 6, 8, 14, 17, and 21	MuV	IFN-α6 showed higher antiviral activity	Markusic et al. (71)
IFN α-01/13, 2, 6, 8, 14, 16, 23, 24, 25, 26, 27, 28, and 29, IFN-β, IFN-ω, and IFN-λ1	SIV	IFN-α01/13, 2, 6, 8, 14, 16, 23, 24, 25, 26, 27, 28, 29, IFN-β, IFN-ω, and IFN-λ1 were significantly increased in lymph nodes at day 10 postinfection compared to restricted expression in PBMC (IFN-α01/13 and IFN-λ1) and jejunum (IFN-α1, 6, 8, 14, and 23, IFN-ω, and IFN-λ1). Primary source of all subtypes were dendritic cells (DC)	George et al. (3)
Pegylated IFN-α	HIV	Treatment with pegIFN-α and ribavirin reduced HIV DNA and increased frequencies of NK cells in HIV-1/HCV-infected patients	Hua et al. (90)

HIV, human immunodeficiency virus; hMPV, human metapneumovirus; IFN, interferon; MEV, murine encephalomyelitis virus; MuV, mumps virus; TRAIL, TNF-related apoptosis-inducing ligand.

in combination with other factors appear promising. Micci et al. (83) reported that a combination of recombinant IL-21 and pegylated-IFN-α2a limited residual inflammation and viral persistence in SIV-infected rhesus macaques and significantly delayed viral rebound after withdrawal of antiretroviral therapy. Others (84) have shown that pretreatment of CD4 T cells with IFN-α and IFN-β reversed HIV latency in T-cells both *in vitro* and *ex vivo* and was associated with a reduction in the number of latently infected cells. Azzoni et al. (81) demonstrated that pegylated-IFN-α2 monotherapy successfully suppressed HIV-1 replication and reduced cell-associated HIV DNA.

Recent studies by Lavender et al. (70) showed that IFN-α14 when delivered at the same clinical dose as IFN-α2 to humanized mice significantly suppressed HIV replication and proviral loads and reduced immune activation that was accompanied by induction of high levels of APOBEC3G, MX2, and tetherin that have been shown to interfere with HIV replication (85–88). Abraham et al. (66) showed that gene therapy with plasmids encoding IFN-β and IFN-α14 significantly suppressed HIV-1 replication in mice for longer periods of time compared to other commonly used subtypes such as IFN-α2. Interestingly, all treated mice rebounded after cessation of IFN-α14 treatment. Additional studies are warranted to determine if the protective efficacy of IFN-α14 activated specific innate defenses during antiretroviral therapy that were different from those induced by other subtypes tested. These studies, however, raise the possibility that IFN subtypes such as IFN-α14 could be a potent adjunct to current approaches exploring functional cure strategies in HIV-infected subjects.

Other studies have shown that specific IFN subtypes were more potent at activating NK cells that could be harnessed to eradiate latently infected cells after reactivation. Gibbert et al. (89) demonstrated that IFN-α11-activated NK cells that enabled cytolytic killing of Friend retrovirus-infected cells compared to other subtypes such as IFN-α2 and IFN-α5. Hua et al. (90) recently reported that the treatment of HIV-1/HCV co-infected subjects on HAART with pegylated-IFN-α induced activation of $CD56^{bright}CD16^{-}$ and $CD56^{bright}CD16^{+}$ NK cells expressing NKG2D an NKp30 that significantly correlated with a decrease in level of HIV-1 viral reservoir in CD4 T cells. Song et al. (67) examined that the effect of IFN-α subtypes on HBV infection and found that IFN-α4 and IFN-α5 correlated with expansion of effector NK cells in both liver and spleen that was associated with better control of HBV replication. Treatment of HIV-infected humanized mice with IFN-α14 was found to increase the expression of cytotoxic molecule TRAIL in NK cells, whereas Stegmann et al. (91) showed that induction of TRAIL on NK cells by IFN-α was associated with better control of hepatitis C infection. NK cells play an important role in the control of HIV infections (92) and strategies that can enhance NK cell activity could be beneficial in eradicating latently infected cells.

The studies described above suggest that a subset of IFN subtypes may be more effective at controlling infection than the others although there is a significant gap in our knowledge regarding the timing of administering these subtypes in the context of suppressive HAART that could potentially impact their efficacy. Sandler et al. (32) treated SIV-infected rhesus macaques with IFN-α2 during the acute phase of infection and reported that IFN-α2 initially upregulated the expression of antiviral genes, whereas continuous treatment was accompanied by desensitization and an increase in the viral reservoir size. Although

the effect of initiating IFN therapy early in infection appears to be apparent, it is not clear if subjects under suppressive HAART regimens when treated would be unresponsive to treatment with various IFN subtypes.

Two exciting new studies (93, 94) using the humanized mouse model have reported that that blocking IFN signaling and reducing IFN-induced activation by treating with an antibody to the IFN receptor could reduce the size the HIV reservoir and delay viral rebound after cessation of HAART. These studies appear to be in contrast to what has been reported earlier using non-human primate models where blockade of IFN-αR was found to have the opposite effect (32). Audige et al. reported that treatment with anti-IFN-αR antibody was associated with increased HIV replication (13). On the other hand, blockade of chronic IFN signaling was shown to decrease immune activation and clear persistent LCMV infection in mice (55). Additional studies are needed to better clarify and confirm these findings in HIV infected subjects.

CONCLUSION

Functional cure strategies that can eradicate the viral reservoir are urgently needed. A number of approaches are being currently explored to achieve this goal. Although IFN-α therapy has been attempted in the field, there is new evidence suggesting that specific subtypes such as IFN-α8 and 14 may display more potent efficacy against HIV infection than the subtypes such as IFN-α2 that have been used in the past. Whether these subtypes can enhance innate immune defense during suppressive HAART and if these innate defenses would be sufficient to eradicate the reactivated latent reservoir remains to be seen. Studies that use a combination of approaches such as specific IFN-α subtypes along with therapeutic immunization to activate both the innate and adaptive immune responses during suppressive HAART are likely to be more effective at achieving full remission of HIV.

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

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REVIEW
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The Significance of Type-I Interferons in the Pathogenesis and Therapy of Human Immunodeficiency Virus 1 Infection

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Type-I interferons (IFN-I) are a widely expressed family that could promote antivirus immunity in the process of pathogens invasion. In a human immunodeficiency virus 1 (HIV-1)-infected individual, the production of IFN-I can be detected as early as the acute phase and will persist throughout the course of infection. However, sustained stimulation of immune system by IFN-I also contributes greatly to host-mediated immunopathology and diseases progression. Although the protective effects of IFN-I in the acute phase of HIV-1 infection have been observed, more studies recently focus on their detrimental role in the chronic stage. Inhibition of IFN-I signaling may reverse HIV-1-induced immune hyperactivation and furthermore reduce HIV-1 reservoirs, which suggest this strategy may provide a potential way to enhance the therapeutic effect of antiretroviral therapy. Therefore, we review the role of IFN-I in HIV-1 progression, their effects on different immunocytes, and therapeutic prospects targeting the IFN-I system.

Keywords: type 1 interferons, human immunodificiency virus 1, pathogenesis, immunocytes, immunotherapy

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INTRODUCTION

Human immunodeficiency virus 1 (HIV-1) is a highly pathogenic retrovirus that causes immune system degeneration (1). In the past 30 years, antiretroviral therapy has achieved considerable advances. However, despite remarkable scientific achievements in HIV-1 diagnosis and treatment, acquired immune deficiency syndrome (AIDS) still prevails and there are estimated 35 million people worldwide living with HIV-1 infection or AIDS (2, 3). The innate immune system, a significant alarm system in our body, has been caught more attention on resisting foreigner pathogens in recent years (4–6). One of the key effector molecules in innate system is interferons (IFNs), which rapidly respond to virus infection by a broadly, non-specific manner.

Interferons are classified into three groups based on the structure of their receptors: type I (IFN-α, IFN-β, IFN-κ, IFN-δ, IFN-ε, IFN-τ, IFN-ω, and IFN-ζ), type II (IFN-γ), type III (IFN-λ1, IFN-λ2, and IFN-λ3). Among these three types, IFN-I bind to a cell surface receptor complex known as the IFN-α/β receptor (IFNAR), which consists of IFNAR1 and IFNAR2 chains (7). Contrary to the limited expression of type II and type III interferons receptor, IFNAR is widely expressed on almost all kinds of immunocytes and epithelial tissue (5, 8, 9), suggesting that IFN-I have an extensive influence and are able to arouse quick activation of the whole immune system.

Innate immune responses mainly derive from the recognition of viral pathogen-associated molecular patterns (PAMPs) by host pattern recognition receptors (PPRs) such as toll-like receptors

(TLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), and other DNA-sensing receptors (10–12). Upon sensing PAMPs, several downstream signal molecules and transcriptional factors will be recruited, subsequently production of IFNs, especially IFN-I, is stimulated. At the earliest stage of acute infection, the production of IFN-I and other inflammatory cytokines is an essential event to determine the rate of virus replication and spreads (11, 13, 14). Unfortunately, this response is usually ineffective to suppress HIV-1 activity, due to the ability of this virus to hijack host immune system and evade the IFN-mediated antiviral activities (4). Moreover, the persistent IFN-I secretion greatly disturbs the immune homeostasis, contributing to immune activation-dependent disease progression (15, 16).

The administration of IFN-I, especially IFN-α, as monotherapy or an adjunct to combined antiretroviral therapy (cART), has been intensively reported (17), but the results varied greatly. In addition, as the continuous IFN-I production impedes immune recovery and enhances T cells exhaustion, IFN-I blockade may provide another strategy to weaken the virus-induced immune hyperactivation in the chronic HIV-1 infection (18, 19). Actually, inhibition of IFN-I system is likely to be an efficient way to reverse excessively elevated IFN-I signaling and rescue specific anti-HIV-1 immunity (20). Concerning the extensive impact of IFN-I system, it is unclear whether this method to shut off the IFN-I system is beneficial to pathogenesis or merely a secondary effect.

In this review, we will discuss the stimulation of IFN-I by sensing viral pathogens after HIV-1 infection, the antiviral/immunomodulatory activities of IFN-I on different immunocytes, and the manipulation of IFN-I system as a therapeutic strategy *in vivo*.

THE INDUCTION OF IFN-I IN THE PROCESS OF HIV-1 INFECTION

Recognition of HIV-1 by PRRs in Innate Immune System

At the beginning of infection, HIV infects immunocytes such as dendritic cells, macrophages and $CD4^+$ T cells in the human intestinal mucosal. In this process, virus can be rapidly recognized by innate immune system through a series of complex mechanisms as follows. The initial sensing of HIV-1 is mediated by PRRs. There are four classes of PRRs family have been identified, including transmembrane protein as TLRs and C-type lectin receptors, as well as cytoplasmic proteins as RLRs and NOD-like receptors (21). They recognize conserved structures of HIV-1 nucleic acid, which is called PAMP. The interaction between PAMP and PRRs will result in different level of immunocytes activation. And evidence has been found that plasmacytoid dendritic cells (pDCs) produce the highest level of IFN-I once sensing PAMP, whereas the production of IFN-I is barely detectable in other immune cells (22).

HIV *in vivo* can be divided into two types: cell free virus and cell-associated virus. The former is the virus infected directly from outside; while the latter is produced through viral RNA reverse transcription, integration and packaging in infected $CD4^+$ T cells (**Figure 1**). However, they enter pDCs through separate pathways. Cell free virus are taken up by pDCs through endocytosis mediated by envelope–CD4 interaction (23, 24), while cell-associated virus enter pDCs by fusion or endocytosis (14). Although cell-associated viruses are at a high level *in vivo*, they are a less potent inducer of IFN-I than cell free virus (25). One of the possible reasons is that most of cell-associated viruses are defective viruses which are not able to induce effective immune responses.

Plasmacytoid dendritic cells highly express TLR7, which greatly enhances their ability to produce IFN-I up to 1,000-fold more than other cell types' response to HIV-1 infection (26). In the cytosol of pDCs, HIV nucleic acid is presented to TLR7 located in endosomes, which is transferred from the endoplasmic reticulum (ER) to endosome *via* polytopic membrane protein UNC93B1 and heat shock protein gp96 (27, 28). After the formation of endosomes, TLR7 rapidly catches up with single-strand RNA (ssRNA) (29). But the specific character of these ssRNA has not been identified. Moreover, TLR3 is the other essential TLR expressed in pDCs, which detects both ssRNA and double strands RNA (dsRNA) (30, 31). Similarly, TLR3 also transferred from ER to endosome. In mature endosome, activated TLR7 and TLR3 by pathogenic nucleic acid is phosphorylated by tyrosine kinase-Src (32, 33). Although TLR7 and TLR3 are both explicitly expressed in pDCs, TLR7 plays a more important role in recognition of pathogenic nucleic acid and stimulation of IFN-I in response to HIV-1 infection (34).

Although ways of HIV-1 entering into macrophage and $CD4^+$ T cells are similar to that entering into pDCs, the recognition of HIV nucleic acid by these cells are completely different. In these cells, viral RNA is detected by RIG-I, a cytosolic receptor, without the generation of endosomes. RIG-I is a member of DExD/H box RNA helicases family. The crystal structure of RIG-I can be divided into three distinct domains: N-terminal region consisting of caspase activation and recruitment domains (CARD) to trigger IFN-I secretion; a central DExD/H box RNA helicase domain binding to specific RNA; as well as C-terminal repressor domain (35, 36). Just because of the special structure of RIG-I, PAMP of RNA virus whose sequence is marked with 5′ triphosphorylated (5′ppp) ends could be well recognized (35). It is by 5′ppp marks that RIG-I distinguishes exogenous RNA from their own (37). Various studies confirm that RIG-$I^{-/-}$ mice become more susceptible to RNA virus infection (35, 38, 39). Furthermore, stimulating the RIG-I pathway by retinoic acid effectively reactivates HIV reservoirs and promotes apoptosis of these infected cells, leading to the enhancement of innate immune system to eliminate latent reservoirs (40).

Interferon-Regulatory Factors (IRFs) Regulating the Production of IFN-I

Interferon-regulatory factors are a member of transcription factors that place in the central position of innate immune responses. Actually, they play a crucial role in bridging PRRs and the induction of IFN-I in gene-regulatory network (4). IRFs family consist nine members: IRF 1–9. The common of these transcriptional factors is that they all contain a conserved DNA-binding domain to recognize DNA sequences known as IFN-stimulated response element (41). Especially IRF-3 and IRF-7, they are main regulators

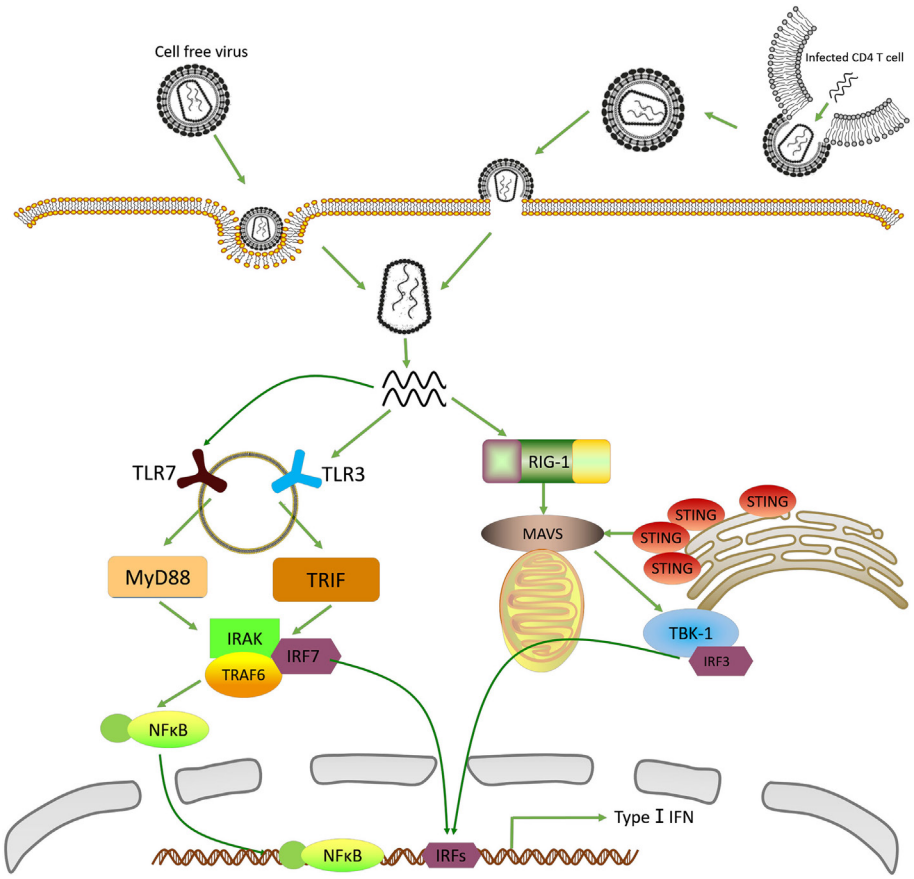

FIGURE 1 | Recognition of human immunodeficiency virus 1 (HIV-1) by innate immune system. In plasmacytoid dendritic cells (pDCs), cell-free HIV is taken up through endocytosis while cell-associated virus enters into pDCs by fusion and endocytosis. The single-strand RNA (ssRNA) released from virus is recognized by TLR3 and TLR7. Then the activated toll-like receptors (TLRs) stimulate MyD88 and TRIF signal pathway, recruit NF-κB and interferon-regulatory factor (IRF)-7, respectively, to trigger type-I interferons (IFN-I) production. In macrophages and $CD4^+$ T cells, HIV-1 enters cells mainly through fusion and endocytosis. But the ssRNA is detected by retinoic acid-inducible gene (RIG)-I, which stimulates mitochondrial antiviral signaling protein (MAVS)–IRF-3 dependent pathway, and next moderately induces the expression of IFN-I.

in producing IFN-I among pDCs, macrophages and $CD4^+$ T cells after the interaction of PAMP and PPRs.

Interferon-regulatory factor-7 has attracted much attention for its function in pDCs. Upon recognition of HIV-1 ssRNA by TLR7 and TLR3 in endosome, a complex including IRF-7, IRAK, TRAF6, and other proteins are rapidly recruited (42, 43). However, the complex is engaged by these two TLR through distinct pathways: TLR7 is mediated by a MyD88-dependent manner whereas TLR3 by TRIF (44, 45). The establishment of complex drives the phosphorylation of IRF-7 by IRAK1 and IKKα. Then phosphorylated IRF-7 translocates from cytoplasm into nucleus, attach to the promoter of IFN-I and increase their expression (42, 46). At the same time, the complex activates NF-κB by a MyD88-TRAF6 dependent pathway, which further stimulates the production of IFN-1. Notably, phosphorylated IRF-7 can form a dimer (a homodimer or a heterodimer with IRF-7), which stimulates the activity of histone-acetyltransferase to loosen chromatin structure and facilitate more efficient transcription of IFN-I (47).

The other significant IRFs that have been intensively studied in HIV infection is IRF-3. Similar to IRF-7, IRF-3 also resides in the cytosol. However, the recruitment of IRF-3 follows the interaction between viral RNA and RIG-I in macrophage and $CD4^+$ T cells (37). During viral infection, 5′ppp RNA PAMPs bind to R-terminal region of RIG-I, which cause the release of CARD to trigger CARD-depended interaction with mitochondrial antiviral signaling protein (MAVS) that is located on the outer mitochondrial membrane (48, 49). The activation of MAVS strongly catalyzes 2,3′-guanosine-adenosine monophosphate (cGAMP), which is the paramount agonist of stimulator of interferon genes (STING). STING initially aggregate around the MAVS, then stimulate the downstream signaling cascades that involve multiple kinases and finally lead to the phosphorylation of IRF-3 (50). Following behind phosphorylation, IRF-3 shares the similar mechanism of facilitating the transcription of IFN-I as IRF-7. Importantly, recent advances show that reverse transcribed HIV-DNA but not its RNA induces IRF-3 activation and IFN-I production depend on cGAMP–STING–IFI16 pathway

in macrophage (51–53). In addition, the polyglutamine binding protein 1 (PQBP1) is recently identified as the co-receptor of HIV-DNA to trigger cell-autonomous antiviral responses (54). Therefore, PQBP1 as an immune regulator provides a pharmacological target to improve the efficiencies of HIV medicine. However, sensing of HIV-DNA by IFI16 cannot induce IFN-I production in $CD4^+$ T cells, resulting the HIV evasion from innate immune system and formation of HIV reservoirs (55).

Interestingly, IRF-3 functions at the initial transcription of IFN-I gene. Whereas IRF-7, the upregulation of which also need the stimulation by IFN-I itself, is involved in the late phase of IFN-I gene induction. That is to say, the induction of IFN-I by IRF-3 is mediated by a two-step activation, which forms a positive-feedback-loop (56). It is likely another reason that pDCs produce the highest level of IFN-I as is mentioned earlier.

EFFECTS OF IFN-I ON IMMUNOCYTES IN CHRONIC HIV-1 INFECTION

Unlike the effective response in other infectious diseases, IFN-I in HIV-1 infection becomes rapidly dysfunctional and unable to purge the virus finally. Inversely, as the widely expression of IFNAR, prolonged virus replication and sustained stimulation of IFN-I progressively induce a generalized immune activation, injured inflammation, as well as T cell exhaustion (2, 46). Moreover, a strong correlation between the levels of IFN-I and disease progression has already been observed. In the models of simian immunodeficiency virus (SIV) infection, the common character of long time non-progression macaques is that they can induce rapid and transient high levels of IFN-I but declines in the chronic phase (19, 57, 58). Though the administration of cART can effectively suppress the replication of HIV-1, it cannot completely reverse the immune hyperactivation caused by IFN-I (59, 60). In the lymphocytic choriomeningitis virus (LCMV) mouse models, persistent production of IFN-I exacerbates $CD4^+$ T cell exhaustion and is detrimental to its antiviral response (61–63). Other researches also indicate that chronic infection of LCMV induces inhibitory molecules expression and apoptosis of Treg (64, 65). These studies suggest that compare to a positive role of IFN-I on restricting virus spread and replication at the acute phase of infection, IFN-I tend to exert a negative effect on different immunocytes in chronic HIV-1 infection.

Plasmacytoid dendritic cells are a special dendritic cell subset that produces a large amount of IFN-I in the process of HIV-1 infection. With the progression of HIV-1 infection, pDCs gradually decrease in blood while accumulate in lymph nodes. pDCs from these lymph nodes secrete higher titer of IFN-α spontaneously but not express co-stimulatory molecular (66). These nonfunctional cells are continuously produce IFN-I but cannot develop into mature antigen presentation cells. Instead, the redistributed pDCs increase staining of Annexin V and thus exhibit apoptosis (67). Even for cART treatment patients, the frequency and function of pDCs in peripheral blood is decline and accumulate in gut-associated lymphatic tissue (68). Moreover, in the chronic phase of HIV-1 infection, the excessive IFN-I may result in the dysregulated activation and even depletion in pDCs. In SIV models, a negative correlation between the decline of circulating pDCs and overexpression of IFN-I are observed during pathogenic SIV infection of macaques, but not in natural ones (69). Furthermore, pDCs with the upregulation of β7-integrin and CD103 are aggregated to the colorectum in chronic HIV-1 infected patients, which facilitates much more production of IFN-I (70, 71). Unfortunately, the amount of IFN-I produced by these lymphatic tissues is much more than what organisms really need to fight infection (71). Furthermore, these redundant IFN-I will lead to the activation of innate immune system and conversely damage the normal function of pDCs. However, the specific mechanism how IFN-I interacts with pDCs has not been clearly elucidated. There are two possible reasons for this phenomenon: On the one hand, pDCs that are persistently stimulated by IFN-I express low levels of migration receptors and regulatory factors, such as CCR7, CD40, and CD86. These pDCs produce IFN-I since pathogenic nucleic acid traffics to the endosome (72, 73), which induces apoptosis to these out-of-control cells mediated by the TNF-related apoptosis-inducing ligand (TRAIL) (74). On the other hand, IFN-I activates the non-canonical NF-κB signaling in pDCs. This pathway will promote the expression of indoleamine 2,3-dioxygenase, the most essential factor to gather regulatory T cells (Treg) (75). These pDCs-induced Treg intensively inhibit the maturation of pDCs through the engagement of cytotoxic T-lymphocyte antigen (CTLA)-4 and PD-1 on these activated pDCs (6).

Natural killer (NK) cells play a crucial role in innate immune system that act as the first line to defense HIV-1. First, the interaction between killer immunoglobulin-like receptors expressed on the surface of NK cells with their cognate HLA ligands sets a guarantee recognize specific HIV-derived peptides and eliminate HIV-1 infected cells (76, 77). Second, antibody-dependent cellular cytotoxicity (ADCC) is the other way of NK cells to control HIV-1 infection. Notably, ADCC activity was associated with the modest protective efficacy in the RV144 HIV vaccine trial (78). Moreover, new data show that the levels of NK cells activation is tightly associated with HIV-1 virological suppression in patients receiving cART (79). Patients who initiate ART early during infection obtain the improvement of cytotoxic function of the NK cells while decline of the levels of ADCC mediating antibodies (80, 81). These data suggest that NK cells. However, during the whole phase of HIV-1 infection, the relation between NK cells and IFN-I is complicated. In the acute infection, IFN-I has been observed to promote NK cells survival, expansion, maturation, activation and enhance their cytotoxic activity against virus (82, 83). In $IFNAR^{-/-}$ mice, there are barely detectable mature NK cells in peripheral blood (84). Identically, impaired cytotoxicity ability as well as a loss of highly activated subset of NK cells has been found in rapid progressors (85). But in the chronic HIV-1 infection, IFN-I may greatly disturb the normal function of NK cells. First, despite viral control well, NK cells are consistently activated in the chronic HIV-1 infection after the establishment of reservoirs. In the presence of high level of IFN-I, activated NK cells attenuate the cytotoxicity of $CD8^+$ T cells response to virus infection. As the suppressed function of NK cells and $CD8^+$

T cells, HIV escape from ADCC and CTL effect (86). Another research demonstrates that NK cells-depletion mice promoted virus-specific T cells response and contributed to viral control (87). Second, IFN-I disturbs the balance between STAT1 and STAT4, two downstream transcriptional factors of IFNAR in NK cells. In HCV/HIV coinfected patients, high level of IFN-I is relevant to the upregulation of STAT1 while downregulation of STAT4, which increase the expression of perforin induced by interleukin (IL)-12 (88). This mechanism further prompts the nonfunctional activation of NK cells. Thirdly, IFN-I decreases the production of IFN-γ through upregulating the expression of IL-10 and PD-L1 in NK cells (89), which further weaken their cytotoxic effects.

It is well established that HIV-infected patients maintain persistently high circulating $CD8^+$ T cells number, in spit of many years of therapy (90). The CD4/CD8 ratio often fails to become normal despite CD4 count normalization. Notably, new data showed that the majority of $CD8^+$ T proliferation and activation was induced in an antigen-independent manner (91). The great disturbing of T cell homeostasis makes the immune system dysfunctional and exhausted in the chronic HIV-1 infection. It is intensely investigated that IFN-I is responsible for the expansion of $CD8^+$ T cells. On the one hand, IFN-I has been shown to induce memory $CD8^+$ T cells proliferation and differentiation through bystander effect (92, 93). Thus, it is hypothesis that sustained exposure to IFN-I could contribute to $CD8^+$ T cell persistence. On the other hand, during the chronic infection, IFN-I favor the formation of terminally differentiated $CD8^+$ T cells that do not renew but enhanced cytotoxic function. This subset skewing likely contributes to the progressive IFN-I-mediated immune dysregulation (61, 89).

As we all known, HIV-1 features as the destroyer of $CD4^+$ T cells. It is the death of this kind of cell that propels the late phase of clinical procession, AIDS (2, 26). Although IFN-I could silence T cells to limit viral replication and program cells death to get rid of HIV-1 infection, IFN-I also influence the differentiation of T cells and induce death of HIV-1 uninfected cells *via* bystander effect (92, 94). On the one hand, depending on cytokine environment, naïve $CD4^+$ T cells mainly differentiate into Th1, Th2, Treg, and follicular T helper (Tfh) cell populations that have different biological functions (95). Actually, continuously stimulated by IFN-I facilitate naïve $CD4^+$ T cells differentiate toward Th2 cells, leading to a severe disproportionality of Th1 and Th2 (96, 97). On the other hand, IFN-I induces apoptosis of uninfected $CD4^+$ T cells in peripheral blood and in secondary lymphatic tissue by upregulating TRAIL expression on $CD4^+$ T cells, leading to the destruction of lymph node in gastrointestine in the acute phase of HIV-1 infection (98). Recent studies revealed that the death of 95% resting, non-permissive $CD4^+$ T cells are caused by caspase-1-mediated pyroptosis, a highly inflammatory form of programmed cell death (99, 100). With the stimulation of IFN-I, the expression of PD-1 is upregulated in exhausted T cell. Instead, blockade of PD-1/PD-L1 pathway helps to restore the function of T cells and decrease the viral load (101). Another studies also show that IFN-I significantly suppress HIV-1-specific $CD4^+$ T response, while blockade of IFN-I signaling pathway inactivates immune system, downregulates the expression of negative immune-regulatory factors and maintains the lymphoid structure after chronic LCMV infection (89, 102). In the model of humanized mice, blocking IFNAR rapidly enhances $CD4^+$ T recovery and reduces HIV-1 reservoirs (103).

Recently, a special subset of $CD4^+$ T cells, Tfh cells, has been intensively reported for its function to induce memory B cells activation, survival, differentiation, as well as assist B cells to produce antigen-specific neutralizing antibodies. At the same time, Tfh cells act as the major $CD4^+$ T cells compartment for HIV-1 infection, replication, and long-lived viral reservoirs (104, 105). In the chronic HIV-1 infection, the differentiation of Tfh cells impairs greatly as the repressive effect by excessive production of IFN-I and the activation of signal transducer and activator of transcription 3 (106). But this effect can be partly conversed by blocking the IFNAR in mice (107). Moreover, the population of HIV-specific Tfh cells expand during the chronic phase in patients who have a relatively high level of plasma HIV-RNA and IFN-I, which in turn leads to perturbation of B-cell differentiation, resulting in dysregulation antibody production (108). While in elite controllers, a stronger capacity to induce B cells maturation in Tfh cells is always tightly correlated to the low level of IFN-I (109, 110).

In addition, several studies indicate that the IFN-I also damage the normal function of B cells in the consistent presence of IFN-I. Indeed, IFN-I, especially IFN-α, is one of the most essential factor that contribute to the B-cell hyperactivation and exhaustion in HIV-1 viremic individuals (111–113). The loss of CD21 expression on exhausted B lymphocytes is a reliable marker of HIV-1 disease progression (114). It has been demonstrated that these cells that express low levels of CD21 is associated with the high expression of inhibitory markers as PD-1 and CTLA-4 (115, 116). Interestingly, IFNAR also express highly in these cells, which suggests that IFN-I plays a role on B cells dysfunction (117, 118). Another subpopulation of lymphocytes response to IFN-I is Treg, who work as a dominant role in immunosuppressive function to protect body from unwanted immune responses and maintain the homeostasis of immune system (119). However, the function of Treg is suppressed during the process of HIV-1 infection. When IFN persistently stimulated by IFNAR expressed on Treg, downstream signaling molecules will have impact on the expansion and suppressed function of Treg in turn (120). On the one hand, IFN-I inhibits Treg proliferation through a higher phosphorylation of STAT1 and a lower expression of suppressor of cytokine signaling 1 (121). Notably, this inhibition is tightly correlated with lower frequency of virus-specific $CD8^+$ T response and viral clearance (122). On the other hand, chronic HIV-1 infection also leads to the downregulation of Foxp3. Foxp3 appears to act as a master regulator on the development and suppression function of Treg (123, 124). The possible mechanism is that IFN-I induces the secretion of IL-10 and decreases transforming growth factor-β. The overexpression of inhibitory inflammatory factors will competitively inhibit the suppressive function of Treg to rectify the disorder in immune system, which causes dysregulation in Foxp3 (125–127).

Overall, these observations confirm that the detrimental effect of IFN-I on immunocytes is evident during chronic HIV-1

infection. Despite the effective suppression of HIV viral load achieved by cART, it cannot completely reverse the immune hyperactivation and negative function induced by IFN-I. How to take advantage of IFN-I to restrict HIV-1 replication while restore the normal function of immunocytes is a key point we must balance.

THERAPEUTIC STRATEGIES TARGETING IFN-I SYSTEM *IN VIVO*

As is mentioned earlier, although it is apparent for the importance of IFN-I on limiting HIV-1 in the acute phase of infection, dysfunctional IFN-I is more likely to disturb the balance of immune system and be detrimental to the function of pDCs, NK cells, $CD4^+$ T cells and Treg in the chronic phase. Considering the complexity of IFN-I system, manipulating this system may have unpredictable consequences *in vivo*. Thus, it is reasonable to use or block this system in the treatment and prevention of HIV infection, but the virological and immunological effects must weigh against the possible adverse events. Nowadays, therapeutic strategies toward IFN-I system consists of two aspects: administration of IFN-I or inhibiting its signaling pathway. Next, we will summarize the related studies about IFN-I system in clinical trials or in HIV-1/SIV/LCMV models.

Clinical Effects of Administration of IFN-I in HIV-1 Infection

The administration of IFN-I to resist HIV infection undergoes two stages. In the era of pre-cART, IFN-I can be used as a monotherapy on HIV/AIDS. In 1990, a randomized, double-blind clinical trial conducted in 34 HIV-infected patients with the treatment of IFN-α 2b showed that early administration of IFN-I could

TABLE 1 | Studies of IFN-I administration in human.

Participants	Intervention	Conclusions	Reference
261 HIV-infected patients failing current cART treatment with plasma HIV-1-RNA >2,000 copies/mL	0.5, 1.0, 1.5, and 3.0 μg/kg pegylated IFN-α or placebo with current cART × 4 weeks followed with optimized cART × 24 weeks	IFN-α greatly decreases HIV-RNA level No significant changes in $CD4^+$ and $CD8^+$ T cells count between treatment and control arms	Angel et al. (130)
174 HIV-infected patients without receiving cART with $CD4^+$ T cells count ≥500 cells/μL	a. 200 mg/4 h AZT × 52 weeks b. 1 MIU/day IFN-α 2b with IFN-dose escalation × 52 weeks c. 200 mg/4 h AZT in combination with 1 MIU/day IFN-α 2b × 52 weeks	In combination with IFN-α greater decreases HIV-RNA level than AZT alone IFN-α transiently increase the $CD4^+$ T cells count	Tavel et al. (131)
13 HIV-infected patients without receiving cART with $CD4^+$ T cells count ≥300 cells/μL and plasma HIV-1-RNA >5,000 copies/mL	180 μg/week of pegylated IFN-α 2a × 12 weeks	Pegylated IFN-α 2a slightly decreases HIV-RNA and increases in $CD4^+$ T cells count	Asmuth et al. (132)
168 HIV-infected patients receiving cART with $CD4^+$ T cells count ≥350 cells/μL and plasma HIV-1-RNA <400 copies/mL	1.5 μg/kg/week pegylated IFN-α 2a from day 15 followed by cART interruption to day 8 after each cART resumption	IFN-α greatly decrease the $CD4^+$ T cells count and do not prolong the time to treatment resumption	Boué et al. (134)
89 HIV-infected patients without receiving cART	a. 1 μg/kg/week pegylated IFN-α 2a × 14 weeks + cART b. 1 μg/kg/week pegylated IFN-α 2a × 14 weeks + cART × 36 weeks followed by interruption at week 36, 48, and 60 c. 1 μg/kg/week pegylated IFN-α 2a × 14 weeks + cART × 36 weeks followed by interruption at week 36, 48, and 60 with IFN-α	Viral rebound and HIV-DNA is lower in IFN-α group but no difference after 6-month interruption $CD4^+$ T cells count is higher in IFN-α group but also no difference after 6-month interruption	Goujard et al. (133)
23 HIV-infected patients receiving cART with $CD4^+$ T cells count >450 cells/μL	a. 180 μg/week of pegylated IFN-α 2a with cART × 5 weeks + pegylated IFN-α 2a with cART interruption × 12 weeks b. 90 μg/week of pegylated IFN-α 2a with cART × 5 weeks + pegylated IFN-α 2a with cART interruption × 12 weeks	Pegylated IFN-α 2a results in a sustained control of viral replication in 45% of subjects with cART interruption and a significant reduction of integrated HIV-DNA in $CD4^+$ T cells	Azzoni et al. (135)
12 HIV/HCV-coinfected patients receiving cART with suppressed HIV-1 viremia	180 μg/week of pegylated IFN-α 2a and ribavirin 500–600 mg twice daily	Approximately twofold decreases of total and integrated HIV-DNA in $CD4^+$ T cells during and after IFN-α/ribavirin therapy	Sun et al. (138)
a. 15 HIV/HCV-coinfected patients b. 17 HIV-infected patients	a. 180 μg/week IFN-α and ribavirin 900 mg twice daily × 48 weeks + cART b. cART	IFN-α obviously decreases $CD4^+$ T cells count and HIV-DNA, especially 2-LTR circular HIV-DNA	Jiao et al. (136)
162 HCV treatment naïve or experienced patients coinfected with HIV-1	d. ca. 750 mg/8 h telaprevir + 180 μg/week IFN-α and ribavirin 800 mg/day × 18 weeks + cART	Telaprevir and IFN-α decreases $CD4^+$ T cells count and three patients had a viral load increase ≥200 copies/mL	Montes et al. (137)

IFN, interferon; IFN-I, type-I interferons; HIV-1, human immunodeficiency virus 1; cART, combined antiretroviral therapy.

decrease the frequency of viral isolation and slow the progress of disease despite a few side-effects such as flu-like symptoms and granulocytopenia (128). Another trial fixed attention on patients with AIDS-associated Kaposi's sarcoma. Although the absolute $CD4^+$ T cells count in these patients was relatively low, 50% of them were observed on tumor regression and reduction in HIV after treatment with IFN-α for 12 weeks (129).

However, with the development of cART, IFN-I is more likely to act as an adjunct therapy instead of monotherapy. In recent 10 years, most of trials were designed to treat patients with IFN-α in association with cART, but the results varied greatly (summarized in **Table 1**). In 2009, more than 200 treatment failure patients were enrolled to receive the administration of IFN-α before optimization of their antiretroviral therapy, finally they were observed a significant decrease in HIV-RNA, but no effect on $CD4^+$ T cells count compared with placebo-controlled group (130). Subsequently, several studies carried out in treatment naïve patients, although the majority of these patients had a high level of HIV-RNA at first, administration of IFN-α resulted in a great decline in viral load as well as a transient increase in $CD4^+$ T cells count (131, 132). At the same time, IFN-α was added to current treatment of cART, but the results seemed paradoxical: one indicated that IFN-α had a negative effect on disease progression (133), while the other suggested IFN-α decreased HIV reservoirs and delayed virus rebound after treatment interruption (134). Recently, two researches recruited patients who was coinfected with HIV/HCV and received IFN-α in combination with ribavirin, the result of which showed a reduction in integrated HIV-DNA and 2-LTR circular HIV-DNA (135–137). Altogether, administration of exogenous IFN-α intends to decrease HIV-RNA or HIV-DNA transiently in these studies. But the mechanism of IFN-α-induced reduction of HIV-RNA or HIV-DNA remains uncertain. Notably, it is well recognized that IFN-α treatment is related to decreasing $CD4^+$ T-cell counts, raising the possibility that reductions of HIV viral load during IFN-α therapy may result from its unspecific lymphocellular toxicity (138). Although IFN-α could decrease the viral burden, adding IFN-α to the current antiretroviral therapy is not likely to enhance T cells reconstitution and improve clinical outcome. Due to the high variability of HIV, the effects of exogenous IFN-α will be rapidly compromised and dysfunctional. Indeed, these IFN-α further disturbs the balance of immune system and even have a negative effect to some extent.

Influences of Inhibiting IFN-I Signaling *In Vivo*

Faced with challenge about immune disorder in long-term treatment of cART, more attention is fixed on inhibiting IFN-I signaling pathway to reverse hyperactivation and exhaustion of immune system caused by redundant production of IFN-I.

TABLE 2 | Studies of TLR and IFNAR blockade *in vivo*.

Species	Virus	Methods	Conclusions	Reference
Human	HIV-1	13 patients without receiving cART with $CD4^+$ T ≥250 cells/μL treated with chloroquine or placebo for 2 months in chronic HIV-1 infection	Significantly reducing $CD4^+$ and $CD8^+$ T cells activation	Murray et al. (140)
Human	HIV-1	83 patients without receiving cART with $CD4^+$ T ≥400 cells/μL treated with hydroxychloroquine or placebo for 48 weeks in chronic HIV-1 infection	a. Hydroxychloroquine tolerated well b. No effect on $CD8^+$ T cells activation c. Increasing viral load and declining $CD4^+$ T cells count	Paton et al. (141)
Human	HIV-1	19 patients on cART with $CD4^+$ T ≤350 cells/μL and undetectable viral load treated with chloroquine in combination with cART for 24 weeks in chronic HIV-1 infection	a. Chloroquine tolerated well b. Increasing the level of IFN-α2 production c. No effect on $CD4^+$ and $CD8^+$ T cells recovery, T cell activation and inflammation markers in plasma	Routy et al. (142)
Rhesus macaques	SIV	Chloroquine to inhibit TLR7 and TLR9 signaling in acute in acute SIV infection	a. No changing in the level of cell activation b. Temporary increasing the expression of interferon-stimulating genes c. Decreasing $CD4^+$ T cells recovery	Vaccari et al. (143)
Rhesus macaques	SIV	IFNAR antagonist to block IFN-α2 activity or exogenous IFN-α treatment in acute SIV infection	Higher viral load and accelerating disease progression whether by administration of IFNAR antagonist or induction of an IFN-tolerate state	Sandler et al. (17)
Mice	LCMV	Anti-IFNAR (MAR1-5A3) and clodronate liposomes in chronic LCMV infection	Preserving the function of virus-specific B cells and accelerating neutralizing antibody production	Moseman et al. (145)
Hu-mice	HIV-1	Using a monoclonal antibody to block IFNAR2 (clone MMHAR-2) in chronic HIV-1 infection	a. Reversing immune exhaustion b. IFNAR blockade in combination with cART achieving faster viral suppression and lower HIV-1 reservoirs	Zhen et al. (144)
Hu-mice	HIV-1	Using a monoclonal antibody to block IFNAR1 (extracellular domain and transmembrane domain) in chronic HIV-1 infection	a. Greatly suppressing aberrant immune activation b. Reducing the exhaustion of T cells c. Decreasing HIV-1 reservoirs and delaying virus rebound after cART discontinuation	Cheng et al. (103)

HIV-1, human immunodeficiency virus 1; IFNAR, IFN-α/β receptor; TLR, toll-like receptor; cART, combined antiretroviral therapy; SIV, simian immunodeficiency virus; LCMV, lymphocytic choriomeningitis virus.

Recent studies *in vivo* about manipulating IFN-I system consists of two aspects: inhibiting TLR to decrease the production of IFN-I and blocking IFNAR to interfere with its signaling (summarized in **Table 2**).

As is mentioned earlier, pDCs play a significant role in producing IFN-I. It may be an effective way to administrate chloroquine, a novel endosomal inhibitor in blocking TLR7 and TLR9 in pDCs, to reduce IFN-I products (139). Unfortunately, the results of several clinical trials about this strategy seem complicated. First, after receiving chloroquine for 2 months in 13 cART-naïve patients, decreased $CD4^+$ and $CD8^+$ T cells activation were observed (140). Although the result was encouraging, later research indicated that receiving hydroxychloroquine seemed to be no effect on $CD8^+$ T cells activation while hindered the recovery of $CD4^+$ T cells (141). Similarly, chloroquine had a negative influence on immunological non-responders (142). Therefore, the impact of reducing IFN-I production through chloroquine is still controversial, which needs further research.

On the other hand, antagonist of IFNAR has been used to specifically block IFN-I signaling in recent years. In 2014, a study showed that IFNAR blockade in the acute SIV infection resulted in increasing viral load, down-expression of antiviral genes and leading to the depletion of $CD4^+$ T cells (17). Two studies conducted in the model of hu-mice also found that IFNAR blockade was an effective way to diminish T cells exhaustion and restore immune function in the chronic HIV-1 infection. More importantly, declined in HIV-1 reservoirs was also observed in these researches (103, 143, 144). However, in the model of LCMV, blocking IFNAR can completely reverse the depletion of LCMV-specific B cells and promote the secretion of neutralizing antibody to resist infection (145). These data suggest that IFNAR blockade in combination with cART may provide a potential therapeutic strategy for HIV-1 infection. On account of the complex impact of blocking IFNAR *in vivo*, the assessment of anti-immunological consequences and side-effects must be carried out before widespread implementation.

CONCLUSION

Innate immune system provides an immediate defense against pathogens to protect our body from infection by other organisms. As the most important effector molecule, IFN-I is powerful to suppress HIV and stimulate the expression of a bunch of antiviral genes replication at the early phase of infection. Although IFN response seems to be effective, HIV evades the IFN-mediated antiviral activities later since the stimulation of resistance factors. According to the results of clinical trials mentioned earlier, administration of IFN-I may have no benefit on clinical outcome, confirming that it fails to restrict and clear HIV *in vivo*. However, several aspects about this process still require further investigation: in addition to IFN-α and IFN-β, the role of the other subtypes of IFN-I in this process has not been clear; the separate pathways to recognize HIV nucleic acid (ssRNA, dsRNA, or cDNA) needs to be identified in all types of infected cells; and the exact mechanism of HIV escape also have to be clarified. Future studies should address these uncertain questions.

Even if IFN-I do function in the early HIV infection, continuously production promotes immune activation and ultimately exhaustion of immune system in the chronic phase. The detrimental effects of IFN-I on different lymphocyte have been intensely reported. But the regulatory mechanism in different lymphocytes has to be figured out.

Although the long-term clinical results of the administration of exogenous IFN-α seem to be invalid in the acute phase of HIV-1 infection with or without cART, it has been identified that this treatment could reduce the viral load transiently. Future studies should lay emphasis on whether supplementation of IFN-I to cART therapy during chronic infection could further decrease the HIV-1 reservoirs.

Moreover, in animal infectious model, IFN-I blockade strategy contributing to the recovery of T cells and decline in HIV reservoirs have been observed, suggesting that animals obtain benefits from this strategy. Although the results are encouraging, this approach has not been tried in human for its complicate effects. Further proof needs to be provided whether it is effective to decrease the morbidity of AIDS and reduce reservoirs through blocking IFN-I signaling, as this strategy may decrease immune activation whereas increase T cell responses. What is more, if it is possible, clinical trials about effective monoclonal antibody toward IFN-I blockade are expected to conduct in human.

In summary, despite long terms of research, the exact relationship between the production of IFN-I, the pathway of viral evasion, and the induction of pathogenic cellular immunological injury has not been clearly deciphered. Numerous questions remain to answer. A better understanding of the role of IFN-I in HIV pathogenesis will aid in managing this pathway for therapeutic purposes.

AUTHOR CONTRIBUTIONS

BW reviewed the mechanisms about the production of IFN-1 in the acute phase of HIV-1 infection; WK provided the data of the clinical trials by administration of IFN-1 in HIV patients; JZ gathered the information of negative effect of IFN-1 on different immunocytes in the chronic phase of HIV-1 infection. As corresponding authors, WK and YS carefully checked the whole manuscript.

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