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Spatial Alterations between CD4⁺ T Follicular Helper, CD8⁺ T Cells during Simian Immunodeficiency Virus Infection: T/B Cell Homeostasis, Activation, and Potential Mechanism for Viral Escape

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HIV/SIV infections induce chronic immune activation with remodeling of lymphoid architecture and hypergammaglobulinemia, although the mechanisms leading to such symptoms remain to be fully elucidated. Moreover, lymph nodes have been highlighted as a predilection site for SIV escape in vivo. Following 20 rhesus macaques infected with SIVmac239 as they progress from pre-infection to acute and chronic infection, we document for the first time, to our knowledge, the local dynamics of T follicular helper (TFH) cells and CD8⁺ T cells in situ. Progression of SIV infection was accompanied by increased numbers of well-delineated follicles containing germinal centers (GCs) and TFH cells with a progressive increase in the density of programmed death-1 (PD-1) expression in lymph nodes. The rise in PD-1⁺ TFH cells was followed by a substantial accumulation of Ki67⁺ B cells within GCs. However, unlike in blood, major increases in the frequency of CD27⁺ memory B cells were observed in lymph nodes, indicating increased turnover of these cells, correlated with increases in total and SIV specific Ab levels. Of importance, compared with T cell zones, GCs seemed to exclude CD8⁺ T cells while harboring increasing numbers of CD4⁺ T cells, many of which are positive for SIVgag, providing an environment particularly beneficial for virus replication and reservoirs. Our data highlight for the first time, to our knowledge, important spatial interactions of GC cell subsets during SIV infection, the capacity of lymphoid tissues to maintain stable relative levels of circulating B cell subsets, and a potential mechanism for viral reservoirs within GCs during SIV infection. The Journal of Immunology, 2012, 188: 000–000.

The differentiation of B cells into memory B cells and their affinity maturation and differentiation into long-lived plasma cells both take place within germinal centers (GCs) of secondary lymphoid follicles. After the establishment and maintenance of GC in the presence of activated helper T cells, these T cells enhance B cell proliferation, isotype switching, affinity maturation, and differentiation into memory B cells (1, 2). GC-associated CD4⁺ helper T cells are distinguished from other CD4⁺ T cell subsets phenotypically by their expression of readily detectable levels of CXCR5, ICOS, Bcl-6, and programmed death-1 (PD-1) (3) and functionally by their synthesis of high levels of IL-21 (4). IL-21 is a common γ-chain cytokine that is critical for GC B cell survival and differentiation (5). PD-1 belongs to the family of CD28 costimulatory receptors that are generally thought to be upregulated primarily upon chronic activation. Ligation of PD-1 after cross-linking with its cognate ligands PD-L1 or PD-L2 generally delivers inhibitory regulatory signals to T cells (6, 7). Murine and human GC T cells, compared with T cells from other lymphoid tissues, express relatively high levels of PD-1 (8, 9). The GC helper T cells not only serve as helper cells for humoral immune responses, but also, due to their expression of CD4, these T follicular helper (TFH) cells may serve as targets for HIV and SIV and serve as potential viral reservoirs. Several reports have shown that at these sites, GC CD4⁺ T cells are exposed to and are highly susceptible to HIV (10, 11). The follicular dendritic cells (FDCs) are thought to trap and transfer virus particles to susceptible T cells, leading to HIV replication in GC CD4⁺ T cells (12, 13). However, the significance and/or potential role of PD-1⁺ GC-associated CD4⁺ T cells during the various phases of infection remains to be elucidated. In this study, we investigated the distribution and concentration of PD-1⁺ TFH cells in lymph nodes collected sequentially from rhesus macaques before and after SIV infection. In addition, the cellular distribution of SIV and alterations in the levels of B cell activation at various stages of infection were also studied. Our findings suggest that GC CD4⁺ T cells may not only promote B cell activation and maturation but also serve as a uniquely “protected” reservoir during chronic infection.

Materials and Methods

Animals

Twenty-seven adult Indian rhesus macaques were used in this study. All animals were maintained at the Yerkes National Primate Research Center of Emory University in accordance with the regulations of the Committee on the Care and Use of Laboratory Animal Resources. The experiments were performed in accordance with the Principles of Laboratory Animal Care as formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985). This work was supported by National Institutes of Health Grants R01AI078775 and R01AI077192; and R24016988 (to F.V.) and DRR000165 (to Yerkes National Primate Research Center) and by the Emory Center for AIDS Research.

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Abbreviations used in this article: DC, dendritic cell; FDC, follicular DC; GC, germinal center; PD-1, programmed death-1; TFH, T follicular helper.
activated by incubating with 1% Triton X-100 for 30 min, and then captured onto Maxisorp plates previously coated with 2.5 μg ConA/well. After a washing step, the wells were blocked with buffer containing 4% whey, 5% dry milk, and 0.01% Tween 20 in PBS for 1 h. Serial plasma dilutions were added to the wells containing the captured virions and incubated for 1 h. After extensive washing, biotin-conjugated goat anti-human IgG or IgM (Millipore, Billerica, MA) diluted 1:5000 in blocking buffer was added and incubated for 1 h followed by the addition of 1:1000 dilution of streptavidin–HRP (Southern Biotech, Birmingham, AL) in blocking buffer. The plate was then incubated for 1 h, washed, and 100 μl TM Blue substrate (KPL, Gaithersburg, MD) was added to each well, and the reaction was stopped by the addition of 2 N sulfuric acid and the OD450 recorded in an automated ELISA plate reader.

Immunofluorescence staining and quantitative image analysis

Inguinal lymph nodes were biopsied from SIV-naive monkeys and during acute (14 d postinfection) and chronic (112–131 d postinfection) SIV infection and embedded unfixed in OCT medium. Seven-micrometer-thick sections were fixed with 4% paraformaldehyde for 10 min, followed by washing in PBS–0.1% BSA. The sections were blocked (FcR blocker; Innova Biosciences, Richmond, CA) in PBS–0.1% BSA–0.1% Triton-X–4% donkey serum for 30 min. Subsequently, the sections were incubated with goat anti-human PD-1 (R&D Systems, Minneapolis, MN), mouse anti-human Ki67 (clone MM1; Vector, Burlingame, CA), rabbit or mouse anti-human CD20 (Thermo Scientific, Rockford, IL; or clone L26, Novocastra), mouse anti-human CD4 and CD8 (clone CD3-12; AbD Serotec, Oxford, U.K.) Abs diluted 1:20 to 1:100 in blocking buffer for 1 h. Thereafter, the sections were washed three times with blocking buffer and incubated with Alexa Fluor 488, Cy3 or Cy5 conjugated appropriate donkey anti-mouse, rat or goat Abs, respectively, diluted 1:2000 (Jackson ImmunoResearch, West Grove, PA) in blocking buffer for 30 min. Tissue sections treated in parallel with mouse and goat IgG isotype Abs were used as controls to confirm the staining specificity. Finally, the sections were washed and mounted using warmed glycerol gelatin (Sigma) containing 4 mg/ml n-propyl gallate (Fluka, Switzerland). Images were collected with an Axio Imager Z1 microscope (Zeiss) using various objectives. For quantification, the area stained with CD20 (follicle) was calculated using the AxiosVision V4.8.1.0 program (Zeiss). Positive fluorescence signal of PD-1, Ki67, and PD-L1 within follicles was measured using Image J1.43u (National Institutes of Health). PD-1/CD3 and CD3/CD8 double-labeled cells were manually counted using FluoView software 1.7 (Olympus). The scoring was performed in a blinded fashion by three independent observers.
Immunohistochemistry

In situ histochemistry was performed on 4-μm tissue sections that were dewaxed and rehydrated with ddH₂O as previously described (15). Heat-induced epitope retrieval was performed with DIVA Decloaker and then blocked with SNIPER Reagent (Biocare, Walnut Creek, CA) for 15 min. The sections were incubated with an SIVgag mAb (clone FA2; AIDS Repository Program) (16) and rabbit anti-CD4 and anti-CD8 polyclonal Abs (Abcam) for 1 h, followed by Mach2 Double-stain detection kit (Biocare Medical) for 30 min. Abs were detected by development of the chromogens (Vulcan Fast Red and Vina Green Chromogen Kit; Biocare Medical). Images were collected with the Axio Imager Z1 microscope.

FACS analysis

Inguinal lymph node cells (1 million) isolated as previously described (17) were incubated with optimized concentrations of anti-CD3–Alexa Fluor 700 (clone SP34-2), anti-CD8–Pacific blue or anti-CD8–allophycocyanin–Cy7 (clone RPA-T8), anti-CD20–allophycocyanin (clone 2H7), anti-CD21–PE–Cy5 (clone B-ly4), anti-CD27–Pacific blue (clone M-T271) (BD Biosciences), anti-rhesus CXCR5–FITC (clone 710D82.1), anti-CD4–AmCyan (clone L200) (National Institutes of Health Nonhuman Primate Reagent Resource), anti–PD-1–PE–Cy7 (clone EH12.2H7), and anti-CCR7–allophycocyanin–Cy7 (clone 3D12) (BioLegend). After washing, cells were fixed with 1% paraformaldehyde. Data were acquired on an LSRII flow cytometer (BD Biosciences) and the data analyzed using FlowJo software (version 9.2; Tree Star, Ashland, OR).

Statistical methods

All statistical analyses were performed by using GraphPad Prism (version 5.03) and GraphPad instat (version 3.10). For the comparison of two time points, the Mann–Whitney U test (two-tailed p value) and the Wilcoxon matched pairs test (two-tailed p value) were used. The level of correlation was assessed by Spearman’s rank correlation test.

Results

Follicular lymph node CD4⁺ T cells within GCs show intense expression of PD-1

To address the normal distribution of PD-1–expressing cells within lymph nodes, we first performed in situ analyses for PD-1 expression in CD20⁺ cell-rich areas, termed as follicles (Fig. 1A), within which GCs form for optimal Ag presentation to B cells in SIV-naive rhesus macaques. As expected in these animals, few GCs were seen that contained a modest number of T₁FH cells. However, even in SIV-naive monkeys, T₁FH cells appeared to express relatively high levels of PD-1 compared with low to undetectable expression of PD-1 on T cells in the paracortical areas of lymph nodes (Fig. 1B–G). Moreover, the PD-1⁺CD3⁺ T cells were not uniformly distributed and appeared clustered in one area of the GC in tissues from these animals (Fig. 1B, 1D). Similar staining patterns were seen in histological sections of the spleen (data not shown). Most PD-1⁺ T cells in follicles were positive for CD4, but not CD8 (Fig. 1H–M), suggesting that they are CD4⁺ T₁FH cells expressing high relative levels of PD-1 (3) even in healthy animals. In fact, CD8⁺ T cells seem to be essentially excluded from GCs (Fig. 1H–J).

Marked accumulation of PD-1ʰ T₁FH cells within GC during chronic SIV infection

Chronic immune activation is a hallmark of HIV/SIV infection (18, 19) characterized by increased frequencies of lymphoid follicles and GC development postinfection. However, the modulation and distribution of PD-1ʰ T₁FH cells has not formally been investigated in this context. We therefore investigated whether SIV infection induced alterations of GC-associated immune architecture, as hypergammaglobulinemia and polyclonal B cell activation are a common occurrence in HIV-1/SIV infection (20). Although a slight increase in the frequency of PD-1ʰ T₁FH cells was observed in lymph node sections during peak viremia (day 14 postinfection), the values were not significantly different from those of tissues from SIV-naive animals. However, during chronic infection (day 133 postinfection), marked differences were noted. Thus, the number of follicles containing GC and PD-1ʰ–expressing T cells was markedly increased in lymph node sections from chronically infected animals compared with that in healthy and acutely infected animals (Fig. 2A, 2B), and the number of follicular PD-1ʰ T cells positively correlated with the size of lymph node follicles from acutely and chronically SIV-infected rhesus macaques, respectively.

FIGURE 2. Immunohistological evaluation of PD-1ʰ–expressing CD3⁺ T cells in lymph node sections of a representative rhesus macaque as a function of time after SIV infection. (A and B) The staining profile of CD20⁻ (blue, Cy5), CD3⁻ (red, Cy3), and PD-1⁻ (green, Alexa Fluor 488) expressing cells within lymph node sections from macaques during acute infection (day 14) (A) and during chronic infection (day 133) (B) (montage original magnification ×20). White arrowhead indicates follicle containing GC and PD-1ʰ–expressing T cells in (A) and (B). Lymph node sections from 10 SIV-infected macaques obtained during acute infection (day 14, blue diamond) and during chronic infection (day 133, red open square) were examined for the frequency of PD-1ʰ–expressing T cells based on follicle size (mm²) as seen in (C). The correlation was assessed by Spearman’s rank correlation test. The p value <0.05 was considered statistically significant. (D) Comparative analysis of PD-1ʰ–expressing T cells in seven uninfected (UN) and the same 10 SIV-infected macaques shown in (C). Plasma viral load of the 20 SIV-infected animals used for this study is depicted in (E). Statistical analyses were performed using Mann–Whitney U test (two-tailed p value) and Wilcoxon matched pairs test (two-tailed p value). A p value <0.05 was considered statistically significant.

Please refer to the figure for detailed analysis and results.
In addition, the frequencies of PD-1+ T cells/mm² were significantly higher within lymphoid follicles from chronically SIV-infected macaques compared with those in acutely infected or SIV-naive animals (Fig. 2D, \( p = 0.0059 \)). Of note, most if not all PD-1\(^{hi} \) TFH cells enumerated from the follicles in Fig. 2C and 2D were indeed positive for CD4 (data not shown). There was no significant difference in the frequencies of PD-1\(^{hi} \)-expressing cells in sections from lymph nodes of SIV-naive and acutely infected macaques (\( p = 0.2065 \)). After i.v. infection, a typical viral load profile with a peak around week 2 was observed, followed by a decline to stable viral load set points of >10⁵ copies/ml of plasma during the chronic phase of infection (except one animal that showed spontaneous virus control) (Fig. 2E). These data suggest that PD-1\(^{hi} \) T\(_{FH} \) cells accumulate during hyperplasia of follicles induced by continuous SIV replication in the setting of an organized immune response. We also found that most follicular PD-1\(^{hi} \) T cells expressed CD4, whereas only a few follicular PD-1\(^{hi} \) T cells were CD8\(^{+} \) within GCs (data not shown).

**Confirmation of levels of PD-1 expression on T\(_{FH} \) cells during chronic SIV infection by flow cytometry**

Single-cell suspensions of CXCR5 positive/negative subsets of lymph node cells from macaques prior to and after SIV infection were analyzed for levels of PD-1 expression by T\(_{FH} \) cells in efforts to confirm the in situ data. Subsets of T cells infiltrating B cell areas of lymphoid tissues were defined by the expression of CXCR5 (21, 22) (Fig. 3A). Of note, none of the CXCR5 reagents currently available were able to detect macaque CXCR5 in situ, preventing us from verifying that GC CD4\(^{+} \) T cells all expressed CXCR5. As expected, whereas the frequency and density of PD-1 expression increased significantly on all T cells as a function of time after SIV infection (Fig. 3B–E), the values for PD-1 expression did not markedly differ between CXCR5\(^{+} \) and CXCR5\(^{-} \) subsets of CD4\(^{+} \) T cells in samples from SIV-naive monkeys (Fig. 3B–E). During acute infection, the frequency and density of PD-1–expressing cells were already significantly higher in both CXCR5\(^{+} \) and CXCR5\(^{-} \) total and CD4\(^{+} \) T cells, suggesting that overall activation of lymph node T cells had already been initiated (Fig. 3B–E, \( p < 0.01 \) for total T cells and \( p < 0.02 \) for CD4\(^{+} \) T cells). Similar differences were observed in samples collected 3 mo later, during the early chronic phase of infection. The frequencies and density of PD-1 increased during chronic infection in both CXCR5\(^{+} \) and CXCR5\(^{-} \) total and CD4\(^{+} \) T cells compared with values observed pre-infection and during the acute infection. In addition, the difference in PD-1 expression on CXCR5\(^{+} \) T cells and CD4\(^{+} \) T cells versus their CXCR5\(^{-} \) counterpart was even more prominent (Fig. 3B–E).

The high frequency of PD-1\(^{hi} \) T\(_{FH} \) cells within GC correlates with the generation of CD27\(^{+} \) memory B cells and Ab production during chronic SIV infection

**GC interactions dependent on CD4\(^{+} \) T\(_{FH} \) cells contribute to the establishment of B cell memory and effective humoral immune response**

**FIGURE 3.** Flow cytometric analysis of lymph node cells from seven SIV-naive (UN) and 20 SIV-infected rhesus macaques after acute (day 14) and chronic (day 112–133) infection. (A) The gating strategy to identify the frequency of CD3\(^{+} \), CD4\(^{+} \), CXCR5\(^{+} \) cells that express PD-1 in a representative sample on day 14 and day 112–133 postinfection. The isotype controls are also shown. (B and C) The mean frequency of PD-1–expressing CD3\(^{+} \) (total) T cells and CD3\(^{+} \)CD4\(^{+} \) T cells that are CXCR5\(^{-} \) and CXCR5\(^{+} \) in lymph node cells from seven SIV-naive (UN) and 20 SIV-infected animals after acute (day 14) and chronic (day 112–133) infection. (D and E) Mean fluorescence intensity (MFI) values obtained on the same samples. Statistical analyses were performed using Mann–Whitney \( U \) test (two-tailed \( p \) value) and Wilcoxon matched pairs test (two-tailed \( p \) value). Horizontal lines indicate medians. A \( p \) value <0.05 was considered statistically significant.
responses (21, 23), and although PD-1 has been linked to immune exhaustion, it is also a marker of T cell activation (7). We investigated the proliferative status of GC T and B cells in situ as a function of SIV infection. Ki67+ was readily detectable in GCs but absent from T cell zones within the lymph nodes (Fig. 4A, 4B), suggesting a general but not strict overlay of PD-1 and Ki67+ cells within the follicles. However, the expression of PD-1 seemed more restricted geographically than that of Ki67, suggesting that these two molecules are expressed by different cell lineages. The fact that most Ki67+ cells were also CD20+ (Fig. 4C–F) suggests that the majority of the Ki67 signal was associated with B cells in GCs rather than Tfh cells. However, there was a strong positive correlation between PD-1 and Ki67 coexpression within each follicle from healthy and SIV-infected animals ($r = 0.8454$, $p < 0.0001$), suggesting an association between Ki67+ and PD-1+ expressing cells (Fig. 4G). The frequency of naive and memory B cell subsets were also evaluated based on the expression of CD21 and CD27 (24, 25) (Fig. 5A). First, we confirmed that the relative distribution of B cells in the blood of SIV-naive rhesus macaques markedly differed from humans, comprising ~35% naive (CD21+/CD27−), 5% resting memory (CD21+/CD27+), 40% activated memory (CD21+/CD27+), and 20% tissue memory B cells (CD21−/CD27−) (Fig. 5C) similar to the values reported by Titanji et al. (25). These values did not change significantly during acute infection with slightly elevated levels of activated memory B cells in blood during chronic infection (Fig. 5C). Although the definitions of B cell subsets used for blood do not adequately apply to lymphoid tissues (26), using the same markers to analyze B cells in lymph nodes pre-infection and postinfection showed dramatic differences. In SIV-naive animals, the relative frequencies of B cell subsets differed substantially from blood (Fig. 5B), with CD21+/CD27− B cells accounting for >70% of lymph node B cells and CD21+/CD27− B cells accounting for 12 and ~5% each of CD21+/CD27− and CD21−/CD27− B cells. Upon infection, significant decreases in the frequency of lymph node CD21+/CD27− B cells was readily seen as early as during acute infection ($p = 0.0001$), which decreased further during the chronic phase ($p = 0.002$). This decrease was compensated for by significant increases in CD27+ memory B cells while the relative representation of CD21+/CD27− B cells did not change (Fig. 5B). In particular, the transition from acute to chronic phase was associated with an increase in CD21−/CD27+ B cells ($p < 0.01$), but it is uncertain whether these cells are equivalent to activated memory B cells observed in blood or plasma cells/plasmablasts that would be consistent with the observed hypergammaglobulinemia. Although a better definition of lymphoid B cells in macaques is still being assessed using new Abs specific for various B cell markers (CD19, CD38, CD77, etc.), the results have important implications for the homeostasis of B cells during SIV infection. After SIV infection, whereas two fast progressor monkeys failed to generate detectable anti-SIV Abs, the remaining normal progressors developed detectable SIV Ab titers with relatively modest changes in their frequency of peripheral blood B cells subsets (25) (Fig. 5C). However, within lymph nodes, marked B cell proliferation occurred with the observed shifts in subset distribution (Fig. 5B), likely resulting in increased B cell turnover and death as they emigrate into the peripheral circulation. Thus, in normal progressor monkeys, there appears to be a balance between the supply of new B cells and decrease of at least the activated memory B cell subsets. To determine the biological significance of the increase in memory B cell subsets within lymph nodes, we measured the levels of total IgM, IgG, and SIV-specific IgG in the plasma of the 20 animals as a function of time postinfection. As expected, the monkeys demonstrated statistically relevant increases in total plasma IgG including SIV specific IgG during the chronic stage (Fig. 5D and Supplemental Fig. 1A), which reflects the hypergammaglobulinemia characteristic of both HIV/SIV infection. IgM levels showed a non-significant decrease over the same time period (Fig. 5E). Notably, the frequency of follicular PD-1+ T cells detected in situ positively correlated with total IgG levels, but not...
with IgM (Fig. 5F, 5G). A similar but non-significant trend was seen between the frequency of PD-1+ T cells and SIV-specific Abs ($r = 0.6667$, $p = 0.0589$) (Supplemental Fig. 1B).

GC predominantly exclude CD8+ T cells, providing for a replication site and a potential reservoir and escape site for SIV in follicular CD4+ T cells

GCs induced in secondary lymph nodes are thought to be important sites of HIV/SIV replication, suggesting that activated Tfh cells could serve as viral targets throughout infection. Moreover, a recent publication has highlighted lymph nodes as a primary site for viral escape (27). As outlined in Fig. 1, whereas CD4+ T cells and B cells comprise the most abundant cell populations within GC, CD8+ T cells appear restricted to the T cell zone but largely excluded from the GC in SIV-naive monkeys. The absence of CD8+ T cells within GC was also true in lymph node sections from SIV-infected animals (Fig. 6D–F). Of interest was the finding of readily detectable SIVgag p27 staining both within GCs and T cell zones of lymph nodes collected during the chronic infection stages (Fig. 6A, 6D), as previously reported (28). These data indicate that recruitment of CD8+ T cells into GCs does not occur or is very inefficient, which is further supported by the lack of correlation between frequencies of CD8+ T cells in GC and frequencies of PD-1+ T cells (Fig. 6H), while the concurrent increase in the frequency of PD-1hi Tfh cells generates additional potential targets for the virus to replicate. Thus, whereas CD8+ T cells may control infected T cells within the T cell zone, the few CD8+ T cells present within GCs may have little impact on the control of localized infection, allowing for continuous replication, the maintenance of viral reservoirs, and perhaps also contributing to viral escape.

SIV infection promotes PD-L1–PD-1 contact within GCs, potentially negatively regulating Tfh cells

Several laboratories including ours have reported that PD-1–PD-L1 interaction limits effector functions of not only CD8 but also CD4+ T cells in vitro and likely in vivo (29–32). We therefore examined the lymphoid tissue of our animals for the presence of PD-L1+ Tfh cells. As illustrated in Fig. 7, lymph node cells from control SIV-naive monkeys exhibited PD-L1 mostly as single scattered cells throughout the node and largely outside of GCs. In contrast, lymph node cells from SIV-infected monkeys showed a markedly increased expression of PD-L1 as single scattered cells throughout the node and largely outside of GCs.
Of note, we found that whereas cells coexpressing PD-1 and CD3 presented primarily a lymphocytic morphology as expected, cells expressing PD-L1 had more of an interdigitating morphology consistent with FDC (Fig. 7C–E). Moreover, there was a strong positive correlation between the magnitude of PD-L1 and PD-1 expression within each follicle from healthy and SIV-infected animals ($r = 0.8651, p < 0.0001$) (Fig. 7F). Recent studies have shown that the PD-1–PD-L1 pathway plays a key role in the suppression of proliferation and cytokine production by CD4+ and CD8+ T cells during chronic viral infections including SIV (31, 33). The relationship between such PD-1–expressing T cells and PD-L1–expressing cells with dendritic cell (DC)-like appearance on chronic immune activation and synthesis/release of cytokines remains to be defined. It is of interest to note that even though PD-L2 can be induced on bone marrow-derived DCs in vivo (34, 35), the in situ detection of this ligand was essentially negative in lymph nodes of monkeys tested before and after infection (data not shown), suggesting that this ligand may not play a significant role within this compartment.

**Discussion**

In the current study, we examined the in vivo spatial and temporal relations of GC-associated Tfh cells, their relative expression of PD-1, and the activation of B cell subsets during early SIV infection. Although flow cytometry-based investigations provide invaluable detail as to the subset and potential function of disassociated lymph node cells, these techniques fail to provide an appreciation for the spatial distribution of these various subsets. In addition, the relative expression of receptors across cell subsets may not be readily apparent. Thus, although PD-1 upregulation was seen on follicular B cells postinfection using flow cytometry, the density was very modest compared with Tfh cells (data not shown), and therefore such upregulation was eclipsed by the far brighter expression on Tfh cells using immunofluorescence staining. However, this method provides a direct visual comparison of relative expression of molecules expressed on various cell lineages. In this regard, the in situ techniques used in the current study have allowed us to identify three important findings. First, PD-1+ Tfh cells accumulate within follicles during SIV infection. Second, the presence of PD-1+ Tfh cells has relevance to local/systemic humoral immune responses. Third, there is likely insufficient recruitment of CD8+ T cells into follicles, particularly GCs, which may contribute toward the development of viral reservoirs (27).

Recent findings have advanced our understanding of the role of PD-1+ Tfh cells, located within the GCs, both in mice and humans (4, 8, 9, 36). These Tfh cells also express high levels of CXCR5, ICOS, and Bcl-6 (3), and their PD-1 expression was selectively elevated compared with that of T cells in other areas of lymphoid tissues, suggesting that GC-associated PD-1+ CD4+ T cells form a distinct population among T cell subsets. These Tfh cells are characterized by the production of IL-21, which is a critical cytokine for GC B cell survival and differentiation (5). To our knowledge, a systematic evaluation of the changes that may be induced within GCs of lymph nodes after pathogenic lentivirus infection has not been undertaken.
infection has not yet been defined. In this report, we have focused on the study of PD-1 expression by CD4+ T<sub>FH</sub> cells and the dynamics of GC B cell activation and the effect of SIV infection. It has been previously suggested that SIV infection may in fact disrupt the adequate maturation of high-affinity memory B cells and plasma cells (37) and hence the rationale for the studies presented in this report. As expected, we identified CD4+ T cells showing relatively high levels of PD-1 expression within GCs compared with that in T cells in other areas of lymphoid tissues of SIV-naive macaques (Fig. 1). However, the extent of the changes in the expression of PD-1 in T<sub>FH</sub> cells clearly highlights the profound changes that occur within the lymphoid environment after SIV and by extension HIV infection. A significant number of PD-1<sup>hi</sup>-expressing follicular T cells accumulate within hyperplastic follicles of SIV-infected macaques during chronic infection, relative to SIV-naive or SIV-infected macaques during acute infection (Fig. 2). Of note, these changes were not correlated with viral loads, as PD-1 expression increased further during chronic infection while viral loads decreased from their peak level. This lack of correlation may be secondary to the fact that lymph nodes may not be the major source of virus production compared with, for example, the gastrointestinal tract (38, 39), a fact that is also suggested by the rapid destruction of T<sub>FH</sub> cells in the lymph nodes of rapid progressors, despite very high viral loads (Supplemental Fig. 2). We confirmed the enhanced expression of PD-1 by CXCR5<sup>+</sup>CD4<sup>+</sup> T cells after SIV infection using flow cytometry, but could not do so in situ, due to the lack of binding reagents (data not shown). CXCR5 is the chemokine receptor for CXCL13 produced by FDCs (40, 41). When CD4<sup>+</sup> T cells encounter DCs bearing Ag in the T cell zone, they transiently upregulate CXCR5 and downregulate CCR7, resulting in their infiltration into B cell follicles (42). Although one chemokine alone is not indispensable for the migration of T cells to lymphoid follicles (8), CXCR5 expression is a specific molecular marker to identify GC-homing T cells (21, 22). CXCR5<sup>+</sup>CD4<sup>+</sup> T cells showed a higher level of PD-1 expression compared with that of CXCR5<sup>-</sup> T cells, but only after SIV infection (Fig. 3). We also found a progressive increase in the levels of PD-1 expression by the CXCR5<sup>+</sup> T cell subsets as a function of time after SIV infection. Besides its role as an inhibitor of T cell response, PD-1 also plays an important role in the regulation of humoral immune responses specifically within GCs as elegantly reported by the laboratory of Dr. Shlomchik (43). Thus, mice deficient in PD-1 or its ligands presented markedly lower numbers of long-lived plasma cells (43). Of interest however was the finding that the remaining plasma cells in the absence of PD-1 regulation exhibited higher affinity for their Ag. Although it remains to be established whether such findings translate to humans and primates, the findings nevertheless provide potential insights into the mechanisms leading to the hypergammaglobulinemia commonly observed in HIV-1<sup>+</sup> patients and SIV-infected monkeys (20), as not only was PD-1 expression upregulated within GCs but also PD-1<sup>-</sup> on follicular cells with DC morphology (Fig. 7). The marked upregulation of PD-1 on T<sub>FH</sub> cells, which in our studies accompanied the upregulation of Kit67 on B cells colocalized with PD-1<sup>-</sup> T<sub>FH</sub> cells during chronic infection, may lead to activation and differentiation of increased numbers of long-lived plasma cells. We submit that such interaction likely drives the activation and differentiation of B cells leading to the diverging distribution of B cell subsets in blood (Fig. 5C) (25) and corresponding lymph nodes during SIV infection. Unfortunately, a precise phenotype of plasma cells, plasmablasts, and the various subpopulations of B cell precursors in lymph nodes of rhesus macaques is still being delineated. Thus, these populations could not be evaluated and compared at this time. However, although our studies confirmed that only minor changes occur in circulating B cell subsets in clinically stable SIV infection (25), marked changes were noted in lymph nodes, even using our relatively limited phenotypic definitions. Thus, the marked increase in CD21<sup>+</sup>/CD27<sup>+</sup> B cells at the acute/chronic stage of infection followed by CD21<sup>-</sup>/CD27<sup>-</sup> B cells at the chronic phase in lymph nodes suggest that the output of these
subsets likely increases to maintain frequencies of circulating memory B cells during normal SIV progression. In contrast, a rapid and prolonged decrease in circulating activated memory B cell has been reported in fast progressor monkeys (25), which is characteristic by lymphoid exhaustion with poor GC formation (14, 44). The functional role of PD-1 upregulation in this context remains to be determined, and our study included too few fast progressor animals (Supplemental Fig. 1A). Finally, the local expansion of follicular PD-1+ T cells correlated with the levels of circulating IgG during chronic SIV infection (Fig. 5). Thus, our findings collectively confirm that GC-associated PD-1+CD4+ helper T cells may drive B cell proliferation, while affinity maturation may be impaired due to the constant presence of high levels of Ag during SIV infection. These observations are not surprising because of recent reports that GC PD-1+ T cells from human (45) and mice (46) mainly produce IL-21, which is an important cytokine known to stimulate B cells to undergo proliferation and differentiation (47, 48). Furthermore, coculture of tonsillar CD4+, CXCR5+ T and B cells has also been shown selectively to enhance the synthesis of IgG and IgA, but not IgM production, suggesting B cell Ig class switching and/or memory formation to enhance the synthesis of IgG and IgA, but not IgM production.

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Disclosures

The authors have no financial conflicts of interest.

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References

ACTIVATION OF FOLLICULAR T AND B CELLS BY SIV


Supplemental Figure 1: (A) SIV specific antibody titers in the plasma of rhesus macaques prior to (day 0), during acute (day14) and chronic (day133) SIV infection. (B) There was a weak correlation between levels of SIV specific antibody titers and levels of PD-1^{hi} follicular T cells at day 133 pi. * indicates animals that succumbed to AIDS-related illness within 5 to 10 months.
**Supplemental Figure 2:**

**Supplemental figure 2.** PD-1$^{hi}$ expressing T cells within lymphoid follicles of a rapid disease progressor (REi8). (A to C) representative image of lymph nodes stained with CD20 (blue), CD3 (red) and PD-1 (green). (D) Serial adjacent section (10μm) showing the same lymph node (A to C) stained with Ki67 (blue).