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Compartmentalization of Simian Immunodeficiency Virus Replication within Secondary Lymphoid Tissues of Rhesus Macaques Is Linked to Disease Stage and Inversely Related to Localization of Virus-Specific CTL


We previously demonstrated that HIV replication is concentrated in lymph node B cell follicles during chronic infection and that HIV-specific CTL fail to accumulate in large numbers at those sites. It is unknown whether these observations can be generalized to other secondary lymphoid tissues or whether virus compartmentalization occurs in the absence of CTL. We evaluated these questions in SIVmac239-infected rhesus macaques by quantifying SIV RNA+ cells and SIV-specific CTL in situ in spleen, lymph nodes, and intestinal tissues obtained at several stages of infection. During chronic asymptomatic infection prior to simian AIDS, SIV-producing cells were more concentrated in follicular (F) compared with extrafollicular (EF) regions of secondary lymphoid tissues. At day 14 of infection, when CTL have minimal impact on virus replication, there was no compartmentalization of SIV-producing cells. Virus compartmentalization was diminished in animals with simian AIDS, which often have low-frequency CTL responses. SIV-specific CTL were consistently more concentrated within EF regions of lymph node and spleen in chronically infected animals regardless of epitope specificity. Frequencies of SIV-specific CTL within F and EF compartments predicted SIV RNA+ cells within these compartments in a mixed model. Few SIV-specific CTL expressed the F homing molecule CXCR5 in the absence of the EF retention molecule CCR7, possibly accounting for the paucity of F CTL. These findings bolster the hypothesis that B cell follicles are immune privileged sites and suggest that strategies to augment CTL in B cell follicles could lead to improved viral control and possibly a functional cure for HIV infection. The Journal of Immunology, 2014, 193: 000–000.

In the absence of antiretroviral therapy, HIV-1 replication continues inexorably and results in progressive depletion of CD4+ T cells, immunodeficiency, and ultimately death of the untreated host. The majority of HIV-1 replication in vivo during the chronic phase occurs in secondary lymphoid tissues within CD4+ T cells located in B cell follicles (1–5). SIV replication is also concentrated in CD4+ T cells located primarily in B cell follicles in lymph nodes of chronically infected rhesus macaques (6), which develop a disease similar to HIV-1 infection in humans that progresses to simian AIDS (SAIDS) and death. Mechanisms underlying the compartmentalization of HIV-1 and SIV replication in B cell follicles of lymphoid tissues are not fully understood. Within germinal centers of B cell follicles, the presence of follicular dendritic cells (FDC) laden with extracellular virions (7, 8) that are potently infectious to CD4+ T cells (9) likely plays a significant role in HIV-1 propagation at those sites. Nevertheless, it is unknown why the host immune response is unable to fully suppress HIV-1 replication in the F compartment.

CD8+ CTLs play a key role in control of HIV-1 and SIV replication. CTL develop shortly after primary HIV-1 (10–12) and SIV (13, 14) infection, concurrent with declines in viremia. Diminished HIV-1–specific CTL responses are associated with progression of
HIV-1 and SIV infection to AIDS (15, 16) and SAIDS (17), respectively, and are thought to be the result of mutations in CTL epitopes leading to immune escape (18) as well as loss of CD4+ Th cells that are essential to maintenance of CTL number and function (19, 20). Depletion of CD8+ cells from chronically SIV-infected macaques increases plasma viremia by as much as 1000-fold (21–23), further supporting the notion that CD8+ T cells exercise substantial antiretroviral activity in vivo. Nevertheless, efforts to augment virus-specific CTL through infusion of autologous ex vivo–expanded virus-specific CTL (24–27), structured treatment interruption (28), and therapeutic vaccination (29–33) have failed to substantially reduce virus replication. Furthermore, HIV-1 and SIV replication often take place despite high frequencies of HIV-1– and SIV-specific CTL in PBMC. Thus, numerical deficiencies of CTL are not the fundamental cause for ongoing virus replication.

Previously, we demonstrated that virus-specific CTL fail to accumulate in large numbers in B cell follicles in lymph nodes from chronically HIV-1–infected individuals without AIDS (1), and we hypothesized that follicles are immune privileged sites. Limited studies indicate that SIV replication is concentrated in B cell follicles in rhesus macaques during chronic disease (6) and that CTL directed at a Mamu-A1*001:01–restricted Gag epitope (Gag CM9) fail to accumulate in high concentrations in follicles (34). Whether CTL directed at other epitopes also fail to accumulate in large numbers within B cell follicles in chronic SIV infection is unknown. Furthermore, although some studies reported that most virus replication occurs in extrafollicular (EF) regions of lymphoid tissues during acute SIV infection (35, 36), the magnitude of virus replication in F and EF compartments has not been quantified in either early or advanced disease. More concrete information on the distribution of virus-producing cells in early lentivirus infection, when the nascent CTL response has had minimal impact on virus replication (37), or in advanced disease, when CTL may be dysfunctional or present at a low frequency, could provide additional insight into the role of CTL in the control of lentivirus replication.

To address these questions, we investigated patterns of virus replication and distribution of virus-specific CTL that target multiple SIV epitopes within diverse types of secondary lymphoid tissues of SIV-infected rhesus macaques during acute and chronic SIV infection including some animals with SAIDS. We hypothesized that SIV replication is concentrated in B cell follicles within all secondary lymphoid tissues during chronic disease prior to SAIDS and that virus-specific CTL directed at multiple SIV epitopes are primarily located in EF regions of secondary lymphoid tissues, resulting in high in vivo virus-specific CTL (effector) to SIV RNA+ (target) cell ratios (E:T) in EF compartments and low E:T in follicles. We further hypothesized that there is less compartmentalization of virus replication within B cell follicles 14 d after SIV infection, when the newly evolving virus-specific CTL response has had minimal impact on virus replication (37), or during SAIDS, when the CTL response is often attenuated (17).

Materials and Methods

Tissue collection

Lymph nodes, spleen, and intestinal tissues including ileum, cecum, and colon, were obtained from SIVmac239–infected and uninfected Indian rhesus macaques. Axillary and/or inguinal lymph nodes were obtained from all animals. Mesenteric lymph nodes, spleen and intestinal tissues were only obtained from animals at necropsy, which are indicated in Table I with the letter “N.” Portions of fresh lymphoid tissues were immediately snap frozen in OCT and/or formalin fixed and embedded in paraffin. Animals with MHC class I alleles known to restrict SIV-specific CTL, portions of fresh lymphoid tissue were also collected in RPMI 1640 medium with sodium heparin (18.7 U/ml) and shipped overnight to the University of Minnesota for in situ tetramer staining.

Localization of SIV RNA+ cells within lymphoid tissues

In situ hybridization for SIV RNA was performed using techniques similar to those previously used by us to detect HIV-1 RNA in lymph nodes from humans (2, 40, 41). This technique does not include a protease treatment step to expose encapsidated virion RNA and so primarily identifies cells that are actively transcribing SIV. Extracellular virions encapsulated in envelope glycoprotein and bound to FDC are detectable in some instances but stain faintly in a dendritic conformation that is readily distinguished from the dark blue/black focal staining seen in productively infected cells. Briefly, 6-μm sections of tissue were thaw mounted onto poly-L-lysine–coated microscope slides, fixed in 3% paraformaldehyde, rinsed PBS, and hybridized with digoxigenin-labeled SIVmac239 antisense and sense probes (Lofstrand Laboratories, Gaithersburg, MD) overnight at 50°C. SIV RNA+ cells were visualized using NBT/5-bromo-5-bromo-4-chloro-3-indolyl phosphate (Roche, Nutley, NJ), as described previously (2, 40). In some instances, formalin-fixed, paraffin-embedded (FFPE) tissues were analyzed instead of or in addition to snap-frozen tissues. FFPE tissues were mounted onto slides, baked for 1 h at 60°C, deparaffinized with xylene and rehydrated through graded alcohols to diethyl pyrocarbonate H2O. FFPE tissues were pretreated with proteinase K prior to hybridization. In animals for which both snap-frozen and FFPE samples were available, similar frequencies of SIV RNA+ cells were detected in both paraffin and FFPE sections but not shown here. Immunohistochemical staining for B cells that are actively transcribing SIV was performed in the same tissues using mouse anti-human CD20 (clone 7D1; AbD Serotec, Raleigh, NC) and detected using HRP-labeled polymer anti-mouse IgG (ImmunPressKIt; Vector Laboratories, Burlingame, CA) and Vector NovaRed substrate (Vector Laboratories). SIV RNA+ cells were counted by visual inspection and classified as either inside or outside of B cell follicles, identified morphologically as a cluster of CD20+ cells, as previously described by us in humans (1, 2). A minimum of three sections (~30–μm apart were analyzed for each tissue specimen from each animal. Total tissue area and area of follicles was determined by quantitative image analysis (Qwin Pro version 3.4.0; Leica, Cambridge, U.K.) and used to calculate the frequency of SIV+ cells per square millimeter. A median of 159 mm2 (range, 71–744 mm2) was evaluated for spleen, 51 mm2 of tissue (range, 13–442 mm2) for lymph node, 223 mm2 (range, 43–359 mm2) for ileum, 80 mm2 (range, 33–212 mm2) for cecum, and 86 mm2 (range, 11–164 mm2) for colon.

Quantification of activated and memory CD4+ cells in lymphoid tissues

Six-micrometer frozen sections of lymph node, spleen, and colon tissue from six animals were thaw mounted onto slides and fixed in 1% paraformaldehyde. Indirect immunofluorescent staining was performed using rabbit anti-human CD20 (Abcam, Cambridge, MA), goat anti-CD4 (R&D Systems, Minneapolis, MN), and mouse anti-Ki67 (clone B56; BD Pharmingen) or mouse anti-CD95 (clone DX2; eBioscience, San Diego, CA) diluted in TBS with 1% BSA and incubated for 1 h. After washing in TBS, secondary Abs of AF488-labeled anti-goat, AF594-labeled anti-mouse, and AF647-labeled anti-rabbit were added and incubated for 30 min. Slides were covered with coverslips using SlowFade Gold with DAPI (Life Technologies, Grand Island, NY). Images of full sections were generated at ×40 using an Olympus VS120 scanner outfitted with an OrcaR2 camera (Olympus Center Valley, PA), and numbers of CD8+ or CD4+ or CD4+CD95+ or CD4+CD95+ cells within and outside of follicles, and their respective areas were determined for each tissue type from 10 × 40 images (0.02 mm2 each) using Qwin Pro 3.4.0 (Leica Microsystems, Wetzlar, Germany).

Localization of SIV-specific CTL in lymphoid tissues

In situ tetramer staining combined with immunohistochemistry was performed as described previously (1, 42). Briefly, biotinylated MHC class I
monomers were loaded with peptides (National Institutes of Health Tetramer Core Facility, Emory University, Atlanta, GA) and converted to MHC tetramers. MHC class I monomers used included Mamu-A*001:01 molecules loaded with SIV Gag CM9 (CTPYDINQ) peptides (43), SIV Tat SL8 (STPESANL) peptides (18) or irrelevant negative control peptide FV10 (FLPSDYFPSV) from the hepatitis B virus core protein; Mamu-B*008:01 molecules loaded with Nef RL10 (RRHRILDYLY) peptides (44), Vif RL9 (RRRAIRGELQ) peptides (44), and Env KL9 (KROQELLQRL) peptides (44); and Mamu-A*01:002:01 monomers loaded with Nef Y99 (YTSGPGYRY) peptides (45). Fresh lymph node and spleen tissues were embedded in low-melt agarose and cut using a vibratome into 200-μm-thick sections. Ileum was cut with a scapel into thin strips. Sections were incubated free floating with MHC tetramers at a concentration of 0.5 μg/ml overnight. Sections were then washed twice in PBS and incubated with preformed antibodies three-times. Sections were then exposed to epitopes, and then permeabilized and blocked with PBS-H containing 0.3% Triton X-100 and 2% normal goat serum for 1 h. For the secondary incubation, sections were incubated with rabbit anti-FITC Abs (BioDesign, Saco, ME) along with rat-anti-human CD3 Abs (clone CD3-12; AbD Serotec) and mouse-anti-human CD20 Abs (Novacastra clone L26; Leica Microsystems, Buffalo Grove, IL) or mouse anti-perforin Abs (Novacastra) at 4°C on a rocking platform overnight. For the tertiary incubation, sections were washed with PBS-H and incubated with Cy3-conjugated goat-anti-rabbit Abs (Jackson Immunoresearch Laboratories, West Grove, PA), Alexa 488–conjugated goat-anti-mouse Abs (Molecular probes), and Cy5-conjugated goat-anti-rat Abs (Jackson Immunoresearch Laboratories) or Dylight 649–conjugated goat anti-human IgM (Jackson Immunoresearch Laboratories) in blocking solution for 1–3 d. For each animal, negative control staining was done that included either the same MHC molecule with irrelevant peptide, a different MHC molecule with irrelevant peptide, or mismatched MHC molecule loaded with SIV peptide. A subset of sections was also stained with mouse-anti-human CD20 Abs (clone L26; Novacastra) and Dylight 649–conjugated goat-anti-human IgM (Jackson Immunoresearch Laboratories) to confirm both Abs colocalized similarly in B cell follicles. Stained sections were imaged using Olympus FluoView FV1000 microscope. Confocal z-series were collected from ~5 μm from surface of the tissue section to as deep as the Ab counterstaining penetrated, ~35–45 μm into the tissue. Three-dimensional montage images of multiple 800 × 800 pixel 200× Z-scans were created using Olympus Fluoview Viewer software.

Quantification of SIV-specific CTL in situ. F areas were identified morphologically as clusters of brightly stained closely aggregated CD20+ cells. Cell counts were performed on a LSRII flow cytometer (BD Immunocytometry Systems, San Jose, CA) and analyzed using FlowJo (Tree Star, Ashland, OR).

Quantification of perforin expressing SIV-specific CTL in situ

Perforin expression within MHC-tetramer-binding cells was determined in confocal images of inguinal and tracheobronchial lymph node sections stained with MHC tetramers (red) and anti-perforin Abs (green) as well as IgM Abs (blue) to identify B cell follicles using an Olympus FluoView FV1000 confocal microscope with a ×20 and 1 μm z-steps. For each section, we collected multiple 800 × 800–μm fields and stitched them together using ImageJ software to acquire complete tissues images using Olympus FluoView software. We used FluoView software to visualize and count tetramer-binding cells that were or were not costained with perforin Abs in F and EF areas. For each sample, an average of 97 (range, 22–300) tetramer+ cells inside follicles and an average of 168 (range, 82–384) tetramer+ cells outside follicles were counted.

Flow cytometry to detect chemokine receptor and granzyme B expression in SIV-specific CTL

Cryopreserved disaggregated lymphoid tissue cells were thawed, and 1–2 × 10^6 disaggregated cells were resuspended in 100 μl tetramer staining buffer (5% FBS in PBS with 0.06% sodium azide) and incubated with allophycocyanin-labeled Gag CM9 tetramer (MBL International, Woburn, MA), allophycocyanin-labeled Nef RL10 tetramer (National Institutes of Health Tetramer Core Facility) or BV421-labeled Nef Y99 tetramer (National Institutes of Health Tetramer Core Facility) concurrently with CXCR5-PE (clone MUSDHE9, eBioscience), CD8-phycoerythrin (clone CD8-FITC, BD Bioscience), CCR7-PTA (clone 150505; R&D Systems), CD3- allophycocyanin Cy7 (BD clone SP34-2), and Aqua Live/Dead viability marker (Life Technologies L34957) in the dark for 40 min at room temperature. Cells were washed twice, fixed and permeabilized at 4°C for 20 min (BD 55028), washed again, and stained with granzyme B PE Cy5.5 (clone GB11; Invitrogen) at 4°C for 30 min. After washing twice, the cells were resuspended and were acquired on a LSRII flow cytometer (BD Immunocytometry Systems, San Jose, CA) and analyzed using FlowJo (Tree Star, Ashland, OR).

Results

Rhesus macaque clinical characteristics

Secondary lymphoid tissue specimens from 6 acutely SIV-infected macaques, 14 chronically infected macaques without SAIDS, and 9 chronically infected macaques with SAIDS were evaluated. Clinical and experimental characteristics of these animals are shown in Table I. Acutely infected macaques had lymph nodes collected 14 d postinfection; they had a median plasma SIV RNA viral load of 8.98 log_{10} copies/ml and a median CD4+ T cell count of 1063 cells/mm^3. Chronically infected animals without SAIDS were infected for a median of 22 wk; they had a median viral load of 5.81 log_{10} copies/ml and a median CD4+ T cell count of 368 cells/mm^3. Seven of the nine animals with SAIDS had a CD4+ T cell count < 200 cells/mm^3. The other two animals had CD4+ T cell counts > 200 cells/mm^3 but were sacrificed because of development of conditions associated with SAIDS to prevent suffering. Animals with SAIDS were infected a median of 26 wk, had a median viral load of 6.2 log_{10} copies/ml, and a median CD4+ T cell count of 142 cells/mm^3.

Distribution of SIV RNA+ cells in secondary lymphoid tissues during chronic asymptomatic SIV infection

SIV RNA+ cells were detected by in situ hybridization in all secondary lymphoid tissue samples from chronically infected animals without SAIDS, as shown in representative images in
Fig. 1. Tissues from uninfected animals that were stained with antisense probes and tissues from SIV-infected animals that were stained with sense probes were uniformly negative (data not shown). Within the same tissue type (i.e., lymph node or intestine) frequencies of SIV RNA+ cells were similar (p = 0.754) (Fig. 2A). Frequencies of SIV RNA+ cells in spleen tended to be lower than those in lymph node, although differences were not statistically significant. Frequencies of SIV RNA+ cells were ~6- and 10-fold lower in intestinal tissues compared with spleen (p < 0.0001) and lymph node (p < 0.0001), respectively.

SIV RNA+ cells were significantly more concentrated in B cell follicles compared with EF regions for all tissue types (Fig. 2B). This pattern was consistent among all animals with one exception (R02076-N). This animal (indicated in closed circles in Fig. 2B) demonstrated similar frequencies of SIV RNA+ cells in F and EF regions in axillary and mesenteric lymph nodes but higher frequencies within follicles compared with EF tissues in spleen, in- guinal lymph node, and intestine. Of note, this animal had one of the lowest CD4+ T cell counts of all the chronically infected, asymptomatic macaques.

The F/EF ratio of SIV RNA+ cells was marginally higher in spleen (geometric mean, 7.1) compared with lymph node (geometric mean, 3.1; p = 0.0005) but was substantially higher in intestinal tissues (geometric mean, 32.0) compared with both lymph node (p < 0.0001) and spleen (p < 0.0001) (Fig. 2B). Differences between intestine and other tissues were largely driven by differences in frequencies of SIV RNA+ cells in EF regions; there were substantially fewer SIV RNA+ cells in EF regions of intestinal tissues (geometric mean, 0.16 cells/mm², 95% confidence interval [CI] 0.081, 0.32) compared with spleen (geometric mean, 0.45 cells/mm², 95% CI 0.21, 0.98; p < 0.0019) and lymph nodes (geometric mean, 1.3 cells/mm², 95% CI 0.68, 2.5; p < 0.0001). Frequencies of SIV RNA+ cells in follicles, however, were not statistically different among the tissue types (p = 0.32) ranging from a geometric mean of 3.2 cells/mm² (95% CI 1.5, 7.0) in spleen to 3.9 cells/mm² (95% CI 2.0, 7.6) in lymph node and 5.1 cells/mm² (95% CI 2.5, 10.2) in intestine. Overall, only a small fraction (geometric mean, 2.8%) of the intestinal tissues consisted of follicles, which was significantly lower than that in spleen (28%) and lymph nodes (40%) (Fig. 2C). The majority of SIV RNA+ cells were found within follicles in both spleen and lymph nodes, whereas a geometric mean of 40% of SIV RNA+ cells was found in follicles in intestine (Fig. 2D). Interestingly, most SIV RNA+ cells observed in EF regions of intestinal tissues were located close to a B cell follicle (Fig. 1E, 1F).

To evaluate whether differences in frequencies of virus-producing cells between F and EF regions or among tissues could be related to target cell availability, frequencies of CD4+ cells, CD95+ (memory) CD4+ cells, and Ki67+ (activated) CD4+ cells were determined within spleen, lymph node, and colon of six animals (Supplemental Fig. 1). Significant differences in frequencies of CD4+ cells and CD95+CD4+ cells were observed between F and EF regions of secondary lymphoid tissues, although the differences varied by tissue (p < 0.0001); CD4+ cells and CD95+CD4+ cells were significantly more abundant in follicles compared with EF regions of spleen (F/EF 1.5 for both;
$p = 0.01$ and $0.02$, respectively) and colon (3.0 and 2.8, respectively; $p = 0.001$ for both), whereas they were significantly less abundant in follicles of lymph nodes compared with EF tissues (F/EF 0.6 for both; $p = 0.01$ and 0.007, respectively) (Supplemental Fig. 1A, 1B). Ki67$^+$CD4$^+$ cells, however, were more abundant in F compared with EF regions of all tissues (F/EF 1.5; $p < 0.003$), and the relationship did not differ across tissues ($p = 0.10$) (Supplemental Fig. 1C). Frequencies of SIV RNA$^+$ cells remained consistently higher within the F compartment compared with the EF compartment across spleen (F/EF 6.3, 95% CI 3.3, 12.0; $p < 0.0001$), lymph node (F/EF 6.6, 95% CI 3.5, 12.4; $p < 0.0001$; Fig. 2E), or Ki67$^+$CD4$^+$ cells (F/EF 6.2, 95% CI 3.2, 12.1; $p < 0.0001$). There were no statistically significant differences in the F/EF ratio of virus-producing cells across spleen, lymph node, and colon after adjusting for frequencies of CD4$^+$ cells ($p = 0.78$), CD95$^+$CD4$^+$ cells ($p = 0.67$; Fig. 2E), or Ki67$^+$CD4$^+$ cells ($p = 0.55$).

**Distribution of SIV RNA$^+$ cells in lymph nodes of SIV-infected macaques during acute SIV infection and SAIDS**

Frequencies of SIV RNA$^+$ cells were significantly lower in lymph nodes from chronically infected animals compared with those from animals 14 d postinfection and tended to be lower compared with those from animals with SAIDS (Fig. 3A). No evidence of compartmentalization of SIV RNA$^+$ cells within B cell follicles was observed in any of the acutely infected animals in marked contrast to findings seen in animals with chronic infection (Fig. 3B). In animals with SAIDS, some demonstrated a F concentration of SIV RNA$^+$ cells in lymph nodes, whereas others did not, and overall concentrations of SIV RNA$^+$ cells were not statistically different between the two compartments (Fig. 3B). The F/EF ratio of SIV RNA$^+$ cells was significantly higher in chronically infected animals compared with those with acute SIV infection ($p = 0.0004$) and tended to be higher compared with those with SAIDS ($p = 0.18$) (Fig. 3B). The percentage of tissue that

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**FIGURE 1.** Localization of SIV RNA$^+$ cells in secondary lymphoid tissues of chronically infected rhesus macaques. Representative images of in situ hybridization for SIV RNA to identify virus-producing cells (blue/black cells indicated by arrows) and CD20 staining (brown) to morphologically identify B cell follicles in spleen (A and B), axillary lymph node (C and D), ileum (E and F), and colon (G and H). Images in (B), (D), (F), and (H) are high magnification (original magnification $\times 252$) images from the fields shown in (A), (C), (E), and (F) (original magnification $\times 63$), respectively.
consisted of follicle did not differ significantly among animals by disease stage (Fig. 3C). Percentages of SIV RNA+ cells that resided in follicles, however, were significantly higher in animals with chronic infection compared with those with acute infection ($p = 0.02$) and tended to be higher compared with those with SAIDS (Fig. 3D). When data from animals at all stages of disease were combined, frequencies of SIV RNA+ cells predicted plasma viral load (Fig. 3E). In an analysis that included all animals, the log10 F/EF ratio of SIV RNA+ cells in lymph nodes predicted log10 plasma viral load (Fig. 3F). Notably, the two animals with the highest degree of compartmentalization (i.e., the highest log10 F/EF ratio in Fig. 3F) were both chronically infected animals that expressed the *Mamu-B*^*008:01* MHC allele.

**Distribution of SIV-specific CTL in secondary lymphoid tissues during chronic SIV infection**

CTL directed at seven different SIV epitopes and restricted by three different MHC class I molecules were identified by in situ MHC class I tetramer binding in secondary lymphoid tissues from eight animals with chronic SIV infection including SAIDS (Supplemental Table I). When SIV-specific CTL were detected at high frequencies in one tissue from an animal, they were also detected in other tissues examined from that animal (Supplemental Table I). Frequencies of SIV-specific CTL were quantified in 23 lymph nodes from eight animals and ranged from 10 to 522 cells/mm². SIV-specific CTL were also quantified in spleens of six of these animals and ranged from 22 to 327 cells/mm². Frequencies of SIV-specific CTL did not differ significantly between lymph nodes and spleen ($p = 0.44$).

SIV-specific CTL were generally distributed diffusely among other CD8+ T cells and in all animals were consistently more concentrated in the EF regions in both lymph node and spleen compared with F regions regardless of the MHC class I type of the animal or the SIV epitope presented by the tetramer (Fig. 4A). The highest frequencies of both F and EF SIV-specific CTL were found in the three animals that expressed the *Mamu-B*^*008:01* MHC allele. In all animals, there was a range of localization patterns of SIV-specific CTL within follicles (Fig. 5). In most instances, SIV-specific CTL were largely absent from the follicles, as illustrated in Fig. 5B and 5C. When SIV-specific CTL were found in B cell follicles, typically they were located near the edge of the follicles (Fig. 5C), although in some instances they were located throughout the follicle cross-section (Fig. 5D). Scanning through three dimensions within B cell follicles revealed that in most follicles there were large contiguous areas that were completely devoid of SIV-specific CTL. The frequency of virus-specific CTL in the EF region was predictive of the frequency in the F region (Fig. 4B). The median ratio of EF/F SIV-specific CTL in both lymph node and spleen was 4 (Fig. 4C). There was no obvious difference in the EF/F ratios of SIV-specific CTL related to MHC class I allele expression or the SIV epitope targeted, although the numbers of animals studied was too small to exclude the possibility that such a difference exists.

In intestinal tissues, in situ tetramer staining analysis was limited to the ileum from two chronically infected animals (Rha10 and Rha18), and one animal with SAIDS (R01106) and results were not
FIGURE 3. Distribution and frequency of SIV RNA+ cells in lymph nodes of rhesus macaques during acute infection (day 14), chronic asymptomatic infection, and SAIDS. A) Frequencies of SIV RNA+ cells in lymph nodes. B) Frequencies of SIV RNA+ cells in B cell F regions compared with EF regions of lymph nodes. C) Percentage of tissue that consisted of follicle did not differ among animals by disease stage. D) Percentages of SIV RNA+ cells within B cell follicles. E) Frequencies of SIV RNA+ cells within lymph nodes predicted plasma viral load. For every 1 log_{10} increase in SIV RNA+ cells, there was a 1.40 log_{10} (95% CI, 0.35, 2.45) increase in plasma viral load. F) The F/EF ratio of SIV RNA+ cells in lymph nodes predicted plasma viral load. On average, viral load decreased by 4.0 log_{10} (95% CI, −5.33, −2.67) copies/ml for each 1 log_{10} increase in F/EF. This relationship did not significantly differ by disease stage (p = 0.30). Furthermore, when the analysis was restricted to animals with chronic, asymptomatic infection, results were still statistically significant (p = 0.0078), with viral load decreases by 3.04 log_{10} (95%CI, −5.12, −0.96) copies/ml for each 1 log_{10} increase in F/EF. Dotted lines link data points from animals that were sampled in more than one disease stage. Horizontal lines indicate geometric mean (GM) values.

quantified because of limited numbers of follicles detected in tissue sections. Rhau10 demonstrated a few B*008:01/Vif RL9- and Nef RL10–specific CTL (Supplemental Fig. 2A) that were localized exclusively in EF regions. Rhaps18 demonstrated a few B*008:01/Nef RL10–specific CTL scattered in the EF regions, and a larger number around and inside the one follicle that was observed (Supplemental Fig. 2B). In the animal with SAIDS (R01106), B*008:01/Env KL9–specific CD8+ T cells (Supplemental Fig. 2C) were detected at higher levels than in the other two animals. In this animal, more abundant EF CTL were observed than in the other two animals, and similar to Rhaps18, large concentrations of CTL were found around and inside the B cell follicles.

Effector SIV-specific CTL to SIV RNA+ target cell (E:T) ratios in secondary lymphoid tissues

The in vivo E:T ratios between the dominant (i.e., most abundant SIV-specific CTL and SIV RNA+ cells) ranged from 2 to 235 (median, 36) in lymph node and 6 to 7509 (median, 69) in spleen. The E:T ratio did not correlate with plasma viral load for either lymph node (r = −0.07; 95% CI, −0.74, 0.67; p = 0.87) or spleen (r = −0.31; 95% CI, −0.90, 0.67; p = 0.544). However, in a mixed model combining data from both lymph node and spleen, log_{10} frequencies of CTL were inversely related to log_{10} frequencies of SIV RNA+ cells in the tissue. On average, for every 1 log increase in CTL, there was a 0.81 log_{10} decrease (95% CI −1.82, 0.20) in SIV RNA+ cells (p = 0.089). Interestingly, when frequencies of CTL in F and EF compartments were evaluated together vis-a-vis SIV RNA+ cells in those compartments, there was a significant inverse relationship (Fig. 6B).

Expression of CXCR5 and CCR7 on virus-specific CTL

It is well established that upregulation of CXCR5 and downregulation of CCR7 are required to enable the F subset of CD4+ T cells to migrate into B cell follicles (46). Assuming that a similar mechanism promotes CD8+ T cell migration into B cell follicles, we hypothesized that absence of CXCR5 and/or presence of CCR7 on SIV-specific CTL might account for low frequencies of these cells in B cell follicles. We first evaluated the distribution of CXCR5 in lymph nodes (n = 5) and spleens (n = 2) from chronically SIV–infected rhesus macaques. We found that the majority of CXCR5+ cells were localized within B cell follicles, as shown in representative images in Fig. 7A and 7B, confirming that the expected localization of CXCR5–expressing cells in B cell follicles exists in SIV infection. We next evaluated CXCR5 and CCR7 expression on virus-specific CTL using cryopreserved,
disaggregated cells from spleen and lymph nodes that were stained with Abs and MHC class I tetramers, and analyzed by flow cytometry (Supplemental Fig. 3). CXCR5+CCR7−SIV–specific CTL were a minority population in all animals and all tissues, whereas the CXCR5−CCR7+ and CXCR5−CCR7−CTL subsets were the most abundant (Fig. 7C).

Expression of CTL effector molecules by virus-specific CTL

To evaluate whether CTL dysfunction might hinder clearance of SIV-producing cells within B cell follicles, granzyme B and perforin expression were determined on SIV-specific CTL. Using disaggregated cells from the same animals as those in Fig. 7E, we determined granzyme B staining within each chemokine receptor expressing CTL subset by flow cytometry (Supplemental Fig. 3). As shown in Fig. 8A, CXCR5 expression correlated with the most granzyme-expressing cells, regardless of whether they expressed CCR7, whereas low levels of granzyme B were observed in CXCR5+ subsets. Perforin expression was evaluated by immunofluorescent staining within virus-specific CTL identified through in situ tetramer staining. As shown in Fig. 8B and 8C, perforin+ and perforin−SIV–specific CTL were observed within both F and EF regions of tissues, respectively. Perforin expression was 15.9% lower within the F population of SIV-specific CTL compared with CTL in the EF region (Fig. 8D).

Discussion

To our knowledge, this is the first study to quantify and compare the distribution of virus replication within multiple secondary lymphoid tissues during chronic SIV disease as well as during acute infection and SAIDS. To our knowledge, this is also the first study to quantify diverse virus-specific CTL within F and EF compartments of secondary lymphoid tissues during chronic disease. We found that virus replication was concentrated in B cell follicles in all secondary lymphoid tissues of chronically infected rhesus macaques prior to SAIDS even after adjusting for differences in frequencies of viral target cells, demonstrating that compartmentalization of SIV replication is a widespread phenomenon. In contrast, the F concentration of SIV replication was uniformly absent in animals at day 14 of SIV infection when the nascent CTL response has had minimal impact.
on virus replication (13, 14). Furthermore, compartmentalization of SIV replication was attenuated or lost in animals with SAIDS when SIV-infected macaques often have substantial reductions in CTL and impairments in CTL function (17). SIV-specific CTL were distributed in a pattern inverse to that of SIV RNA+ cells, with high concentrations of virus-specific CTL in EF regions and low concentrations in B cell follicles during chronic disease. Effector (CTL) to target (SIV RNA+) cell ratios in spleen and lymph node were on average ~40-fold higher in EF regions compared with B cell follicles. Frequencies of virus-specific CTL within F and EF compartments predicted frequencies of virus-producing cells within those sites when analyzed together, providing additional evidence that in vivo levels of CTL are significant determinants of virus replication. Collectively, these data suggest that virus-specific CTL are highly efficient at suppressing lentivirus replication in EF tissues during chronic asymptomatic disease because they are present in those tissues in large numbers but that they are unable to suppress virus replication within follicles because of low frequencies at those sites. These findings further bolster our hypothesis that B cell follicles are immune privileged sites because CTL fail to accumulate in large numbers in that compartment, thereby establishing a reservoir of chronic lentivirus replication (1, 2).

To our knowledge, this study is the first to directly compare frequencies of virus-producing cells among secondary lymphoid tissues during chronic infection. Importantly, we quantified virus-producing cells using a technique that readily distinguishes between virus-producing cells and virions bound extracellularly to FDC because the latter would not be targeted by CTL as they are not productively infected (47) and consequently do not present Ag in the context of MHC class I molecules required for CD8+ T cell recognition. Although the phenotype of SIV RNA+ cells was not determined in this study, previous studies have demonstrated that the majority of SIV RNA+ cells in secondary lymphoid tissues of rhesus macaques are CD4+ T cells (48, 49). Overall, frequencies of SIV RNA+ cells were 6-fold lower in intestinal tissues compared with spleen and 10-fold lower compared with lymph node (Fig. 2A). In light of estimates that intestine contains ~10 times more tissue than spleen (50), these data suggest that intestine harbors ~1.7 times more virus-producing cells in vivo than spleen, an organ which has generally been considered to harbor a minor portion of replicating virus. Recent studies have challenged the notion that intestinal tissues harbor the majority of CD4+ T cells in vivo (50, 51), which has been the primary basis for assertions that the majority of HIV-1 and SIV replication...
occurs in the gut. Furthermore, phylogenetic studies of virus in SIV-infected rhesus macaques indicate that gut is not the major source of plasma virus in vivo (52, 53), and one study suggested that lymph nodes are the major source (54). Collectively, these data suggest that the intestine is a minor source of plasma virus during chronic asymptomatic disease. Further studies to precisely quantify the amount of virus-producing cells in secondary lymphoid tissues by measuring the dimensions of the tissues could be useful in establishing the true contribution of each tissue type to virus production during both acute and chronic infection.

Similar levels of virus replication were seen in B cell follicles in spleen, lymph nodes, and intestinal tissues during chronic infection, suggesting that the factors that promote SIV replication within follicles are consistent across these diverse tissue environments. Location of the virus-producing cells within B cell follicles is highly suggestive that these cells are T F helper cells, but further studies to clarify the phenotype of these virus-producing cells are warranted. Marked differences in virus replication were found among EF regions of secondary lymphoid tissues; frequencies of SIV RNA⁺ cells in the lamina propria of intestine were on average three- to eight-fold lower than those in the EF regions of spleen and lymph node, respectively. These differences appeared to be largely explained by lower concentrations of memory CD4⁺ cells in lamina propria compared with EF regions of spleen and lymph node because differences in virus-producing cells in the EF compartment disappeared after adjusting for frequencies of memory CD4⁺ cells. Relatively low numbers of memory CD4⁺ cells in the lamina propria of the intestine may exist because of either intrinsic differences among the tissues (50, 51) or selective loss during acute SIV infection (55). Importantly, there was no evidence from our studies of a more robust CTL response in the lamina propria of the ileum compared with EF regions of lymph nodes and spleen to account for fewer virus-producing cells in the lamina propria of the intestine. The observation that SIV RNA⁺ cells in the lamina propria of intestinal tissues were usually located near B cell follicles raises the interesting possibility that virus-producing cells in the lamina propria are cells that recently emigrated from follicles, possibly after being infected through interactions with FDC. Indeed, a major difference between the intestinal tissues and the spleen and lymph node was the relative paucity of follicles in the intestine, which could account for the relatively lower level of SIV replication in the lamina propria if most virus-producing cells originate in follicles. It is possible that most SIV RNA⁺ cells originate in follicles in lymph nodes and spleen as well, but because of the close adjacency of follicles in those tissues, a gradient in distribution of EF virus-producing cells vis-a-vis the follicles is less readily observed.

The magnitude of compartmentalization of SIV RNA⁺ cells in lymph nodes, as signified by the F/EF ratio, was strongly related to plasma viral load (Fig. 3E). This was true not only for animals with chronic infection, but also those with acute infection and SAIDS. Compartmentalization of virus replication within B cell follicles has been associated with a beneficial phenotype in rhesus macaques infected with nef-deleted SIVmac239. In these animals, which usually have very low viral loads and a nonprogressor
phenotype, almost all virus-producing cells are found within B cell follicles in secondary lymphoid tissues (56). Mechanisms underlying the profound compartmentalization of virus replication in this model are not clear but could include enhanced CTL killing of virus-producing cells in EF tissues because of abrogation of Nef-induced downregulation of MHC class I molecules (57). Intriguingly, and in contrast to the rhesus macaque model, the nonprogressive phenotype seen in SIV-infected sooty mangabeys is not associated with compartmentalization of virus replication (6). Indeed, these animals, which fail to progress to SAIDS despite high viral loads, demonstrate neither an F concentration of virus replication nor large numbers of virions associated with FDC (6).

Reasons for this distinct distribution of virus-producing cells in sooty mangabeys are unclear but could include lack of permissiveness of T follicular helper cells to SIV, failure of FDC to bind virion Ab complexes, or presence of high concentrations of CTL in the F microenvironment. The failure of sooty mangabeys to develop progressive disease has been attributed to lack of infection of central memory cells (6). It should be noted, however, that frequencies of virus-producing cells in lymph nodes of these animals were substantially lower than those in SIV-infected rhesus macaques (6), which could provide an alternative explanation for their relatively delayed disease progression. Regardless, these findings suggest that mechanisms that underlie nonprogressive SIV disease in sooty mangabeys may be fundamentally different from those in rhesus macaques.

In the current study, the lack of compartmentalization of virus replication during early SIV infection and diminished compartmentalization during SAIDS were interpreted as evidence that CTL are essential to compartmentalization of virus in B cell follicles. Alternative explanations should nevertheless be considered. It is possible that a highly vulnerable target cell population in the EF region is preferentially infected and destroyed during acute SIV infection, resulting in compartmentalization of virus in the B cell follicles in chronic disease because of loss of EF target cells. Nevertheless, an F concentration of virus replication was found even after adjusting for frequencies of total CD4+ cells, memory CD4+ cells or Ki67+CD4+ cells, weighing against this explanation. In addition, the fact that virus replication in the EF regions was elevated in some animals with SAIDS and associated with loss of compartmentalization (Fig. 3B) further argues against a theory of limited target cells in EF regions. Nevertheless, a change in virus coreceptor usage could have increased the permissiveness of target cells in the EF region, causing this loss of compartmentalization in some animals with advanced disease. Studies of virus coreceptor tropism or deep sequencing of the virus envelope to evaluate for mutations in these animals could be useful in excluding this possibility. Furthermore, studies of the effects of CD8 depletion on chronically infected macaques could further address the question of whether the EF target cell population is depleted or if virus replication is suppressed by CTL.

The findings that virus-specific CTL failed to accumulate in large numbers in B cell follicles in both spleen and lymph node during chronic SIV infection confirmed our previous observations in humans (1) as well as one previous description of Mamu-A*007:01/Gag CM9 tetramers (red), anti-perforin Abs (green), and anti-IgM (blue) to define B cell follicles morphologically. Confocal z-scans were collected with a ×20 objective. Scale bar, 50 μm (B) and 10 μm (C). (D) Percentages of perforin+ tetramer-binding cells within and outside of lymph node follicles determined by in situ staining. Using a generalized linear model for a negative binomial distribution was evident for multiple different SIV epitopes presented by several different MHC class I molecules, indicating that this is a generalized phenomenon of SIV-specific CTL and not unique to a few animals, MHC types, or epitopes. Importantly, few SIV-specific CTL expressed the F homing molecule CXCR5 in the absence of the EF retention molecule CCR7, which may account for the paucity of CTL in follicles. It would be important to determine in future studies whether there are differences in the degree of F localization of CTL or their expression of CXCR5 and CCR7 related to MHC restricting molecule or SIV epitope targeted because the current study had insufficient numbers of animals to address these questions. B*008:01 animals in our study demonstrated the largest virus-specific CTL responses as well as the highest degree of compartmentalization of virus replication. These animals had been previously elite controllers and were beginning to lose virologic control at the time that they were studied. It would be important in future studies to determine whether B*008:01 animals with intact elite control demonstrate more compartmentalization as well as higher frequencies of EF.
CTL than B*008:01 animals that do not achieve virologic control or B*008:01 animals that have completely lost virologic control. Studies to evaluate whether F CTL have impairments in effector function were equivocal. Higher percentages of granzyme B+ cells were found in CXCR5+ CTL including the CCR7+ subset, which is presumably representative of F CTL. In contrast, in situ studies demonstrated ~16% fewer F CTL expressed perforin compared with EF CTL, suggesting a possible subtle impairment in effector function in F virus-specific CTL in addition to numerical impairments. Further studies to evaluate the ability of F SIV-specific CTL to suppress virus replication compared with EF CTL are warranted to definitively address this question because phenotypic studies do not necessarily fully reflect effector function. Such studies would require virus-specific CTL to be sorted prior to assay, as CXCR5 is transiently upregulated on T cells after Ag-specific stimulation (Ref. 58 and E. Connick, unpublished observations), which would confound results if CXCR5 is also used to identify F CTL. Additional factors, such as T regulatory cells, may also be affecting virus-specific CTL proliferation and function within and outside of the follicles (59) and are worthy of further investigation.

The present study has provided strong circumstantial evidence that B cell follicles are immune privileged sites where SIV-specific CTL that target multiple different epitopes fail to accumulate at sites of HIV-1 replication in lymphoid tissue. For example, germinal center B cells express high levels of programmed death-ligand 1 in F virus-specific CTL in vivo (60), which is known to inhibit CTL activity (61). Whether this would be functionally relevant in vivo is not clear. A recent study linked the magnitude of the cellular immune response in lymphoid tissues with protection from pathogenic infection in rhesus macaques vaccinated with a live-attenuated SIV that induces CTL (62). Whether CTL in these protected animals entered the follicles, however, is unknown. A better understanding of the immunologic and virologic milieu in B cell follicles during lentivirus infection and in the context of vaccination is essential to the development of effective preventive and therapeutic strategies for HIV-1 infection.

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References


