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Early Lymphoid Responses and Germinal Center Formation Correlate with Lower Viral Load Set Points and Better Prognosis of Simian Immunodeficiency Virus Infection

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We have investigated the dynamics of germinal center (GC) formation in lymphoid tissues following acute SIV infection. SIV induces a marked follicular hyperplasia, associated with an aberrant accumulation of nonproliferating T follicular helper cells within GCs, but with an abundance of cells producing IL-21, demonstrating that the mechanisms involved for these two events appear independent. IL-21–stimulated T follicular helper cells are considered a critical element for GC formation, a physiological process that seems dysregulated and excessive during HIV/SIV infection, contributing to lymphoid pathogenesis. However, the data suggest that the kinetics by which such GCs are formed may be an important predictor of the host–pathogen equilibrium, as early GC hyperplasia was associated with better control of viral replication. In contrast, monkeys undergoing fast disease progression upon infection exhibited an involution of GCs without local IL-21 production in GCs. These results provide important clues regarding GC-related hyperimmune responses in the context of disease progression within various individuals during HIV/SIV infection and may open novel therapeutic avenues to limit lymphoid dysfunction, postinfection. The Journal of Immunology, 2014, 193: 000–000.

Three decades after the discovery of HIV as the causative agent for AIDS, details of the mechanisms of pathogenesis that lead to progressive immune dysfunction observed during the course of HIV infection remain incompletely understood. Chronic immune activation has been highlighted as one of the hallmarks associated with HIV-induced disease progression that appears to be initiated during acute infection (1). As expected, such chronic processes are associated with extensive lymphoid tissue remodeling (2), most apparent in patients after prolonged infection. At earlier time points, however, several changes have been documented in lymphoid tissues, including a profound reorganization of lymphoid architecture with the development of abundant lymphoid follicles and germinal centers (GCs).

Under physiologic conditions, secondary lymphoid organs (e.g., lymph node, spleen, and GALT) comprise spherical aggregates of small lymphocytes, termed primary follicles, which contain numerous immunologically naive B cells. In response to foreign Ags, selected follicles become activated and develop GCs, highly dynamic structures that are surrounded by small naive B cells in the marginal area of the follicles. GCs are mainly composed of CD4+ T cells and B cells, which are communicating amid a network of follicular dendritic cells (FDCs). In terms of host defense against infection, GCs are important sites for the development of adaptive immune responses, including Ag-driven B cell proliferation, isotype switching, affinity maturation, and generation of memory B cells and plasma cells upon encounter with soluble and/or pathogen-associated Ags (3, 4).

Chronic infection with pathogens such as HIV/SIV induces remodeling of the lymphoid architecture throughout the course of infection. Four patterns of structural alteration are reported: follicular hyperplasia, follicular involution, lymphoid depletion of cortex and paracortex, and granulomatous lymphadenitis (e.g., during Mycobacterium infection), any combination of which may be present in an individual case (5). Recently, we demonstrated the dynamics of T and B cell interactions within GCs in hyperplastic follicles, and particularly the expansion of T follicular helper cells (Tfh) (PD-1high CD4+ T cells) during chronic SIV infection (6). These results were confirmed by others in lymph nodes of SIV-infected nonhuman primates (7) and HIV-infected patients (8). In fact, our previous data suggest that the expansion of follicular PD-1high CD4+ T cells positively correlates with the accumulation of proliferating B cells, reflecting follicle size within lymph nodes (6), together with Tfh cell–dependent induction of GCs during chronic HIV/SIV infection. However, these studies did not address the mechanisms by which follicular hyperplasia and involution are regulated by GC Tfh cells and/or the mechanisms that regulate Tfh cells within the GCs during the course of infection.

Herein we define the dynamics of GCs and GC-related immune responses in various lymphoid tissues of rhesus macaques (RM) with a focus on the activation of GC-related T and B cells and its association with the expression of IL-21, which in concert contribute to hyperimmune responses within GCs, during follicular hyperplasia or/and involution of lymph node.

Materials and Methods

Animals and samples

A total of 25 adult Indian RMs were used in this study, including 3 uninfected RMs and 22 SIV-infected RMs. All animals were maintained at the...
Yerkes National Primate Research Center of Emory University (Atlanta, GA) in accordance with the regulations of the Committee on the Care and Use of Laboratory Animal Resources. The experiments were approved by the local institutional animal use and care as well as biosafety review committees. A total of 19 macaques were inoculated with 200 median tissue culture-infective dose SIVmac239, and 3 macaques were inoculated with 250 median tissue culture-infective dose SIVmac239 i.v. Peripheral blood samples were obtained concurrent with the times the biopsy samples were obtained. PBMCs were isolated from EDTA-anticoagulated blood by ficoll-hypaque gradient. Plasma viral RNA load was examined in the corresponding blood samples. Samples from inguinal lymph node biopsies were collected at various time points, as previously described (6). Lymphoid tissues, such as inguinal lymph node, spleen, jejunum, ileum, and colon, were collected immediately after euthanasia from all animals for in situ techniques and for flow cytometric analysis. Tissue samples were fixed by immersion in freshly prepared 4% paraformaldehyde and embedded in paraffin. Selected samples were also frozen unfixed in Tissue-Tek cryo-embedding medium (Fisher Scientific) immediately upon removal, and stored at −80°C until use. Lymph node cells were collected by mincing the tissues and passing the cells through 40-μm nylon cell strainers (Fisher Scientific) with a 10-ml syringe plunger. All isolated cells were > 90% viable by trypan blue exclusion.

Ethics statement
All animals were born and maintained at the Yerkes National Primate Research Center of Emory University in accordance with the rules and regulations of the Committee on the Care and Use of Laboratory Animal Resources and according to the guidelines of the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council and the Department of Health and Human Services guidelines titled Guide for the Care and Use of Laboratory Animals. The animals were fed Purina (supplemented daily with fresh fruits or vegetables and water ad libitum. Additional social enrichment, including the delivery of appropriate safe toys, was provided and overseen by the Yerkes enrichment staff, and animal health was monitored daily and recorded by the animal care staff and veterinary personnel, available 24 hours, 7 days a week. Monkeys were caged in socially compatible same-sex pairs to facilitate social enhancement and well-being. Monkeys showing signs of sustained weight loss, disease, or distress were subject to clinical diagnosis based on symptoms and then provided standard dietary supplementation, analgesics, and/or chemotherapy. Monkeys whose symptoms could not be alleviated using standard dietary supplementation, analgesics, and/or chemotherapy were humanely euthanized using an overdose of barbiturates according to the guidelines of the American Veterinary Medical Association. The studies reported herein were reviewed and approved by the Emory University Institutional Animal Care and Use Committee. The Yerkes National Primate Research Center has been fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International since 1985.

Quantitation of SIV RNA in plasma
The viral load in plasma was determined by quantitative RT-PCR by the Center for AIDS Research Virology Core Laboratory of Emory University School of Medicine, as previously described (9).

Immunofluorescence staining and quantitative image analysis
Immunofluorescence staining was performed on serial sections for CD4, CD20, Ki67, PD-1, IL-21, and Hoechst dye, using a modified protocol previously described (6). In brief, 4-μm mouse paraffin-embedded tissue sections were deparaffinized and rehydrated. Heat-induced epitope retrieval was performed with a Dima Decloaker and then the tissue was blocked with the Snaper reagent (Biocare) for 15 min followed by PBS/0.1% Triton X-100/4% donkey serum for 30 min at room temperature. Subsequently, the sections were incubated with optimized concentrations of goat anti-human PD-1 (polyclonal Ab; R&D Systems), mouse anti-human Ki67 (clone MM1; Vector), rabbit anti-human CD20 (polyclonal Ab; Thermo Scientific), mouse anti-human CD4 (clone BC/1F6; Abcam), IL-21 (polyclonal Ab; AbD Serotec), and rat anti-human CD3 (clone CD3-12; AbD Serotec) Abs diluted 1:20 to 1:100 in blocking buffer for 1 h at room temperature. Thereafter, the sections were washed and incubated with secondary Abs (Alexa Fluor 488/Cy3/Cy5–conjugated appropriate donkey anti-mouse/rabbit/ goat Abs) diluted 1:1000 (Jackson Immuno-technology). Blocking buffer for 30 min (PBS); Sections were stained with Hoechst 33342 (Invitrogen) diluted in water to 1 μg/ml for 10 min at room temperature, rinsed, and mounted in warmed glycerol gelatin (Sigma-Aldrich) containing 4 mg/ml n-propyl gallate (Fluka). Between each step, the sections were washed three times with blocking buffer. The quantitative image analyses were performed as described previously (6, 10). Immunohistology was evaluated in a blinded fashion by two independent observers. Digital 8-24 images were randomly collected from each section with an Axio Imager Z1 microscope (Zeiss) at ×100 or ×200 magnification. The size of the area and the profile data for the intensity plots were calculated using the AxioStitch V4.8.10 program (Zeiss) and Image J 1.43m (National Institutes of Health). Labeled cells were manually counted using FlowJo software 1.7.1 (Olympus). T cell, B cell, medulla, and follicular B cell zones were determined based on CD20 and CD3 staining. The GC size was based on the definition of a less intensely stained area than marginal B cells focusing on the Hoechst nuclei stain. The GC area was also confirmed on the basis of accumulation of Ki67+ B cells (6, 10).

Polychromatic flow cytometry analysis
Flow cytometry was performed as described previously (6). Isolated cells were incubated with predetermined optimized concentrations of anti-CD3–Alexa Fluor 700 (clone SP34-2; BD Pharmingen), anti-CD8–Pacific Blue or PE-C594 (clone RPA-T8; BD Pharmingen), anti-CD20–APC–Cy7 (clone l27; BD Pharmingen), anti-CCRX5–PE (Clone M5U5/BEE; eBioscience), anti-CD200–APC–Cy7 (clone 1261; BD Pharmingen), mouse anti-human CD4 (clone BC/1F6; Abcam), human anti-Ki67 (clone MM1; Vector), rabbit anti-human CD20 (polyclonal Ab; Thermo Scientific), and rat anti-human CD3 (clone CD3-12; AbD Serotec) Abs diluted 1:20 to 1:100 in blocking buffer for 1 h at room temperature. Thereafter, the sections were stained for specific markers. Staining was performed first for LIVE/DEAD fluorochrome (Molecular Probes) and LIVE/DEAD dye–Alexa 430 (Invitrogen). After staining the cell surface, cells were permeabilized and fixed by BD Perm Wash (BD Pharmingen) and washed twice. Finally, cells were incubated with anti-Ki67–FITC (B56; BD Pharmingen), washed twice, and diluted in 1% paraformaldehyde. Data were acquired on an LSR II flow cytometer (BD Biosciences) and the data analyzed using FlowJo software (version 9.2 TreeStar).

Preparation of SIVmac239 for use in ELISA
Con A was dissolved in 10 mM HEPES buffer with 0.15 M NaCl, 1 mM CaCl2, 1 mM MgCl2, pH adjusted to 7.5, and sterile filtered, and aliquots were stored at 4°C. A stock of SIVmac239 was grown in day 3 Con A (10 μg/ml) activated rhesus PBMCs in serum-free medium. The medium was pooled and stored to contain 7.5 ng/ml of p27.

ELISA assay
SIV-specific Abs to detergent-disrupted SIVmac239 viral proteins were determined in a Con A ELISA. SIVmac239 viral proteins were disrupted by incubation with Sigma-Alpha-X1 (Sigma) with 1% Triton X-100 on ice for 1 h at room temperature. The disrupted viral proteins were then captured on Nunc Maxisorp microtiter plates previously coated with 2.5 μg Con A per well, for 1 h at room temperature. After a washing step with PBS containing 0.05% Tween 20, all the wells were blocked by the addition of buffer containing 4% whey, 5% dry milk, 0.1% Tween 20 in PBS for 1 h at room temperature. After the wash step, plasma samples to be assayed were diluted 1:1000 in 1% Tween 20 in PBS for 1 h at room temperature. After a final washing step, all wells were incubated with 100 μl TX Blue substrate (KPL); the reaction was stopped by the addition of 50 μl 2N sulfuric acid to each well, and the wells were read at an OD of 450 nm (OD450), using an automated ELISA plate reader. Controls consisted of wells coated with Goat anti-mouse IgG, IgA, and IgM (Rockland #617-101-130) at 2 μg/ml. After blocking and washing as above, known amounts of purified monkey IgL (Life Diagnostics) was added in 4-fold serial dilutions. The SIV-specific Ab was determined not as an absolute value but relative to the standards used.

Analysis of SIVgag proliferative and cytokine responses
Cell proliferation assay and intracellular cytokine staining (ICS) were performed as described in our previous study (11). Briefly, to measure Ag-specific proliferation, the cells were labeled with 3 μM CFSE and incubated at 37°C for 5 min; then excess CFSE was washed away with RPMI 10 medium containing 10% FBS. The labeled cells were then incubated in 24-well plates in the presence or absence of a peptide pool spanning the entire SIV gag (1 μg/ml) for 6 d, after which the cells were harvested and stained for specific markers. Staining was performed first for LIVE/DEAD marker (Alexa 430; Invitrogen A10109) and, after washing, incubated with a mixture of Alexa Fluor 700–conjugated anti-CD3 (clone SP34-2; BD), peridinin–chlorophyll protein–conjugated anti-CD4 (clone L200; BD), and
BD Horizon V450-conjugated anti-CD8 (clone RPA-T8; BD). For the ICS assay, 1 × 10⁶ PBMCs were stimulated with the same pool of SIV gag peptides (1 μg/ml) for 6 h in the presence of brefeldin A (10 μg/ml). The cells were then stained with LIVE/DEAD marker (Alexa 430; Invitrogen A10169) anti-CD3, anti-CD4, and anti-CD8. Following permeabilization, cells were then stained with a mixture containing predetermined optimum amounts of anti–IFN-γ (clone B27; BD), anti–TNF-α (clone Mab11; BD), and MIP-1β (clone D21-1351; BD). Finally, the stained cells were acquired on an LSR II cytometer driven by the FACSDiva software. Analysis of the acquired data was performed using FlowJo software (TreeStar version 9.2). For detecting the single, dual, and triple cytokine producers, Boolean gating strategy was followed.

**Statistical methods**

All analyses were performed 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 the Spearman rank correlation test. A p value < 0.05 was considered statistically significant.

**Results**

The density and size of GCs reflect the level of immune activation in lymphoid tissues of normal uninfected RMs

As found with other species (3), GCs in RMs are clearly identifiable within lymphoid follicles of lymph nodes, spleen, and mucosal lymphoid aggregates based on their typical architecture. The cell types within the GC are predominantly large B cells positive for the proliferation marker Ki67 (which sharply contrasts with the smaller mostly Ki67− marginal zone B cells located within the follicular mantle area around GCs) and substantial populations of PD-1high CD4+ T cells and FDCs (Supplemental Fig. 1) (6). In a previous study (10), GC areas containing proliferating (Ki67+) B cells were measured using Hoechst staining of nuclei, which show markedly less intense staining than the smaller marginal B cells. The less intense Hoechst-stained nuclei correlated with the expression of Ki67 on CD20+ B cells within the center of GCs in lymph nodes, spleen, and gastrointestinal lymphoid tissue (GALT; Fig. 1, Supplemental Fig. 2). These findings suggest that the frequency of cells expressing Ki67 within lymphoid follicles represents a parameter by which the size of GCs may be evaluated, including both the dark and light zones. Anatomical features of GCs within the spleen and GALT appeared generally similar to those found in lymph nodes (Fig. 1, Supplemental Fig. 2). As expected, a positive correlation was found between the size of GCs relative to the entire lymphoid follicle area and the magnitude of proliferating cells within follicles of all lymphoid tissues, as a measure of the expansion of GC B cells (Fig. 2).

Follicular hyperplasia and GCs markedly expand during chronic SIV infection, but accumulated Tfh cells stop proliferating

A total of 14 SIV-infected monkeys were followed up during both the acute and early chronic infection period. Lymph nodes were collected from all monkeys at 14 d (acute) and at 112–133 d (early chronic) following SIV infection and analyzed for the presence of GCs and changes in lymphoid architecture. This immunohistochemical analysis was performed on sections from inguinal lymph node biopsies, using Abs against Ki67, PD-1, CD20, and Hoechst dye. The clinical profile and disease course of 12 of the 14 monkeys followed a standard normal progressor course characterized by clearly detectable humoral and cellular responses to SIV (11). However, differences in the kinetics of lymphoid reorganization were observed in this group. Thus, lymph node sections from 4 of these 12 normal progressor monkeys showed evidence of follicular hyperplasia as early as 14 d postinfection in response to the extensive replication of SIV (Fig. 3B). Lymph nodes from each of these same 12 normal progressor monkeys all showed follicular hyperplasia by 112–133 d postinfection, with a trend toward larger GCs and more abundant Ki67+ staining within follicles relative to day 14 postinfection (Fig. 3B, 3C). In contrast, lymph nodes from the two fast progressor animals displayed no evidence of follicular hyperplasia, during either the acute or the chronic stage, as well as poor GC size and organization (Fig. 3B). These animals displayed little, if any, anti-SIV Ab responses (Fig. 4D) and succumbed to AIDS at 3 and 10 mo postinfection, whereas normal progressors developed AIDS between 1 and 2.5 y postinfection. We next evaluated the distribution and quantity of Tfh cells in animals with follicular hyperplasia, as previously defined by flow cytometry and in situ techniques (6, 7, 12). Most PD-1high CD4+ T cells were of the memory, and predominantly the central memory, phenotype, based on the expression of CD28 and CD95 (data not shown). As expected, lymph node sections from all 12 animals displayed significantly greater frequencies and densities of Tfh cells within GCs at 112–133 d postinfection relative to corresponding sections of lymph nodes collected at 14 d postinfection (Fig. 3E). However, the frequency of proliferating Ki67+ Tfh cells at 112–133 d postinfection was significantly decreased compared with 14 d postinfection when analyzed by flow cytometry (p = 0.0015) (Fig. 3F). In contrast, the density of proliferating Ki67+ Tfh cells per cubic millimeter within follicles was not decreased, but slightly increased, albeit not significantly, when measured by in situ analyses (Fig. 3F). These data suggest that differences exist in the identification of Tfh by these two methods. The mean fluorescence intensity (MFI) of Ki67 expression by Tfh cells did not correlate with their frequency (r = 0.1437, p = 0.4657) (Fig. 3G), suggesting that although increased numbers of Tfh are recruited to hyperplastic follicles during chronic SIV infection, the relative number of dividing Tfh appears to decrease.

Plasma viral loads are statistically lower in macaques with early hyperplasia during chronic infection, but B cell immune parameters and SIV-specific Ab titers are comparable between groups with and without early lymphoid hyperplasia

To investigate whether early lymphoid hyperplasia was beneficial or detrimental to the infected host, immune parameters and viral loads were compared. Plasma SIV RNA levels at 2 wk postinfection showed peak viral loads between 1.7 × 10⁵ and 3.6 × 10⁶ in the normal progressors with early hyperplasia, 1.4 × 10⁵ and 3 × 10⁵ in the normal progressors with late hyperplasia, and 3.1 × 10⁵ and 2.3 × 10⁷ in the fast progressors (Fig. 4A). These data suggest that the early hyperplasia was not likely to be a result of higher levels of viremia during acute infection. However, the monkeys displaying early lymphoid hyperplasia tended to have lower plasma viral load set points during the early chronic phase, and this difference reached statistical significance from 8 wk postinfection onward, although plasma viral loads in this group of monkeys were still above 1 × 10⁵ viral RNA copies per milliliter (Fig. 4A). Next, we examined whether a correlation exists between the viral loads and the generation of memory (CD27+ CD21+ CD20+) B cells and the production of SIV-specific Ab in longitudinal peripheral blood samples from macaques with early and/or late hyperplasia. PBMCs from all the normal progressor macaques showed a similar increase in the frequency of circulating memory B cells from 4 wk postinfection (Fig. 4B), the frequencies of Ki67+ expression on memory B cells by 3 wk postinfection (Fig. 4C), and SIV-specific Ab titers by 8 wk postinfection. There did not appear to be any detectable difference in these values in samples from macaques with early and those with
late hyperplasia. In contrast, PBMCs from fast progressor monkeys failed to show a similar increase in the levels of memory B cells seen in the other two groups of monkeys that developed lymphoid hyperplasia. This failure in memory B cell generation correlated with lower levels of SIV-specific Ab responses (Fig. 4B–D). In addition, determinations of SIV-specific T cell responses were performed on PBMCs isolated from the blood of the same cohort of monkeys at 12 wk postinfection, as previously described (11). Overall, the levels of SIVgag-specific CD4+ and CD8+ T cell proliferative responses, as measured by CFSE, and cytokine synthesis by ICS, were higher in cells from animals with early hyperplasia, compared with those with late hyperplasia, in-

**FIGURE 1.** Representative GC responses in lymphoid tissues. Immunohistological profile of Hoechst, CD20+, CD3+, and Ki67+ cells within lymph node and spleen sections from SIV-naive RMs. The sections were stained with Hoechst dye for cell nuclei, anti-CD20 for B cell aggregates (lymphoid follicles), anti-CD3 for T cells, and anti-Ki67 for proliferating cells. Active follicles (B) in lymphoid tissues of RMs include GCs that can be anatomically demarcated by Hoechst (less intensely stained), follicular CD3+ cells, and Ki67+ B cells (intensely accumulated). Inactive follicles shown in (A) do not contain GCs. Scale bars = 50 μm.

**FIGURE 2.** Increased Ki67 expression in follicles in the lymphoid tissues of normal RMs correlates with GC size. Correlations between Ki67 expression in follicles and GC size relative to follicles in lymph nodes (A), spleen (B), jejunum (C), ileum (D), and colon (E) were shown. The data represent samples from three uninfected RMs. The correlation was assessed by the Spearman rank correlation test. A p value < 0.05 was considered statistically significant.
including multifunctional CD4 and CD8 T cell responses, although the differences did not reach statistical significance (Fig. 5). Nevertheless, the observed differences in the levels of SIV-specific cellular immune responses suggest that there may be a beneficial effect of early lymphoid responses for the outcome of pathogenic SIV infection.

**IL-21 expression in lymphoid tissues**

To investigate potential mechanisms underlying the discordant activation of resident GC Tfh cells during follicular hyperplasia, we investigated IL-21 production within various lymphoid tissue compartments during acute and early chronic infection. IL-21 is a pleiotropic cytokine that regulates the function of a variety of cell lineages but is clearly essential for the formation of GCs within secondary lymphoid follicles (3). Sections from lymph node biopsies were incubated with anti-CD20, -CD3, and –IL-21 Abs, followed by fluorochrome-conjugated secondary Abs (Fig. 6). As illustrated in Fig. 6A, 6B, IL-21 was abundantly expressed in the secondary follicles within GCs and the medulla of lymph nodes of macaques relative to primary follicles and the paracortical T cell zones. Of note, the cells producing IL-21 predominantly appear along the GC periphery, suggesting functional heterogeneity of Tfh cells, depending on their location within GCs. A gradual and significant increase in IL-21–expressing cells was seen in follicles throughout the course of infection (Fig. 6C, 4.0 ± 0.7% in acute infection and 7.8 ± 1.1% in early chronic infection, *p < 0.05).
suggesting that IL-21 expression is associated with follicular hyperplasia during chronic SIV infection. The expression of IL-21 within the paracortical T cell zones was relatively low, compared with other lymph node compartments, and did not vary significantly during chronic infection (Fig. 6C, 2.3 ± 0.3% in acute infection and 2.6 ± 0.6% in early chronic infection). In the lymph node medulla, a diffuse pattern of IL-21 expression was noted, similar to secondary follicles with GCs; but unlike GCs, IL-21 expression in the medulla did not vary significantly during chronic SIV infection (Fig. 6C, 8.5 ± 0.9% in acute infection and 9.6 ± 0.8% in early chronic infection). In SIV-naive macaques, IL-21 was also abundantly expressed within secondary follicles with GCs and the medulla of the lymph node, relative to primary follicles and the paracortical T cell zone, similar to findings in samples studied during acute infection (3.8 ± 2.7% in follicles, 1.1 ± 0.2% in the T cell zone, and 8.0 ± 2.0% in the medulla).

The expansion of GCs in hyperplastic follicles is associated with delayed disease progression in SIV+ animals

In our SIV model, lymph node samples from the two fast progressor animals displayed no follicular hyperplasia and poor GC development, compared with normal progressors. Therefore, a key question is whether the Tfh cell expansion seen in large GCs of hyperplastic follicles is beneficial for or detrimental to the control of virus replication and pathogenesis following SIV infection because these cells also serve as viral targets. Samples from additional fast progressor monkeys were tested (n = 8, time to clinical AIDS between 76 and 311 d postinfection, without evidence of seroconversion to SIV) and compared with the 12 normal progressors with hyperplastic follicles (time to clinical AIDS from 385 to 881 d postinfection). All fast progressor monkeys exhibited poor GC development, concomitant with a paucity of Ki67+ B cells within follicles. In contrast, as indicated above, normal progressors had expanded GCs and a dramatic accumulation of Ki67+ B cells within follicles. As expected, the absence of GCs was coincident with a lack of IL-21 expression within follicles (Fig. 7Q, IL-21 expression: 2.9 ± 2.0% versus 16.9 ± 10.6%; *p < 0.05, fast versus normal progressors). As a consequence of the former correlation, a positive correlation was observed between plasma viral load set points and GC size at the early chronic time point, when considering all animals (normal progressors plus fast progressors), whereas no correlation was observed within the groups of animals (Supplemental Fig. 3A, r = 0.2697 and p = 0.3912 in normal progressors, r = −0.1172 and p = 0.7756 in fast progressors, and r = −0.5867 and p = 0.3912 in total animals). As a consequence of the former correlation, a positive correlation was also observed between disease progression and GC size at the chronic time point, when combining all monkeys (Supplemental Fig. 3B, r = −0.5867 and p = 0.3912 in total animals). However, although such correlation was valid for fast progressor monkeys alone, it was not significant when normal progressor monkeys were evaluated by themselves, suggesting higher heterogeneity in this group (Supplemental Fig. 3B, r = −0.1172 and p = 0.7756 for fast progressors, and r = −0.2697 and p = 0.3912 for normal progressors).

Discussion

After HIV/SIV infection, four different patterns of alterations of lymph node architecture appear to develop. These include 1) follicular hyperplasia, 2) follicular involution, 3) diffuse follicular/paracortical depletion, and 4) granulomatous lymphadenitis (5). These alterations were highlighted by follicular hyperplasia that was associated with exaggerated GCs and GC immune responses. The SIV-infected RM model provides unique insights into our ability to investigate GC dynamics during acute and chronic SIV infection. Previously, we reported that the accumulation of Tfh cells within hyperplastic follicles is associated with enhanced humoral immune responses (6). However, Tfh cells can be infected by HIV/SIV, and thus potentially serve as a long-lived viral reservoir (7, 8), and also become functionally impaired. These Tfh cells express high levels of PD-L1 in association with the expression of PD-L1 on FDCs (6). A key issue raised in this article is whether the expansion of Tfh cells noted within large GCs of hyperplastic follicles is beneficial for or detrimental to the control
of SIV replication and disease progression. Therefore, we investigated the dynamic relationships between plasma viral loads and GC scores (GC size, Ki67 expression, and density of Tfh cells in follicles) from sequential lymph node biopsies of 14 animals chronically infected with SIVmac239. The results of such analyses showed several observations and associations. RMs with follicular hyperplasia during acute (n = 4) and early chronic infection (n = 12) had larger GCs than those without follicular hyperplasia, as expected. Of interest, the four monkeys that exhibited lymphoid hyperplasia already at the 2-wk postinfection time point collectively presented better cellular antiviral immune responses and a log lower plasma viral load set point throughout infection relative to the other monkeys (Figs. 4, 5), suggesting a potential benefit of early immune responses in vivo. However, no significant difference of virus-specific humoral immune responses was noted between early and late hyperplasia, which may be potentially relevant to GC development. Therefore, a definite causative link for such association is suggested and will require further investigation.

Another novel finding was the observation that although Tfh cells markedly expanded during follicular hyperplasia, the majority of these cells during the chronic stage of infection no longer proliferate based on their expression of Ki67. Thus, although flow cytometry and immunohistochemical analyses concurred in outlining increased frequencies and density of Tfh cells during chronic infection, there was a parallel decrease in the frequency of Ki67

**FIGURE 6.** IL-21 expression is increased in hyperplastic follicles during SIV infection. (A) Representative IL-21 expression in primary and secondary follicles, T cell zone, and medulla in lymph nodes. Scale bars, 50 μm. (B) Intensity profile along the yellow line between A and B in the montage image of lymph node. (C) The expression of IL-21 was significantly increased in the follicles during early chronic infection (n = 14), compared with those during acute infection (n = 14). T cell zone and medulla of lymphoid tissues showed no difference. IL-21 expression was measured as the ratio of fluorescent pixels within lymph node compartments. *p < 0.05.
expression by these Tfh cells, with <10% of such cells expressing Ki67 (Fig. 3F). Moreover, a lack of correlation was noted between the intensity of Ki67 expressed by Tfh cells and the frequencies of this lineage during infection (Fig. 3G). In situ data also demonstrated little to no increase in the density of Ki67+ PD-1<sup>high</sup> cells within GC follicles during the same chronic time points with the accumulation of GC PD-1<sup>high</sup> cells (Fig. 3F). This discrepancy was not a result of an overall diminution of immune activation because in contrast to Tfh cells, there was also an increase in GC B cells, with the majority of these cells positive for Ki67. In fact, a direct correlation was observed between the level of GC Ki67 expression and GC size in the follicles of these animals that appeared to be associated with the expression of Ki67 almost exclusively by B cells (Fig. 3) (6). This finding raises a number of interesting hypotheses regarding the regulation of Tfh in the context of HIV/SIV infection. These cells express the highest density of PD-1 among T cell lineages tested (Supplemental Fig. 1) (13). In a previous report, we showed that PD-L1, but not PD-L2, was abundantly expressed by FDCs in GCs of SIV-infected RMs (6). A negative regulation of GC PD-1<sup>high</sup> T cell function by PD-L1-expressing GC B cells has also recently been reported by Cubas et al. (14). Their in vitro coculture assay demonstrated that PD-L1 engagement led to a decreased level of proliferation and survival and a decreased synthesis of cytokines by Tfh cells (14).

These current findings suggest that the proliferation and potentially select functions of Tfh may be markedly suppressed by this regulatory mechanism in vivo as well. The precise level of such functional inhibition remains to be delineated, as humoral responses are clearly upregulated during HIV/SIV infection, leading to higher overall circulating levels of IgG (6). It is nevertheless possible that Tfh dysfunction may affect B cell differentiation and maturation, leading to the release of plasma cells producing Abs of lower affinity, suboptimal class switching, and so on. In that regard, the role of IL-21, a common γ-chain signaling cytokine, has gained prominence in
its role in promoting the reorganization of lymphoid architecture and humoral immunity via enhancement of class switch recombination and differentiation into memory B cells and plasma cells (15). In HIV-infected individuals, circulating IL-21 has been reported to be lower than in HIV-seronegative individuals, and IL-21 production progressively decreases following infection (16). Our in situ data support this finding whereby the majority of IL-21–producing cells are seen to surround GCs (Fig. 6). It will be of interest to understand how such cells position themselves within the lymphoid follicles. Of note, the studies reported herein (Figs. 4, 5) did not use in vitro activation to delineate the frequency of IL-21–producing cells but showed the expression of IL-21 by cells in vivo, which, to our knowledge, has not been previously reported in the context of HIV or SIV infection. Indeed, recent findings reported that IL-21+ CD4+ T cells were significantly depleted in both blood and rectal mucosa, consistent with a decrease in IL-21 expression in the colon of RMs chronically infected with SIV (17), and immunotherapy with recombinant IL-21 improved mucosal immune function in the same model (18). However, our data show that in lymph nodes undergoing follicular hyperplasia during infection, there is a clear increase in IL-21–producing cells in vivo, suggesting perhaps altered trafficking of such cells during chronic lentivirus-induced immune activation. In contrast, Shi et al. (19) reported increased numbers of IL-21–producing CD4+ T cells in blood during primary SIV infection of RMs. Several findings within this context need to be addressed. First, a unique population of PD-1hiCD44hi CD4+ T cells with a Tfh cell phenotype capable of secreting IL-21 has been reported to be present within secondary lymphoid tissues, but is nearly absent in the peripheral blood (Supplemental Fig. 1) (13). Second, during HIV and SIV infection, an expansion of Tfh cells has been observed (6–8). Third, the frequency of CXCR5+PD-1hiCD4+ T cells secreting IL-21 was significantly higher than that of other CD4 subsets in lymph nodes of HIV-infected individuals (8). In the current study, we found that IL-21 was abundantly present in secondary follicles with GCs (Fig. 6). More to the point, during the course of infection, IL-21 expression was significantly increased within GCs of hyperplastic follicles (Fig. 6), pointing to the fact that if PD-1 engagement limits Tfh cell proliferation, it does not appear to affect IL-21 production to the same extent. It is possible that the elevated frequencies and density of Tfh cells capable of secreting IL-21 result from vigorous recruitment of T cells into the hyperplastic follicle.

Subsequent T cell interactions with GC PD-L1–expressing cells may lead to the downregulation of Tfh cell function in vivo. In addition to cross-linking of PD-1 with PD-L1, the presence ofFoxp3+ regulatory T cells could also contribute to the downregulation of GC-related immune response. A series of recent studies in mice and humans showed that CXCR5+Bcl-6+ Foxp3+ T cells are present and suppress differentiation of GC B cells in follicles (20). However, this population within follicles appeared to be a minor population during the course of infection (7). Indeed, IL-21 may actually suppress the frequency of regulatory T cells in select chronic inflammatory situations (21). Overall, these findings suggest that follicular regulatory T cells are present, but this specialized population does not expand within GCs during SIV infection–induced immune activation. Although the apparent IL-21–associated hyperimmune responses within GCs correlated with delayed disease progression, at the opposite end of the clinical spectrum, fast progressor monkeys showed a poor quality and quantity of GCs, and paucity of IL-21–expressing cells especially surrounding GCs (Fig. 7). Of note, though, in other lymph node areas, such as the T cell zone and medulla, IL-21–producing cells were still observed (Fig. 7), suggesting that perhaps cells are not fully depleted but that the disease is associated with a lack of adequate cell trafficking within lymphoid tissues, or that there is a lack of IL-21 induction in a specific subset. We submit that our data are consistent with previous reports linking higher viral loads, poor GC formation, and loss of Thf and proliferating GC B cells with an absence of, or severely decreased production of, SIV-specific Abs in rapid progressor macaques (22–25). Possibly, individual variations in the inherent ability to rapidly induce GC formation may be at the origin of the differences in disease progression in animals given the same viral infection, although the mechanisms underlying such differences in kinetics remain to be understood.

In addition to the effect of IL-21 on B cells, IL-21 serves multiple functions in the Ag-dependent and -independent activation of CD8+ T cells, such as proliferation, expansion, survival, and cytotoxicity. In humans (26) and RMs (27), IL-21 enhances the expression of perforin and granzyme B in CD8+ T cells and NK cell lineages. In contrast, GCs, which are rich in IL-21–producing cells, are thought to be the largest reservoirs of HIV virions (28, 29), and active viral replication occurs in GC resident CD4+ T cells (30, 31). Moreover, our previous study reported that SIV Gag p27 was detected in GC CD4+ T cells in hyperplastic follicles of RMs chronically infected with SIVmac239 (6). Petrovas et al. (7) also reported that cell-sorted purified Tfh cells from lymph nodes of SIV-infected animals were positive for SIV, suggesting that Tfh cells serve as targets and potentially as viral reservoirs, which are markedly expanded during infection. One important question that deserves to be addressed is the paucity of follicular CD8+ T cells likely leading to poor control of viral replication within the GC environment, with the abundance of IL-21 present during chronic SIV infection. Indeed, several investigators have attempted to study this issue. First, studies by Connick et al. (32, 33) reported that the virus-specific CD8+ T cells were excluded from follicles in HIV-infected humans. Their observations were consistent with our recent study in macaques (6). Second, IL-21 could play a pivotal role in the contraction of CD8 T cell responses by inducing apoptosis (34). Third, IL-21 alone may be sufficient for effective activation of CD8+ T cells, but not for their traffic within GCs. More work will be needed to understand the mechanism(s) involved in the selective exclusion of effector CD8+ T cells within GCs. However, we submit that the delineation of the dynamics of Tfh during SIV infection reported herein provides data on the additional steps toward our understanding of mechanisms and immune modulators involved in GC expansion and the development of antiviral responses to SIV/HIV.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 1:
**Supplemental Figure 1.** (A and B) Representative flow cytometry analysis showing the gating strategy for defining the percentage of PD-1\textsuperscript{neg}, PD-1\textsuperscript{interm} and PD-1\textsuperscript{high} populations of CD4 and CD8 T cells from blood and lymphoid tissues. (C) The expression of CXCR5, ICOS, CD200, CCR7, and CD95 was shown in PD-1\textsuperscript{neg} PD-1\textsuperscript{int} and PD-1\textsuperscript{high} CD4 T\textsuperscript{+} cells from inguinal lymph node. PD-1\textsuperscript{high} CD4\textsuperscript{+} T cells are dense within GCs in lymphoid follicles of SIV-uninfected macaques. The sections were stained with CD20 (blue), CD4 (red) and PD-1 (green). Scale bars = 50 microns.
Supplemental Figure 2. Representative GC responses in lymphoid tissues: Immunohistological profile of Hoechst, CD20⁺, CD3⁺, and Ki67⁺ cells within ileum and colon sections from SIV-naïve rhesus macaques. The sections were stained with Hoechst dye for cell nucleus, anti-CD20 for B cell aggregates (lymphoid follicles), anti-CD3 for T cells and anti-Ki67 for proliferating cells. Like lymph node and spleen, active follicles (B panels) in lymphoid tissues of rhesus macaques include GCs that can be anatomically demarcated by Hoechst (less intensely stained), follicular CD3⁺ cells and a high density of Ki67⁺ B cells. Inactive follicles shown in A panels do not contain GCs. Scale Bars = 50 micron
Supplemental Figure 3.

Supplemental Figure 3. Correlations between plasma viral load and GC size (A) and between disease progression and GC size (B) of lymph node follicle in normal progressors (open circle), fast progressors (solid square), and both of them was shown in here. The correlation was assessed by Spearman’s rank correlation test. A P-value of less than 0.05 was considered statistically significant.