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

Jung Joo Hong,* Praveen K. Amancha,* Kenneth Rogers,* Aftab A. Ansari,† and Francois Villinger*†

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 B 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: 3247–3256.

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 CXCVR5, 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 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

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J.J.H., P.K.A., K.R., and F.V. performed the experiments; and J.J.H., P.K.A., A.A.A., and F.V. wrote the paper.

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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.

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approved by the local institutional animal use and care as well as biosafety review boards. Twenty animals were inoculated intravenously with 200 TCID$_{50}$ (50% tissue culture infective dose) SIVmac239 and served as a source of blood and inguinal lymph nodes at various time points postinfection.

**Quantitation of SIV RNA in plasma**

Plasma SIV viral load was determined by quantitative RT-PCR by the National Institutes of Health Center for AIDS Research sponsored Virology Core Laboratory of Emory University School of Medicine (14).

**Plasma levels of IgM, IgG, and SIV-specific Abs**

The ELISA and all staining techniques were performed at room temperature. Plasma levels of total IgM and IgG were determined using commercially available ELISA kits (Life Diagnostics). To quantitate SIV-specific Abs, SIVmac239 virions were grown in serum-free media, inactivated 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 (Miliopore, 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 OD$_{450}$ 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 (F(ab’)$_{2}$ blocker; Innovex Biosciences, Richmond, CA) in PBS–0.1% BSA–0.1% Triton X–40% 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 BC/1F6 and LT8; Abcam, Cambridge, MA), and rat anti-human CD3 (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 AxioVs40 V4.8.1.0 program (Zeiss). Positive fluorescence signal of PD-1, Ki67, and PD-L1 within follicles was measured using Image OVs40 V4.8.1.0 program (Zeiss). Positive fluorescence signal of PD-1, Ki67, and PD-L1 within follicles was measured using Image J 1.43u (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 Reagent 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 7D10), 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 7D10D21), 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 TFH cells. However, even in SIV-naive monkeys, TFH 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⁺ TFH 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 T FH 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 T FH 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 T FH 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.
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-1hi 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-1hi-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-1hi TFH 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-1hi T cells expressed CD4, whereas only a few follicular PD-1hi T cells were CD8+ within GCs (data not shown).

**Confirmation of levels of PD-1 expression on TFH 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 TFH 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).

The high frequency of PD-1hi TFH cells within GC correlates with the generation of CD27+ memory B cells and Ab production during chronic SIV infection

GC interactions dependent on CD4+ TFH cells contribute to the establishment of B cell memory and effective humoral immune responses.

**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 node 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 anti-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

**FIGURE 4.** Identification of CD20+ cells that are predominantly Ki67+ in lymph node sections from SIV-infected rhesus macaques during chronic infection. (A) Lymph node section from a representative chronically infected animal was stained with anti–CD3 (red), anti–PD-1 (green), and anti–CD20 (blue). (B) The next consecutive section of the same lymph node was stained with anti–Ki67, and (C)–(E) represent the profiles seen with anti–PD-1 (C), anti–CD20 (D), and anti–Ki67 (E). (F) The merged image of (C)–(E). The boxed area in (F) is shown to highlight the expression of Ki67 by CD20+ cells. Scale bar, 50 μm. The intensity of individual images of single follicles obtained using anti–PD-1 (green) and anti–Ki67 (blue) was measured in lymph node section of SIV-naive and SIV-infected animals after acute and chronic infection. A correlation between Ki67 and PD-1 expression within follicles was determined. Data of individual follicles from the SIV-naive (red square), day 14 SIV-infected (green triangle), and day 133 SIV-infected (blue diamond) animals is shown in (G). The correlation was assessed by Spearman’s rank correlation test. The p values <0.05 were considered statistically significant.
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 ($r = 0.0002$, $p = 0.9687$, $r = 0.0002$, $p = 0.9687$, $r = 0.0002$, $p = 0.9687$, $r = 0.0002$, $p = 0.9687$, $r = 0.0002$, $p = 0.9687$). The $p$ values <0.05 were considered statistically significant.

SIV infection promotes PD-L1–PD-1 contact within GCs, potentially negatively regulating T effector 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+ follicular T cells. As illustrated in Fig. 7, lymph node cells from control SIV-naive monkeys showed a markedly enhanced expression of PD-L1 that was almost exclusively localized to GCs and often next to PD-1+ T cells.
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+ T cells and PD-L1+ 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 Bel-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-1hiCD4+ 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 yet been defined. In this report, we have focused on
the study of PD-1 expression by CD4+ TFH 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 TFH cells clearly highlights the
profound changes that occur within the lymphoid environment
after SIV and by extension HIV infection. A significant number
of PD-1hi-expressing follicular T cells accumulate within hyper-
plastic follicles of SIV-infected macaques during chronic infec-
tion, 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 TFH 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+CD4+ 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+ 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+CD4+ T cells showed a higher level of
PD-1 expression compared with that of CXCR5− 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+ T cell subsets as
a function of time after SIV infection. Besides its role as an in-
hibitor 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 re-
mains 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+ patients and SIV-infected monkeys
(20), as not only was PD-1 expression upregulated within GCs but
also PD-L1 on follicular cells with DC morphology (Fig. 7). The
marked upregulation of PD-1 on TFH cells, which in our studies
accompanied the upregulation of Kif67 on B cells colocalized with
PD-1+ TFH 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 defini-
tions. Thus, the marked increase in CD21+/CD27+ B cells at the
acute/chronic stage of infection followed by CD21−/CD27− B cells
at the chronic phase in lymph nodes suggest that the output of these

FIGURE 7. PD-L1–expressing cells are localized within GCs of lymph nodes interdigitating with CD4+PD-1hi cells. Immunohistological profile of PD-
L1– (red, Cy3), PD-1– (green, Alexa Fluor 488), and CD3– (blue, Cy5) expressing lymph node cells with a representative section from an SIV-naive animal (A) and chronically SIV-infected animal (B). F. follicle. The boxed image in (B) was magnified, and profiles of PD-L1 (C), CD3 and PD-1 (D), and merged image of PD-L1–PD-1–CD3 (E) are shown. Scale bar, 20 μm. A correlation between the intensity of PD-1 and PD-L1 expression by lymph node cells
within follicles of representative specimens obtained prior to (red squares, day 0) and during acute infection (green triangles, day 14) and chronic infection
(blue diamonds, day 133) is shown in (F). The correlation was assessed by Spearman’s rank correlation test. A p value <0.05 was considered statistically
significant.
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 characterized 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-1hi CD4+ 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 B cell activation is dependent on helper T cells (21). Therefore, our data suggest that GC PD-1hi CD4+ T cells most likely contribute to B cell proliferation in follicles and increased Ab production during chronic viral infection, a potentially important mechanism of B cell homeostasis and humoral immune responses during SIV infection.

Another level of complexity in SIV infection is the presence of virus within the follicular environment, which can also alter GC function and TFF-B cell interactions. In HIV and SIV infection, FDCs trap and stably harbor large quantities of infectious virus for many months (49–51). Furthermore, GC CD4+ T cells are highly susceptible to HIV infection in coculture with FDC (13). Consistent with a previous report (28), we found an abundance of virus within GCs (Fig. 6). However, our studies demonstrate a paucity of follicular CD8+ T cells within GCs, whereas they are abundantly represented in the T cell zone. This situation was unaltered within the follicular sites of lymph nodes during chronic infection (Fig. 6G), implying that antiviral functions exerted by follicular CD8+ T cells are predominantly lacking or very limited within this defined compartment, a finding that has recently been confirmed by Vojnov et al. (52) for SIV-specific effector CD8+ T cells. This may create an ideal environment for virus replication and reservoir formation and perhaps facilitate escape if the few effectors present in GC promote the expansion of escape mutants, as a recent report suggests that lymph nodes provide an important source of early viral escape (27). Additional studies and experiments on follicular CD8+ T cells are still required better to understand their traffic and role within lymph nodes during acute and chronic infection and the relative role of GCs versus T cell zone as the source for escape mutants.

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Disclosures

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References


