Persistent Simian Immunodeficiency Virus Infection Drives Differentiation, Aberrant Accumulation, and Latent Infection of Germinal Center Follicular T Helper Cells

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ABSTRACT

CD4⁺ follicular T helper (T_{fh}) cells play a prominent role in humoral immune responses, but the mechanisms of their accumulation and infection in AIDS remain unclear. Here we found that germinal center (GC) T_{fh} cells, defined here as CXCR5⁺ PD-1^{HIGH} CD4⁺ T cells, do not express the HIV coreceptor CCR5 yet serve as a latent reservoir in GCs. With disease progression, an expansion of GC T_{fh} cells is accompanied by increases in dysfunctional CD8⁺ T cells. In contrast, T_{fh} precursor (CXCR5⁺ CD4⁺ T) cells in lymph nodes do express CCR5 and differentiate into GC T_{fh} cells following interleukin-6 (IL-6) and IL-21 stimulation, and viral DNA is detectable in fully differentiated GC T_{fh} cells *ex vivo*. This suggests that SIV-infected GC T_{fh} cells may be derived from T_{fh} precursor cell subsets that become infected in marginal zones and then migrate into GCs as fully mature GC T_{fh} cells that serve as persistent virus reservoirs. These findings suggest that viral persistence in lymph nodes drives compensatory differentiation, aberrant accumulation, and latent infection of GC T_{fh} cells, resulting in marked impairment of humoral immune responses.

IMPORTANCE

Generation of antibodies that can effectively eliminate viruses requires interactions of B cells with highly specialized T cells in GCs of lymphoid tissues called follicular T helper cells. Here we show that in simian immunodeficiency virus infection, these cells are initially infected in a precursor stage that leads to alterations in their homing, accumulation, and function that may be responsible for the inability of human immunodeficiency virus-infected patients to generate effective antibody responses.

Follicular T helper (T_{fh}) cells are a class of helper CD4⁺ T cells specialized in promoting antigen-specific B cell immunity. An absence of T_{fh} leads to B cell apoptosis during priming, thereby preventing final B cell differentiation and maturation (1). Subsets of “immature” T_{fh} cells (precursors) are distributed in interfollicular zones of lymph nodes (LNs), where their differentiation is initiated (2), and they eventually migrate into germinal centers (GC), where they perform effector functions. Effector GC T_{fh} cells form stable contacts with GC B cells to provide direct help to naive B cells, promoting their development and maturation into antigen-specific, high-affinity memory B cells or antibody-secreting plasma cells, thereby ensuring long-term effective humoral immune responses (3). Classical GC T_{fh} cells are defined as CD4⁺ T cells expressing high levels of CXCR5, PD-1, SLAM-associated protein, GL7, ICOS, and Bcl-6 (4, 5). T_{fh} cells are also a primary source of interleukin-21 (IL-21) in GCs, which provides help for B cell differentiation and proliferation (6). Notably, PD-1, a potent T cell inhibitory receptor when expressed on CD8⁺ T cells (7), is also highly expressed on GC CD4⁺ T_{fh} cells and contributes to the regulation and survival of GC B cells through interaction with its ligands (8). Our previous study showed that CXCR5⁺ T_{fh} cells include both PD-1^{NEG/INT} and PD-1^{HIGH} CD4⁺ T cell populations, but the latter likely represents specific GC T_{fh} cells residing within the LN GC, and these play the central role in promoting B cell maturation and IgG production (9). As previously shown (9–11), PD-1 intensity measurement by flow cytometry can distinguish GC T_{fh} cells from total CXCR5⁺ T_{fh} cells in lymphoid tissues. Recent reports suggest that T_{fh} cells accumulate in simian immunodeficiency virus (SIV)/human immunodeficiency virus (HIV) infections (10, 12–14), and LN-derived CXCR5⁺ T_{fh} cells

serve as reservoirs for productive viral infection and replication (10, 15, 16). We recently reported that CXCR5⁺ PD-1^{HIGH} CD4⁺ T cells in LNs are transiently reduced in acute infection (day 14 after SIV infection) and accumulate in chronic infection yet are ultimately depleted in animals with AIDS (17). However, the mechanisms behind their infection and this accumulation remain unclear. Here, we examined the distribution and localization of GC T_{fh} (CXCR5⁺ PD-1^{HIGH} CD4⁺ T) cells in peripheral blood and organized lymphoid tissues and investigated the mechanisms of T_{fh} cell expansion and infection during SIV infection, including levels of GC T_{fh} cell differentiation and viral infection status. The results demonstrate that GC CD4⁺ T cells were enriched in LNs of chronically SIV-infected animals, accompanied by a reduction of PD-1^{NEG} CD4⁺ T cells and increases in PD-1⁺ CD8⁺ T cells. Notably, mature GC T_{fh} cells did not express CCR5 but still served as a persistent viral reservoir, having relatively higher levels of SIV DNA and lower levels of RNA/proteins than the immature PD-1^{INT} CD4⁺ T cells in mantle T cell zones, which did coexpress

Received 28 September 2015 Accepted 17 November 2015

Accepted manuscript posted online 25 November 2015

Citation Xu H, Wang X, Malam N, Aye PP, Alvarez X, Lackner AA, Veazey RS. 2016. Persistent simian immunodeficiency virus infection drives differentiation, aberrant accumulation, and latent infection of germinal center follicular T helper cells. *J Virol* 90:1578–1587. doi:10.1128/JVI.02471-15.

Editor: G. Silvestri

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TABLE 1 Animals used in this study

Infection state and no. of RMs	Pathogen	% of CD4 T cells (avg)		Pathologic findings
		LNs	Jejunum	
Uninfected, 38		49.3–80.5 (66.05)	31/6–76.8 (48.18)	None
Chronically infected				
18	SIVmac251	10.2–76.4 (36.31)	2.06–40.7 (16.33)	Lymphoid hyperplasia and dysplasia, chronic inflammation
16	SHIVsf162P3	60.4–79.2 (68.61)	10.4–59.7 (35.17)	Lymphoid hyperplasia and inflammation, thymic atrophy
6 ^a	SIVmac251	23.2–73.2 (58)	7.66–48.6 (25.91)	Lymphoid hyperplasia, dysplasia, mild inflammation

^a Vaccinated.

CCR5. Further, these Tfh precursors (CXCR5⁺ CD4⁺ T cells) in LNs could be induced to become CXCR5⁺ PD-1^{HIGH} GC Tfh cells by IL-6 and IL-21 stimulation *in vitro*, and when they were isolated from SIV-infected macaques, SIV DNA was detectable in these fully differentiated PD-1^{HIGH} CD4⁺ T cells. Together, these findings suggest that Tfh precursors are infected as they migrate toward GCs, where they accumulate as latently infected yet CCR5-negative, mature GC Tfh cells. Thus, persistent viral replication drives the differentiation, pathological accumulation, and latent infection of GC Tfh cells, accompanied by an increase in dysfunctional cytotoxic T cells (CTLs), resulting in failure of effective humoral immune responses and disease progression in HIV/SIV infection. Thus, antiviral therapy, in combination with anti-inflammatory agents and perhaps inhibitors of cell differentiation, could be considered as an early intervention strategy to reduce viral reservoirs in HIV-infected patients.

MATERIALS AND METHODS

Ethics statement. All of the animals in this study were housed at the Tulane National Primate Research Center in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care International standards. All studies were reviewed and approved by the Tulane University Institutional Animal Care and Use Committee under protocol no. P0049. Animal housing and studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH, AAALAC no. 000594) and with the recommendations of the Weatherall report on the use of nonhuman primates in research (https://royalsociety.org/~media/Royal_Society_Content/policy/publications/2006/Weatherall-Report.pdf). All clinical procedures were carried out under the direction of a laboratory animal veterinarian. All procedures were performed under anesthesia with ketamine, and all efforts were made to minimize stress, improve housing conditions, and provide enrichment opportunities (e.g., objects to manipulate in the cage, varied food supplements, foraging and task-oriented feeding methods, and interaction with caregivers and research staff).

Animals and virus. Tissues from a total of 78 adult Indian-origin rhesus macaques (*Macaca mulatta*; RMs) were utilized to examine Tfh cells in LNs. Of these, 38 were uninfected controls and the others were chronically infected with SIVmac251 with no overt signs of disease (chronic asymptomatic; $n = 18$) or animals that became infected with SIVmac251 despite vaccination with various gag/pol/env vaccines ($n = 6$). Finally, 16 animals infected with less pathogenic simian-human immunodeficiency virus strain SHIVsf162P3 or RT-SHIVsf162P3 only were examined (Table 1). Blood from three animals was prospectively monitored at different time points after SIV infection. Blood and LNs were collected at necropsy from uninfected controls or chronically infected RMs (SIV infected for >3 months), processed into single-cell suspensions, and analyzed by flow cytometry. Numbers of animals and tissues used for individual experiments are provided in the figure legends.

Tissue collection and phenotyping. Flow cytometry for surface and intracellular staining was performed in accordance with standard protocols (18). Cells were stained with CD3 (SP34), CD4 (SK3), CD8 (SK1), CD20 (2H7), CXCR5 (MU5UBEE, eBioscience), PD-1 (EH12.2H7, BioLegend), CCR5 (3A9), and the LIVE/DEAD Fixable Aqua Dead Cell Stain kit (Invitrogen, Grand Island, NY). Isotype-matched controls were included in all experiments. All antibodies and reagents were purchased from BD Biosciences Pharmingen (San Diego, CA) unless otherwise noted. Samples were resuspended in BD Stabilizing Fixative (BD Biosciences) and acquired on a Fortessa fluorescence-activated cell sorter (Becton Dickinson, San Jose, CA). Data were analyzed with FlowJo software (Tree Star, Ashland, OR).

Multicolor confocal microscopy analysis and immunohistochemistry analysis. LNs were obtained from RMs within 30 min of necropsy. Tissues were then processed and stained as previously described (7). In brief, tissues were embedded and snap-frozen in optimum cutting temperature compound and 7- μ m frozen sections were stained with unconjugated primary antibodies, including CD3, CD4, CD20, PD-1 FDC (Dako, Carpinteria, CA), and p28 (Microbix Biosystems Inc.), followed by appropriate secondary antibodies conjugated to the fluorescent dye Alexa 488 (green), Alexa 568 (red), or Alexa 633 (blue) (Molecular Probes, Eugene, OR). Confocal microscopy was performed with a Leica TCS SP2 confocal microscope equipped with three lasers (Leica Microsystems, Exton, PA). Individual optical slices representing 0.2- μ m and 32- to 62- μ m optical slices were collected at a resolution of 512 by 512 pixels. Image version 1.63 (NIH, Bethesda, MD) and Photoshop CS5 (Adobe, San Jose, CA) were used to assign colors to the channels collected.

Detection of SIV-infected cells in LNs by *in situ* hybridization. To identify the numbers and distribution of productively infected cells in LNs of chronically SIV-infected macaques, nonradioactive *in situ* hybridization for viral RNA was performed with formalin-fixed, paraffin-embedded sections of mesenteric LNs as previously described (19). Briefly, 5- μ m sections were cut and adhered to sialinized glass slides. After deparaffinization in xylene, rehydration in phosphate-buffered saline, and antigen retrieval with steam, sections were acetylated and hybridized with digoxigenin-labeled antisense SIV riboprobes (Lofstrand Labs, Gaithersburg, MD) encompassing essentially the entire SIV genome. Labeled cells were visualized with fluorescent dye Alexa 568 (red)-conjugated sheep antidigoxigenin antibodies.

Differentiation of Tfh cells *in vitro*. Differentiation of Tfh precursors into mature GC Tfh cells was assessed by surface marker expression following cytokine stimulation *in vitro* (20, 21). To explore GC Tfh cell differentiation from CXCR5⁺ PD-1^{NEG/INT} CD4⁺ T cells, single-cell suspensions were prepared from LNs of normal animals and cells were resuspended in ice-cold sorting buffer (Miltenyi Biotec). CXCR5⁺ PD-1^{NEG/INT} CD4⁺ T cells (presumably Tfh “precursors”) were sorted, and 5×10^5 cells were cultured for 5 days at 37°C in medium containing anti-IL-4 antibody (10 μ g/ml; BD) in 1 ml/well of a 48-well plate pre-coated with anti-CD3 (10 μ g/ml) antibody and CD28 (5 μ g/ml; BD), with or without IL-6 (100 ng/ml; BD) and IL-21 (50 ng/ml; Cell Signaling Technology). Cells were harvested and stained with CD3, CD4, CXCR5, PD-1, and the LIVE/DEAD Fixable Aqua Dead Cell Stain kit (Invitrogen,

FIG 1 Distribution and colocalization of PD-1^{HIGH} CD4⁺ T/GC Tfh cells in blood and LNs of uninfected RMs. (A) Representative plots of CXCR5⁺ PD-1^{HIGH} T cells gated on CD4⁺ T cells in blood and LNs. (B to E) Colocalization of PD-1^{HIGH} CD4⁺ T cells in LNs of uninfected RMs. PD-1, green (B to E); CD3, blue (D, E); CD20, blue (B and C) or red (D); FDC, red (B, C); CD4, red (E). Note that PD-1^{HIGH} cells are specifically localized within GCs and restricted predominantly to CD4⁺ T cells. PBMC, peripheral blood mononuclear cells.

Grand Island, NY). For other experiments, Tfh precursors were sorted from LNs in chronically SIV-infected macaques and cultured in a manner similar to that described above for assessment of differentiation *in vitro*. Differentiated PD-1^{HIGH} and PD-1^{NEG/INT} CD4⁺ T cells were sorted, and SIV DNA was detected by quantitative PCR (22).

Levels of IL-6 and IFN- γ in plasma determined by microbead immunoassay. Plasma IL-6 and gamma interferon (IFN- γ) levels in macaques after SIV infection were measured by microbead immunoassay (Bio-Rad Inc., Hercules, CA, USA) according to the manufacturer's instructions. In brief, 50 μ l of diluted (1:1) plasma was incubated with specific-antibody-coupled beads. Complexes were washed and incubated with another biotinylated detection antibody and then streptavidin-phycoerythrin. The recombinant human cytokines were used to establish standard curves and maximize the sensitivity of the assay. Cytokine levels were determined with a multiplex array reader from the Luminex Instrumentation System (Bio-Plex Workstation from Bio-Rad Laboratories). Analyte concentrations were calculated with software provided by the manufacturer (Bio-Plex Manager Software).

Statistics. Statistical analyses were performed by nonparametric Mann-Whitney *t* test (two tailed) with GraphPad Prism 4.0 (GraphPad Software, San Diego, CA). Statistically significant differences are indicated, and asterisks denote *P* values (*, *P* < 0.05; **, *P* < 0.01; ***, *P* <

0.001; ****, *P* < 0.0001). The data are presented as the mean and the standard error of the mean. Correlations between samples were calculated and expressed with Spearman's coefficient of correlation.

RESULTS

CXCR5⁺ PD-1^{HIGH} follicular CD4⁺ T helper/GC Tfh cells in RMs. Tfh cells are a heterogeneous population of CD4⁺ T cells distributed in both follicular and extrafollicular regions of LNs. By flow cytometry, CXCR5⁺ CD4⁺ T cells can be found in both systemic and lymphoid tissues; however, PD-1^{HIGH} CD4⁺ CXCR5⁺ T cell subsets are found predominantly in LNs and rarely in peripheral blood. In our analysis, CXCR5⁺ CD4⁺ T cells represented $\sim 36.5\% \pm 5.9\%$ (uninfected RMs) to $\sim 72.5\% \pm 13.8\%$ (chronically infected RMs) of the PD-1^{HIGH} CD4⁺ T cells in LNs. In contrast, all LN-derived PD-1^{HIGH} CD4⁺ T cells are CXCR5 positive in normal, uninfected macaques (Fig. 1A). Further, the CXCR5^{NEG} CD4⁺ T cells (designated Tfh precursors here) isolated from LNs were mostly ($\sim 75\%$) PD-1^{NEG} with smaller proportions (25%) of PD-1^{INT} subsets. Consistent with recent studies, the total CXCR5⁺ Tfh cells are largely composed of

FIG 2 CCR5 expression and viral infection of GC Tfh cells in LNs of RMs. (A) Representative plots of CCR5 expression on PD-1^{HIGH} GC Tfh or CXCR5⁺ Tfh cells (gated on CD4⁺ T cells) in peripheral blood and LNs of uninfected RMs. (B, C) Distribution of SIV-positive cells in LNs in asymptomatic chronic infection. Note that the vast majority of SIV⁺ cells are in the paracortical region, with very few productively infected cells in the GC regions of LNs, as shown by detection of SIV p28 protein (B) and/or SIV RNA *in situ* hybridization (B and C). CD20, blue (B) or green (C); SIV p28 protein, green (B); SIV RNA, red. (D, E) Levels of SIV RNA (D, $n = 10$) and DNA (E, $n = 4$) in sorted PD-1^{NEG}, PD-1^{INT}, and PD-1^{HIGH} CD4⁺ T cells in LNs of chronically SIV-infected RMs. Note that PD-1^{INT} CD4⁺ T cells, which comprise most of the CCR5⁺-expressing cells, support higher levels of viral replication, yet PD-1^{HIGH} CD4⁺ T cells in follicular GCs contain higher levels of DNA. *, $P < 0.05$; **, $P < 0.01$ (two-tailed paired t test). PBMC, peripheral blood mononuclear cells.

PD-1^{NEG/INT} and PD-1^{HIGH} CD4⁺ T cell populations, yet these subsets are found mostly in interfollicular T cell zones and follicular GCs, respectively (9, 11). By immunohistochemistry analysis, PD-1^{HIGH} CD4⁺ T cells were localized predominantly in GCs of LNs in uninfected RMs. These cells, termed GC Tfh cells, were usually in close contact with CD20⁺ B and FDC⁺ follicular dendritic cells (FDCs) residing within GCs (Fig. 1B to E). Mature GC Tfh cells also highly coexpressed ICOS and Bcl-6 and produced IL-21, unlike PD-1^{INT} CD4⁺ T cells, which expressed intermedi-

ate levels of CXCR5 yet also produce IL-21 as previously described (9). Combined, these findings suggest that PD-1^{HIGH} GC Tfh cells represent the mature, functional Tfh cells that are specifically distributed in LN GCs.

GC Tfh cells do not express surface CCR5 but are major viral reservoirs in SIV-infected macaques. We next examined the CCR5 expression and infection status of GC Tfh cells during SIV infection. As shown in Fig. 2A, very few PD-1^{HIGH} GC Tfh cells coexpress CCR5 in uninfected animals. Similarly, CXCR5⁺ Tfh

cells also express low/undetectable levels of CCR5, consistent with another report that Tfh cells express neither CCR5 nor other alternative CXCR6 and GPR15 coreceptors in macaques (16). These findings suggest that mature Tfh cells are not major direct targets of SIV infection. However, CCR5 was found on a subset of PD-1^{INT} CD4⁺ T cells, indicating that LN CXCR5^{NEG} PD-1^{INT} CD4⁺ T cells may be direct targets of infection. We then examined productively infected cells in LNs in chronic, asymptomatic infection by both an immunohistochemistry assay for SIV p28 antigen and *in situ* hybridization for SIV RNA. Immunohistochemistry analysis showed that SIV p28⁺ cells were found predominantly in mantle T cell zones, and very few SIV p28-positive cells were observed in follicles or GCs (Fig. 2B). This was also consistent with the localization of SIV RNA-positive cells by *in situ* hybridization (Fig. 2B and C). Notably, the distribution of productively infected cells was consistent with the absence of CCR5 on the Tfh cell surface in LN GCs. To quantify levels of SIV RNA and DNA in specific GC Tfh cell subsets, three CD4⁺ T cell subsets, including PD-1^{HIGH}, PD-1^{INT}, and PD-1^{NEG}, were sorted from LNs of chronically SIV-infected macaques. Although SIV RNA and DNA were detectable in all three populations, the highest levels of viral RNA were detected in CD4⁺ PD-1^{INT} cells, followed by CD4⁺ PD-1^{HIGH} cells, and the lowest levels were found in PD-1^{NEG} cells (Fig. 2D), indicating that PD-1^{INT} CD4⁺ T cells support the highest levels of active viral replication by paired *t*-test analysis. However, SIV DNA analysis showed that PD-1^{HIGH} CD4⁺ T cells had the highest levels of viral DNA among the three subsets (Fig. 2E). Since PD-1^{HIGH} (GC Tfh) cells contained higher levels of DNA yet relatively lower levels of RNA/viral protein than PD-1^{INT} cells, this suggests that a considerable number of PD-1^{HIGH} GC Tfh cells are latently infected. Combined, this indicates that PD-1^{HIGH} GC Tfh cells are both a major source of viral replication and a major reservoir of viral persistence in SIV-infected macaques, which is consistent with studies of HIV-infected humans (23) and SIV-infected macaques (10).

Accumulation of GC Tfh cells correlates with reduction of PD-1^{NEG} CD4⁺ T cells and dysfunction of CD8⁺ T cells in LNs during persistent SIV infection. To investigate the mechanisms behind GC Tfh cell infection, we examined changes in GC Tfh cells, precursor subsets, and PD-1⁺ CD8⁺ T cells in uninfected and chronically SIV-infected animal cohorts. As shown in Fig. 3A, GC Tfh cells accumulated in chronically pathogenic SIV-infected macaques, yet there were minimal differences in GC Tfh cells among uninfected, vaccinated, and less-pathogenic-SHIV-infected animals than in controls. Interestingly, levels of both PD-1^{NEG} CD4⁺ T cells gradually declined in LNs of animals progressing to disease (chronic infection), accompanied by increased percentages of PD-1⁺ CD8⁺ T cells, which are considered to represent dysfunctional CD8⁺ T cells (24, 25). In SIV-infected macaques, plasma viral loads were maintained at high levels in asymptomatic infection, compared to the lower or undetectable levels in vaccinated or SHIV-infected animals (Fig. 3B and C). In fact, frequencies of PD-1^{HIGH} GC Tfh cells correlated negatively with PD-1^{NEG} CD4⁺ T cells and positively with dysfunctional (PD-1⁺) CD8⁺ T cells in LNs in chronically SIV-infected cohorts (Fig. 3D to F). Although cause and effect could not be determined, conceivably, persistent SIV infection drives differentiation, migration, and abnormal accumulation of GC Tfh cells, resulting in loss of PD-1^{NEG} CD4⁺ T cells. Also, dysfunctional CD8⁺ T cells may be the result of impaired host immunity, which can further

contribute to persistent viral infection, assuming that functional SIV-specific CD8⁺ T cells in vaccinated macaques or controllers effectively limit aberrant expansion of GC Tfh cells within lymphoid tissues (11). Nonetheless, these data demonstrate that there are marked imbalances in LN cellular distribution and phenotype that undoubtedly contribute to the impairment of adaptive immune responses.

Persistent SIV infection and inflammation drive differentiation and persistent infection of GC Tfh cells in LNs. As described above, mature GC Tfh cells may not be direct targets of SIV infection because of their lack of surface CCR5 expression. However, both SIV RNA and DNA were clearly detected in purified Tfh cell subsets from SIV-infected LNs. We speculated that the reductions in PD-1^{NEG/INT} CD4⁺ T cells and increased levels of infected GC Tfh cells in LNs in chronic infection may be due to direct viral infection of the former in T cell zones and subsequent differentiation and migration of fully mature, infected GC Tfh cells in GCs. We thus first investigated whether GC Tfh cells could directly develop from PD-1^{NEG/INT} CD4⁺ T cell precursors obtained from LNs of normal macaques and also whether cells from SIV-infected macaques could similarly develop into infected GC Tfh cells. First, Tfh precursors were sorted from uninfected macaques and cultured with anti-IL-4 antibody in plates with prebound anti-CD3 and CD28 for 5 days at 37°C, with or without IL-6 and IL-21. The results showed that PD-1^{HIGH} GC Tfh cells could be induced from these PD-1^{NEG/INT} Tfh precursors by IL-6 and IL-21 stimulation *in vitro*, as shown in Fig. 4A and B. Similarly, increased numbers of CXCR5⁺ CD4⁺ T cells (differentiated GC Tfh cells) were also observed (Fig. 4C and D). Finally, when Tfh precursors were sorted from LNs from chronically SIV-infected macaques, SIV DNA was indeed detected in mature (PD-1^{HIGH}) GC Tfh cells *in vitro* (Fig. 4E). Notably, levels of proinflammatory cytokines such as IL-6 and IFN- γ , which contribute to abnormal accumulation of Tfh cells and overactivity of GCs under pathological conditions (2, 21), were elevated and maintained during persistent pathogenic SIV infection (Fig. 4F). In comparison, significantly lower levels of proinflammatory cytokines in plasma are observed throughout low-pathogenicity SHIV infection (26). Combined, these data suggest that SIV⁺ GC Tfh cells may be derived from cells infected at previous stages of maturation. In addition, these findings suggest that persistent SIV infection may continuously drive Tfh precursor differentiation toward GC Tfh cells, resulting in excess accumulation, and both latent and productive infections of these GC Tfh cells in chronic infection, which, as previously shown, serve as major reservoirs of viral persistence in HIV-infected patients (15).

DISCUSSION

Tfh cells are central to the development and maturation of T cell-dependent B cell responses. Here we investigated mechanisms of accumulation and viral infection of GC Tfh cells in SIV-infected macaques. The data indicate that persistent viral replication in lymph drives accelerated Tfh differentiation, accumulation, and infection in HIV/SIV infection. This conclusion is supported by the following findings. (i) Mature GC Tfh cells lack CCR5 expression and thus are not major direct targets of SIV infection but may be infected as Tfh precursors that then migrate into GCs, where they lose CCR5 expression yet remain persistently infected, as indicated by high levels of SIV DNA. (ii) Viral persistence, accompanied by increased proinflammatory cytokines and defective (PD-1⁺) CD8⁺ T cells, drives differentiation and depletion of Tfh

FIG 3 Persistent SIV infection is accompanied by reduction of PD-1^{NEG} CD4⁺ T cells and dysfunction of CD8⁺ T cells in LNs. (A) Representative plots of GC Tfh cell differentiation, accompanied by reduced PD-1^{NEG} CD4⁺ T cells and increased PD-1 expression on CD8⁺ T cells in LNs of uninfected or chronically SIV-infected cohorts. Cells were first gated on either total CD3⁺ cells (top row) or CD4⁺ T cells (bottom row). (B) Dynamics of plasma viral loads in animals after SIV or SHIV infection. (C) Viral loads in SIV/SHIV-infected animals at the time LNs were examined for PD-1 expression on T cell subsets *, $P < 0.05$. (D, E) Correlation of GC Tfh cells with PD-1^{NEG} or PD-1^{INT} CD4⁺ T cells in LNs during viral infection. (F) Correlation of GC Tfh cells with PD-1⁺ CD8⁺ T cells in LNs during viral infection. The animals examined were uninfected ($n = 38$), vaccinated and then SIVmac infected ($n = 6$), SHIV infected ($n = 16$), or chronically infected and asymptomatic ($n = 18$).

FIG 4 Proinflammatory cytokines induced by persistent SIV infection drive differentiation and latent infection of GC Tfh cells. (A, B) Representative plots showing PD-1^{HIGH} GC Tfh and CXCR5⁺ Tfh cell differentiation from Tfh precursors (PD-1^{NEG/INT} CXCR5^{NEG} CD4⁺ T cells) *in vitro* with or without IL-6/IL-21 stimulation. (C, D) Statistical analysis of Tfh cell differentiation from LN-derived Tfh precursors. Sorted PD-1^{NEG/INT} CXCR5^{NEG} CD4⁺ T cells from LNs of uninfected animals were cultured in the presence (experimental group) or absence (control) of IL-6 and IL-21 for 5 days. Note that GC Tfh cells could develop from CXCR5⁺ PD-1^{NEG/INT} Tfh precursors and be induced to highly express CXCR5 and PD-1. (E) Detection of SIV DNA from PD-1^{NEG/INT} or GC Tfh cells after differentiation *in vitro* from Tfh precursors isolated from LNs in chronically SIV-infected macaques ($n = 3$). Data represent three independent experiments (C to E). (F) Dynamics of plasma IL-6 and IFN- γ levels in RMs after SIV infection ($n = 5$). *, $P < 0.05$ (two-tailed Mann-Whitney t test). The data in panels C to F are the mean $\pm$ the standard error of the mean.

precursors and accumulation and infection of GC Tfh cells. (iii) Tfh precursors, including those virus-infected cells, could be induced to differentiate into CXCR5⁺ PD-1^{HIGH} GC Tfh cells upon cytokine stimulation *in vitro*.

Tfh cells are crucial to multiple steps in B cell maturation within follicular GCs. The GC is the crucial niche for the optimal expansion and survival of activated T cells and for optimal humoral immune responses, including somatic hypermutation, class switch recombination, and selection of high-affinity B cells (27). Mature GC Tfh cells (PD-1^{HIGH} CD4⁺ T cells) reside predominantly in the GC in the gut-associated lymphoid tissue and other secondary lymphoid tissues (spleen, LNs), where they colocalize with FDCs and B cells, but they are not found in peripheral blood (Fig. 1). These mature GC Tfh cells are most efficient in providing help to B cells (5, 28). However, precursor cells, which are PD-1^{INT}

CD4⁺, CXCR5^{INT}, and ICOS^{INT} and produce IL-21, also have Tfh cell properties, albeit they are less efficient in providing help for B cells than are GC Tfh cells (9, 29). Perhaps the degree and balance of CXCR5 and CCR7 expression on Tfh cells determine their precise homing to and location in LNs (30). Thus, CXCR5⁺ CD4⁺ T cells in LNs represent a heterogeneous population of cells that migrate and relocate into GCs as they are activated and “mature” into functional GC Tfh cells (31–34).

Recent studies have demonstrated that lymphoid tissue compartments are the major sites of HIV replication and latency (35, 36), and the follicular CD4⁺ T cells in GCs serve not only as helper cells for humoral immune responses but also as persistent HIV/SIV reservoirs, resulting in impairment of antibody responses (37–39). These infected Tfh cells within the GC “sanctuaries” might be shielded from CTL responses, allowing them to persist in

GCs even if the plasma viral load is completely suppressed by antiretroviral therapy (16, 23, 40). In fact, the concentration of anti-HIV drugs is markedly lower in the lymphoid tissues than in blood, resulting in persistent viral replication and latent infection in these tissues (41). In this study, we show that surface CCR5 is coexpressed predominantly on PD-1^{INT} CD4⁺ T cells (but not PD-1^{HIGH} GC Tfh cells) in LNs of uninfected or SIV-infected macaques (Fig. 2A), consistent with other reports that Tfh cells do not express CCR5 or either of the known alternative SIV coreceptors (CXCR6 and GPR15) (16). These findings indicate that these Tfh precursors, but not mature Tfh cells, are direct targets of viral infection. This is supported by our data showing higher levels of SIV p28 protein and SIV RNA in this subset than in GC Tfh cells and by the distribution of SIV-infected cells localized mainly in the marginal T cell zones of LNs, with minimal SIV RNA in GCs (Fig. 2B). However, significantly higher levels of viral DNA and considerable levels of SIV RNA were detected in GC Tfh cells (PD-1^{HIGH} CD4⁺) in chronically infected animals (Fig. 2D and E). Interestingly, many GC Tfh cells were also able to produce SIV RNA, suggesting that at least the infected GC Tfh cells are activated, which is supported by their higher levels of HLA-DR and Ki-67 expression (17). These findings suggest that GC Tfh cells are major sources of both latent and productive SIV infection in SIV-infected macaques, consistent with previous studies of HIV-infected humans (23). Although naive CD4⁺ T cells are resistant to productive HIV infection *in vitro*, HIV DNA is also detected in naive CD4⁺ T cells in both viremic and suppressed individuals (42–44). Further, PD-1^{NEG} CD4⁺ T cells also contain ~70% naive and ~30% memory cells, which may explain why RNA levels were so low in the sorted PD-1^{LOW} subsets in Fig. 2D. These findings suggest that PD-1^{NEG} CD4⁺ T cell subsets could be infected by HIV/SIV and contribute to the latent reservoirs, albeit they are clearly a smaller reservoir than the other two PD-1^{INT/HIGH} cells in LNs. However, since GC Tfh cells do not coexpress CCR5 (16), we sought to address how these cells become infected. Since we used CCR5-tropic SIVmac251 in our study, GC Tfh cells were not likely to be directly infected through other coreceptors. Here we propose that infected GC Tfh cells are derived from differentiation of virus-infected precursors in the mantle T cell zones, such as CXCR5⁺ Tfh precursors, which have been shown to be infected in viremic HIV-infected subjects (23). Our *in vitro* Tfh cell differentiation experiments confirmed that highly purified Tfh precursors (sorted CXCR5⁺ PD-1^{NEG/INT} CD4⁺ T cells) from LNs were capable of differentiating into PD-1^{HIGH} GC Tfh cells and CXCR5⁺ Tfh cells after IL-6 and IL-21 stimulation (Fig. 4A to D). Further, we showed that DNA could be detected in these CD4⁺ PD-1^{HIGH} cells following differentiation from Tfh precursors in chronically SIV-infected animals (Fig. 4E). However, viral DNA levels were lower in the differentiated Tfh cells cultured *in vitro* than in Tfh precursors directly analyzed *ex vivo* (Fig. 2). Although it is speculative, this may be attributed to the dynamic and continuous levels of activation, differentiation, proliferation, or even apoptosis of GC Tfh cells *in vivo*, which may be limited or different *in vitro*. Since Tfh cells accumulate in asymptomatic chronic infection, it is unclear if Tfh cells represent a stable reservoir or one that is constantly being regenerated via proliferation, differentiation, and cell migration/motility. Recent studies indicate the viral reservoir is rapidly established during the “eclipse” phase, prior to SIV viremia (45), and this may be associated with increased activation, turnover, and differentiation of CD4⁺ T cells undergoing

proinflammatory responses at this stage. Thus, anti-inflammatory agents and perhaps inhibitors of cell differentiation could be considered in combination with antiretroviral therapy as an early intervention strategy to reduce viral reservoirs in acutely HIV-infected patients. Combined, our data suggest that virus-infected GC Tfh cells are derived mostly from Tfh precursors that coexpress CCR5 and are infected prior to entering the GC. Once these cells migrate into GCs, they fully mature into GC Tfh cells and downregulate CCR5 yet persist as latently or even productively infected cells.

Cellular and humoral immune responses coordinate to play key roles in control of infection. Effective immune responses promote the ultimate resolution of viral infection, whereas immune deficiencies prevent viral clearance, leading to persistent viral infection (7, 46). Interestingly, the gradual loss of PD-1^{NEG} CD4⁺ T cells and aberrant accumulation of Tfh cells in progressing animals are accompanied by increased proportions of dysfunctional CD8⁺ T cells in LNs, as indicated by higher expression of PD-1 on CD8⁺ T cells in chronically SIVmac-infected macaques and lower levels in vaccinated, uninfected, or SHIV-infected cohorts (Fig. 3A, D, and F). This suggests that impaired CD8⁺ T cell responses correlate with the expansion of GC Tfh cells in progressing animals (11). In addition, chronic inflammation may play a role in the expansion of GC Tfh cells. In SIV infection, proinflammatory cytokines such as IL-6 and IFN- γ are induced in acute infection and maintained throughout infection (Fig. 4F). Further, evidence indicates that excessive proinflammatory cytokines, such as IL-6, IL-21, and IFN- γ , etc., could upregulate CXCR5 expression on CD4⁺ T cells and accelerate Tfh differentiation, thus leading to abnormal accumulations of GC Tfh cells under pathological conditions (2, 21), as supported by our *in vitro* GC Tfh cell differentiation assays (Fig. 4B). In addition to inflammatory cytokines, continuous antigen presentation is able to promote Tfh cell development (34, 47, 48) and may even redirect Th1 cell differentiation toward Tfh cell development during persistent LCMV infection (38). However, it remains to be determined whether compromised cellular immunity, chronic activation, proinflammatory cytokines, or all of the above are the major drivers of pathological accumulation, infection, and turnover of GC Tfh cells in persistent HIV/SIV infection. Nonetheless, our combined data suggest that persistent SIV replication may drive abnormal differentiation, accumulation, and productive/latent infection of GC Tfh cells.

In summary, GC CD4⁺ T cells appear to represent specific subsets of Tfh cells within GCs in lymphoid tissues of RMs. Mature GC Tfh cells become infected in prior stages of differentiation outside the GC, yet they eventually migrate into GCs and become major reservoirs of viral replication and persistence residing within the sanctuary of the GC. This viral persistence may drive further Tfh differentiation, resulting in further accumulation of infected GC Tfh cells, all resulting in decreased cellular and humoral responses to infection. Better understanding of the mechanisms behind these immunologic abnormalities may lead to more effective vaccination and therapeutic strategies.

ACKNOWLEDGMENTS

We thank Julie Bruhn and Calvin Lanclos for flow cytometry support and Megan Gardner, Meagan Watkins, and Maury Duplantis for technical support.

H.X. performed and analyzed most of the experiments and wrote the

manuscript; X.W. and N.M. performed flow cytometry and confocal microscopy; P.P.A. and X.A. assisted with manuscript preparation; and H.X., A.A.L., and R.S.V. designed the experiments and edited the manuscript.

This work was supported by NIH grants U19 AI076981, R01 AI084793, R01 DE025432, and R01 AI099795 and by the National Center for Research Resources and the Office of Research Infrastructure Programs (ORIP) of the National Institutes of Health through grant OD011104-51. The funders had no role in study design, data collection and analysis, the decision to publish, or preparation of the manuscript. The authors declare no competing financial interests.

FUNDING INFORMATION

HHS | National Institutes of Health (NIH) provided funding to Huanbin Xu, Xiaolei Wang, Andrew Lackner, and Ronald S. Veazey under grant numbers U19 AI076981, R01 AI084793, R01 DE025432, R01 AI099795, and OD011104-51.

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