Diminished Viral Control during Simian Immunodeficiency Virus Infection Is Associated with Aberrant PD-1hi CD4 T Cell Enrichment in the Lymphoid Follicles of the Rectal Mucosa

Geetha H. Mylvaganam, Vijayakumar Velu, Jung-Joo Hong, Shanmugalakshmi Sadagopal, Suefen Kwa, Rahul Basu, Benton Lawson, Francois Villinger and Rama Rao Amara

J Immunol published online 22 September 2014
http://www.jimmunol.org/content/early/2014/09/20/jimmunol.1401222

Supplementary Material
http://www.jimmunol.org/content/suppl/2014/09/22/jimmunol.1401222.DCSupplemental

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Diminished Viral Control during Simian Immunodeficiency Virus Infection Is Associated with Aberrant PD-1hi CD4 T Cell Enrichment in the Lymphoid Follicles of the Rectal Mucosa

Geetha H. Mylvaganam,*† Vijayakumar Velu,*† Jung-Joo Hong,‡† Shanmugalakshmi Sadagopal,*† Suefen Kwa,*† Rahul Basu,*† Benton Lawson,† Francois Villinger,‡† and Rama Rao Amara*,†

The inhibitory receptor programmed death-1 (PD-1) has been shown to regulate CD8 T cell function during chronic SIV infection; however, its role on CD4 T cells, specifically in the gut-associated lymphoid tissue, is less well understood. In this study, we show that a subset of CD4 T cells expresses high levels of PD-1 (PD-1hi) in the rectal mucosa, a preferential site of virus replication. The majority of these PD-1hi CD4 T cells expressed Bel-6 and CXCR5, markers characteristic of T follicular helper cells in the lymph nodes. Following a pathogenic SIV infection, the frequency of PD-1hi cells (as a percentage of CD4 T cells) dramatically increased in the rectal mucosa; however, a significant fraction of them did not express CXCR5. Furthermore, only a small fraction of PD-1hi cells expressed CCR5, and despite this low level of viral coreceptor expression, a significant fraction of these cells were productively infected. Interestingly, vaccinated SIV controllers did not present with this aberrant PD-1hi CD4 T cell enrichment, and this lack of enrichment was associated with the presence of higher frequencies of SIV-specific granzyme B+ CD8 T cells within the lymphoid tissue, suggesting a role for antiviral CD8 T cells in limiting aberrant expansion of PD-1hi CD4 T cells. These results highlight the importance of developing vaccines that enhance antiviral CD8 T cells at sites of preferential viral replication and support the need for developing therapeutic interventions that limit expansion of SIV+PD-1hi CD4 T cells at mucosal sites as a means to enhance viral control. The Journal of Immunology, 2014, 193: 000–000.

*Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA 30322; †Emory Vaccine Center, Yerkes National Primate Research Center, Emory University, Atlanta, GA 30329; and ‡Department of Pathology, Emory University School of Medicine, Atlanta, GA 30322

Received for publication May 13, 2014. Accepted for publication August 26, 2014.

This work was supported by National Institutes of Health Grants R01 AI074471, R01 AR071852, P01 AI088575, and RC2 CA149086 (to R.R.A.), Yerkes National Primate Research Center Base Grant P51 RR00165, and Emory Center for AIDS Research Grant P30 AI050409.

Address correspondence and reprint requests to Dr. Rama Rao Amara, Room 3024, Yerkes National Primate Research Center, Emory University, 954 Gatewood Road, Atlanta, GA 30329. E-mail address: ramara@emory.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: DM, DNA/MVA SIV; LN, lymph node; MFI, mean fluorescence intensity; NHP, nonhuman primate; NIH, National Institutes of Health; PD-1, programmed death-1; RM, rhesus macaque; Tfh, follicular helper CD4 T.
cells increase significantly in lymph nodes (LNs) of HIV-infected humans and SIV-infected nonhuman primates (NHPs) during the chronic stage (29–32). The reasons for this increase are not yet fully understood. Although human studies suggested a direct relationship between the frequency of PD-1+ or Tfh cells and plasma viremia, this association was not observed in NHP studies. Petrovas et al. (29) demonstrated a direct relationship between higher soluble CD14 levels in plasma and the frequency of Tfh cells, suggesting a role for microbial translocation in the gut in regulating Tfh cells in the lymphoid tissue. However, there is no information available on the status of PD-1+ CD4 T cells in the gut, a preferential site of virus replication in HIV-infected humans or SIV-infected NHPs, and a site that is constantly exposed to high levels of pathogenic and nonpathogenic bacteria. In addition, it is not clear whether vaccine-elicited CD8 T cells have any effect on PD-1+ or Tfh cells in the LN and rectum following SIV infection.

In this study, to understand the influence of chronic SIV infection on PD-1+ CD4 T cells in the gut of RM, we studied the PD-1 expression on CD4 T cells in the rectal mucosal tissue (rectum) and compared it with LNs in the context of SIV-naive, chronic uncontrolled SIV infection, and vaccine-mediated controlled SIV infection. Our results showed a preferential increase in the frequency of PD-1+ CD4 T cells in the rectum and LN of uncontrolled SIV infection and revealed important differences between rectal mucosa and LNs.

Materials and Methods

Animals

Young adult RMs from the Yerkes breeding colony were cared for under the guidelines established by the Animal Welfare Act and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals using protocols approved by the Emory University Institutional Animal Care and Use Committee. Noncontrollers were either unvaccinated or received a DNA/MVA SIV vaccine, and all vaccine controllers received the DNA/MVA SIV vaccine (33, 34). All animals were infected with SIVmac251 intrarectally.

Immunizations and Infections

Indian-origin RMs (Macaca mulatta) were unvaccinated or vaccinated with DNA/MVA SIV (DM) vaccine. Vaccination consisted of two DNA primers on weeks 0 and 8 and two MVA boosts on weeks 16 and 24. Both with DNA/MVA SIV (DM) vaccine. Vaccination consisted of two DNA

Intracellular cytokine staining

Fresh blood, LN, and rectal samples were suspended in RPMI 1640 medium (Life Technologies) with 10% FBS (HyClone, Thermo Fisher Scientific), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Lonza). Stainings were performed in the presence of anti-CD28 Ab and anti-CD49d Ab (1 μg/ml; BD Pharmingen). One million cells were stimulated with either 200 ng/ml PMA and 1 μg/ml ionomycin, CD3/CD28 beads (at a 1:2 ratio beads to cells; Miltenyi Biotec) or pooled peptides spanning the entire SIV Gag protein (single pool of 125 peptides with each peptide at a concentration of 1.0 μg/ml; NIH AIDS Research and Reference Reagent Program catalog number 6204) in the presence of brefeldin A (5 μg/ml; Sigma-Aldrich, St. Louis, MO) and GolgiStop (0.5 μM/μl; BD Pharmingen) after 2 h of stimulation for 4 h at 37˚C in the presence of 5% CO2. At the end of stimulation, cells were washed once with FACS wash (PBS containing 2% FBS and 0.25 g sodium azide) and surface stained with anti-CD3, anti-CD8, anti-CD5, and anti-CD27 (PD-1) at room temperature for 20 min. Cells were then fixed with cytofix/cytoperm (BD Pharmingen) for 20 min at 4˚C and washed with Perm wash (BD Pharmingen) before fixation. Cells were incubated for 30 min at 4˚C with Abs specific to IL-2, IL-17A, IFN-γ, IL-21, and CD4, washed once with Perm wash, once with FACS wash, and resuspended in PBS containing 1% formalin. Cells were acquired on LSRFortessa with four lasers (205, 288, 532, and 633 nm) and analyzed using the FlowJo software (Tree Star). At least 50,000 events were acquired for each sample.

Phenotyping

Mononuclear cells isolated from the blood, LN, and rectum were stained with Live/Dead Near-IR Cell stain (Life Technologies) at room temperature for 15 min in PBS to stain for dead cells. Cells were then washed with FACS wash and stained on the surface using Abs specific to CD3, CD4, CD8, CD28, PD-1, CD95, CCR5, and CCR3 and then treated with FcBlock (BD Biosciences) FcBlock (BD Biosciences), BV-515-conjugated CD3 (clone SP-34-2; BD Biosciences), PerCP-conjugated CD4 (clone L200; BD Biosciences), rPeCy7-conjugated anti-CD279 (PD-1; clone EH12.1; BioLegend), PacBlue-conjugated CD3 (clone SP-34-2; BD Biosciences), allophycocyanin-conjugated IL-2 (clone MQ1-17H12; BD Biosciences), and Alexa 700-conjugated IFN-γ (clone B27; BD Biosciences).

Immunofluorescence staining

Rectal tissues were fixed in SafeFix (Fisher Scientific) and embedded in paraffin. Embedded tissue blocks were cut into five microsections, quenched, hydrated, and rehydrated for immunohistochemical analysis. Some tissues also embedded unfixed in OCT medium (TissueTek, Sakura, Finetek, Torrance, CA) for CD8 staining. Immunofluorescence staining was performed for CD20, CD4, CD8, and CD-1 to examine the distribution of PD-1+ cells in tissues, as described previously (38). In brief, heat-induced epitope retrieval was performed with DIVA Decloaker and then blocked with the SNIPER reagent (Biocare, Walnut Creek, CA) for 15 min and in DIVA Decloaker Triton X-100/4% donkey serum for 30 min at room temperature. Subsequently, the sections were incubated with rabbit anti-human CD20 (Thermo Scientific, Rockford, IL), mouse anti-human CD4 (clone BC/IF6; Abcam, Cambridge, MA), mouse anti-human CD8 (clone L78; Abcam, Cambridge, MA), and goat anti-human-1 (R&D Systems, Minneapolis, MN) Abs diluted 1:20 to 1:100 in blocking buffer for 1 h at room temperature. Thereafter, the sections were incubated with secondary Abs (Alexa Fluor 488, 568, 647, or 700; goat anti-mouse/rabbit/rat, 1:100 dilution blocking buffer for 30 min at room temperature. For the frozen tissues, 5-μm-thick sections were fixed with 4% paraformaldehyde
for 10 min, followed by washing in 1× TBS buffer (Biocare Medical, Concord, CA). The same process was then performed without heat-induced epitope retrieval. Finally, the sections were mounted in warm glycerol gelatin (Sigma-Aldrich) containing 4 mg/ml n-propyl gallate (Fluka, Switzerland). Between each step, the sections were washed three times. All images were acquired and analyzed with an Axio Imager Z1 microscope (Zeiss) using various objectives.

Cell sorting

Mononuclear cells isolated from the LN and rectal tissue were processed and stained with anti-CD3, anti-CD279 (PD-1), anti-CD95, and anti-CD8 for 25 min at 4°C and the CD95^−PD-1^neg, CD95^−PD-1^int, and CD95^−PD-1^hi (naive) CD4 T cell populations were sorted using a FACSAriaII (BD Biosciences). In all sorting experiments, the grade of purity on the sorted cells was >93%. SIV RNA levels were determined using a quantitative PCR (37).

In vitro killing assay

Mononuclear cells isolated from the LNs of SIV-infected Mamu A^*01^ SIV controller RMs were processed, stained with Live/Dead IR, anti-CD3, anti-CD4, anti-CD8, anti-CD95, and anti-CXCR5 Abs, and sorted for CD95^+CD4 T cells and CD95^+CXCR5^hi CD4 T cells (Tfh cells) using a FACSAriaII (BD Biosciences). Tfh cells were then pulsed with P11c peptide for 1 h at 37°C at a concentration of 0.1 μg/ml and washed. CD8 T cells were cocultured with unpulsed or pulsed Tfh cells at a 2:1 ratio of CD8 T cells to Tfh cells with no stimulation or anti-CD3/CD28 stimulation at one bead to two cells (Miltenyi Biotec) for 5 d. Cells were then harvested and analyzed using flow cytometry.

Statistical analysis

Statistical analyses were performed using Prism (version 5.0d; GraphPad Software). Statistical significance (p values) was obtained using non-parametric Mann–Whitney U test (for comparisons between groups/subsets) or Spearman rank test (for correlations). Statistical analyses of global cytokine profiles were performed by partial permutation tests using SPICE software (National Institute of Allergy and Infectious Diseases, NIH) as described previously (39).

Results

PD-1^hi CD4 T cells are predominantly found at preferential sites of SIV replication in SIV-naive RMs

To understand the role of PD-1 on CD4 T cells during chronic SIV infection, we characterized PD-1 expression in the rectum compared with the LN and peripheral blood of SIV-naive RM. We observed three subsets of PD-1 expressing memory (CD95^+^) CD4 T cells namely PD-1^neg, PD-1^int, and PD-1^hi (Fig. 1A). Interestingly, the PD-1^hi CD4 T cells were present predominantly in the rectum and LN, with 2–4% in the LN and 8–12% in the rectum and <1% in the blood (Fig. 1A). Thus, PD-1^hi memory CD4 T cells are enriched at sites of preferential SIV replication.

Because Tfh cells in the LN are known to express high levels of PD-1, we phenotyped the PD-1^hi CD4 T cells in the rectum for CXCR5 and Bcl-6, markers used to define Tfh cells in the LN (Fig. 1B). Interestingly, the majority of PD-1^hi but not PD-1^int and PD-1^neg memory CD4 T cells expressed CXCR5 and Bcl-6, suggesting that PD-1^hi cells in the rectum phenotypically may predominately be Tfh cells. We also accessed PD-1 subsets for the level of CCR5 coreceptor expression. In contrast to PD-1^int cells that expressed high levels of CCR5, the majority of PD-1^hi cells in the rectum and LN did not express the viral coreceptor CXCR5, suggesting that these may not be ideal targets for the virus (Fig. 1C).

**FIGURE 1.** Phenotypic characterization of PD-1^+ subsets in the blood, LN, and rectum of healthy RMs. (A) Flow cytometric analysis of mononuclear cells isolated from the blood, LN, and rectum of a healthy RM. Plots show live CD3^+CD4^+CD95^+PD-1^neg, PD-1^int, and PD-1^hi lymphocytes and scatter plot shows the cumulative data for a group in blood (n = 16), LN (n = 7), and rectum (n = 17). (B) Representative histogram plots of CXCR5, Bcl-6, and CCR5 expression overlaying PD-1^neg, PD-1^int, and PD-1^hi CD95^+ CD4 T cells in the rectum (n = 8) and LN (n = 7, 7, and 6, respectively, for CXCR5, Bcl-6, and CCR5) of healthy SIV-naive RMs. Scatter plots show median. ***p < 0.001, ****p < 0.0001.
PD-1<sup>hi</sup> CD4 T cells increase during uncontrolled SIV infection in the rectum and LN of RMs

We next investigated the influence of SIV infection on PD-1<sup>hi</sup> CD4 T cells in the rectum and LN of unvaccinated and vaccinated animals. For this purpose, we used samples from a cohort of SIV-infected RMs that were either vaccine controllers (<10<sup>3</sup> RNA copies/ml plasma at week 24 postinfection) or noncontrollers (>10<sup>3</sup> RNA copies/ml plasma at week 24 postinfection, which include both unvaccinated and vaccinated animals) (Supplemental Fig. 1A). Please note that all conclusions made below remained true even if we defined controllers based on a set point viral load of less than 10<sup>3</sup> RNA copies/ml. As expected, the frequency of total CD4 T cells was significantly lower in the rectum of noncontrollers compared with vaccine controllers (Supplemental Fig. 1B). However, the frequency of PD-1<sup>hi</sup> cells within the memory CD4 T cell compartment was dramatically higher both in the rectum and LN of noncontrollers compared with SIV-naive and vaccine controllers (Fig. 2A). The frequency of PD-1<sup>hi</sup> cells in the vaccine controllers was comparable to the uninfected RMs (Fig. 2A).

To get an estimate of the cell number, we expressed the frequency of PD-1<sup>hi</sup> cells as a percentage of total lymphocytes and found a significant increase in PD-1<sup>hi</sup> CD4 T cells in the LN as a percentage of lymphocytes (Supplemental Fig. 1C), but this was not observed in the rectum (Supplemental Fig. 1B). This finding suggests that despite a decrease in total memory CD4 T cells in the GALT, PD-1<sup>hi</sup> CD4 T cells remain enriched at this site. Consistent with the increase in the frequency of PD-1<sup>hi</sup> cells as a percentage of memory, the mean fluorescence intensity (MFI) of PD-1 was higher on memory CD4 T cells in the noncontrollers than in uninfected and vaccine controllers (data not shown). Furthermore, the frequency of PD-1<sup>hi</sup> cells in the LN and rectum correlated directly with plasma viremia (Fig. 2C).

To understand the kinetics of expansion of PD-1<sup>hi</sup> cells following infection, we followed the frequency of PD-1<sup>hi</sup> cells longitudinally in the rectum of SIV noncontrollers in a separate study and found a similar increase in PD-1<sup>hi</sup> cells during the course of SIV infection, with the increase being observed as early as 2 wk postinfection (Fig. 2C). These data demonstrated that despite the loss of total memory CD4 T cells, the PD-1<sup>hi</sup> memory CD4 T cells are enriched at preferential sites of virus replication in uncontrolled chronic SIV infection very early postinfection, whereas interestingly such enrichment is not seen in vaccine controllers. We also characterized PD-1 expression in the jejunum of a small group of chronically SIV-infected animals, but we did not find PD-1<sup>hi</sup> CD4 T cells (data not shown) at this site. There are two possible explanations for this observation: PD-1<sup>hi</sup> CD4 T cells are depleted from the jejunum during chronic SIV infection or PD-1<sup>hi</sup> CD4 T cells are not present in the jejunum because of a limited number of GALT structures in this region (40, 41).

Altered CXCR5 expression on PD-1<sup>hi</sup> CD4 T cells in the rectum following SIV infection

Next, we investigated the expression of CXCR5 on the PD-1<sup>hi</sup> cells following SIV infection in the rectum and LN to understand their Tfh phenotype and localization. In contrast to SIV-naive animals, a significant fraction of PD-1<sup>hi</sup> cells in the rectum did not express the Tfh marker CXCR5 following SIV infection (Fig. 3A). This was true for both noncontrollers and controllers, except that it was more pronounced in controllers. However, similar to SIV-naive
animals, the majority of PD-1hi cells in the LN expressed CXCR5, although there was a small decrease in the controllers (Fig. 3A). Although we observed a decrease for CXCR5 expression on PD-1hi cells in the rectum of noncontrollers, because the majority of memory CD4 T cells were PD-1hi (Fig. 2A), the overall frequencies of CXCR5+ and CXCR5− PD-1hi cells within the memory CD4 T cell compartment was also higher in the noncontrollers compared with uninfected RMs (Fig. 3B). Consistent with this increase in CXCR5+ PD-1hi cells in the noncontrollers by flow cytometry, immunofluorescence analysis of rectal tissue revealed B cell follicles with significantly higher density of PD-1hi CD4 T cells in the rectum and LN of healthy, SIV+ vaccine controller, and SIV+ noncontroller RMs (Fig. 3C, 3D) (38). We phenotyped CXCR5+ and CXCR5− cells for the expression of Bcl-6 in a limited number of SIV-infected RMs (Fig. 3E). These analyses revealed that in the LN CXCR5+ cells express lower levels of Bcl-6 compared with CXCR5− cells; however, interestingly in the rectum, both the subsets seem to express Bcl-6 at similar levels. These results argue that the phenotype of Th1 cells in the rectum could be different from that of LN during chronic SIV infection and a thorough characterization of their localization and function is critical before they can be classified as Th1 on the basis of CXCR5 and Bcl-6 expression.

PD-1hi CD4 T cells retain survival potential, show enhanced proliferation and albeit decreased IL-2 production in vivo during chronic SIV infection

The enrichment of PD-1hi cells in noncontrollers could be because of increased proliferation of these cells while maintaining their survival potential, so we studied the expression of Bcl-2 (anti-apoptotic protein) and Ki-67 (marker for proliferating cells) ex vivo. The PD-1hi memory CD4 T cells in the rectum and LN of noncontrollers showed either comparable or higher levels of Bcl-2 expression (Fig. 4A, Supplemental Fig. 2) and markedly enhanced Ki-67 expression compared with uninfected animals (Fig. 4B). However, this was also true for vaccine controllers, suggesting that the observed higher proliferation or Bcl-2 expression of PD-1hi CD4 T cells alone did not markedly contribute to their enrichment in noncontrollers. These results demonstrated that the uncontrolled chronic SIV infection is associated with an enrichment of PD-1hi cells at preferential sites of virus replication with preserved survival potential and high proliferation status.

To assess the cytokine production capability of these PD-1hi memory CD4 T cells, we stimulated cells isolated from the rectum and LN with either PMA/ionomycin (non–TCR-driven cytokine production) or anti-CD3/CD28 (TCR-driven cytokine production)
In general, a significant fraction of PD-1 hi cells failed to produce cytokines following anti-CD3/CD28 stimulation (data not shown). However, following stimulation with PMA/ionomycin, a significant fraction of PD-1 hi cells in the uninfected animals produced cytokines IFN-γ, IL-2, and IL-21 (Fig. 4C) and a small fraction produced IL-17 (data not shown). In the LN, they produced predominantly IL-2, followed by IL-21, IFN-γ, and IL-17. However, in the rectum, they produced predominantly IL-21 and IL-2, followed by IFN-γ. PD-1 hi cells in the noncontrollers and vaccine controllers largely maintained IL-21 production but noncontrollers showed decreased production of IL-2 and IFN-γ. However, this defect was not observed in the rectum (Fig. 4C). These results demonstrated that the PD-1 hi cells that accumulate at the preferential sites of virus replication during uncontrolled SIV infection maintain the potential to produce IL-21, which may contribute to hypergammaglobulinemia by aiding in the maintenance and proliferation of memory B cells. PD-1 hi cells show decreased production of IL-2 and IFN-γ during chronic uncontrolled SIV infection, possibly limiting the potential for the generation of functional Ag-specific humoral responses at these sites. The failure of these cells to express cytokines following TCR-driven stimulation could be because of inhibition by PD-1 signaling and needs further investigation.

PD-1 hi CD4 T cells express low levels of CCR5 yet support ongoing viral replication during chronic SIV infection

We then assessed the expression of the viral coreceptor CCR5 on these cells to see whether these cells can be preferentially infected and killed by the virus (Fig. 5A). In general, only a small fraction (2–5%) of PD-1 hi cells in the uninfected animals produced cytokines IFN-γ, IL-2, and IL-21 (Fig. 4C) and a small fraction produced IL-17 (data not shown). However, following stimulation with PMA/ionomycin, a significant fraction of PD-1 hi cells in the LN and rectum of healthy (n = 6 and n = 14, respectively), SIV + vaccine controller (n = 10), and SIV + noncontroller (n = 12) RMs. The percentage of Ki-67 PD-1 hi cells in the LN and rectum of healthy (n = 9 and n = 13, respectively), SIV + vaccine controller (n = 19 and n = 16, respectively), and SIV + noncontroller (n = 17) RMs. Flow cytometric analysis of cytokine production by PD-1 hi CD4 T cell subsets in the LN and rectum. The scatter plot shows the cumulative data of the percentage of IFN-γ, IL-2, and IL-21–producing PD-1 hi cells in the LN and rectum of healthy (n = 6 and n = 5, respectively), SIV + vaccine controller (n = 6), and SIV + noncontroller (n = 7) RMs, and SPICE analysis on cytokine coproduction is shown on the right. *p < 0.05 and is defined using SPICE software Student t test. †p < 0.05 based on Wilcoxon rank-sum test. Because the Bcl-2 analysis was done on multiple days, we expressed the MFI as a ratio of PD-1 hi to naive to correct for day-to-day variation in cytometer settings. For cytokine production, cells were stimulated with PMA/ionomycin. Scatter plots show the median. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

PD-1 hi CD4 T cell enrichment in the rectum

ABERRANT SIV +PD-1 hi CD4 T CELL ENRICHMENT IN THE RECTUM

FIGURE 4. Phenotypic characterization of PD-1 hi CD4 T cells in the LN and rectum of chronically SIV-infected vaccine controller and noncontroller RM. (A) The MFI of Bcl-2 on PD-1 hi in the LN and rectum of healthy (n = 8 and n = 14, respectively), SIV + vaccine controller (n = 10), and SIV + noncontroller (n = 12) RMs. (B) The percentage of Ki-67 PD-1 hi cells in the LN and rectum of healthy (n = 9 and n = 13, respectively), SIV + vaccine controller (n = 19 and n = 16, respectively), and SIV + noncontroller (n = 17) RMs. (C) Flow cytometric analysis of cytokine production by PD-1 hi CD4 T cell subsets in the LN and rectum. The scatter plot shows the cumulative data of the percentage of IFN-γ, IL-2, and IL-21–producing PD-1 hi cells in the LN and rectum of healthy (n = 6 and n = 5, respectively), SIV + vaccine controller (n = 6), and SIV + noncontroller (n = 7) RMs, and SPICE analysis on cytokine coproduction is shown on the right. *p < 0.05 and is defined using SPICE software Student t test. †p < 0.05 based on Wilcoxon rank-sum test. Because the Bcl-2 analysis was done on multiple days, we expressed the MFI as a ratio of PD-1 hi to naive to correct for day-to-day variation in cytometer settings. For cytokine production, cells were stimulated with PMA/ionomycin. Scatter plots show the median. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

PD-1 hi CD4 T cells express low levels of CCR5 yet support ongoing viral replication during chronic SIV infection

We then assessed the expression of the viral coreceptor CCR5 on these cells to see whether these cells can be preferentially infected and killed by the virus (Fig. 5A). In general, only a small fraction (2–5%) of PD-1 hi cells expressed CCR5 in the uninfected animals, and these levels were even lower in noncontrollers. Interestingly, the fraction of PD-1 hi cells expressing CCR5 increased dramatically in the rectum of vaccine controller compared with SIV naive and noncontroller RMs. As expected, the overall frequency of CCR5 + total memory CD4 T cells declined in the rectum and LN of SIV-infected noncontrolling animals; however, this was not evident in controllers (Fig. 5B).

In an attempt to better understand the contribution of these PD-1 hi cells to viral production and persistence during chronic SIV infection, we further studied the infection status of these cells in the rectum and LN of noncontroller animals (Fig. 5C). The levels of viral RNA were significantly higher in PD-1 + cells than in PD-1 − cells. Within PD-1 + CD4 T cells, viral RNA was present in both PD-1 int and PD-1 hi cells. To approximate the production of virus on a per cell basis, we determined the ratio of viral RNA to viral DNA and observed a significantly higher ratio in PD-1 hi cells in the rectum compared with PD-1 int and PD-1 neg.
cells (Fig. 5C). Cell-associated viral RNA and DNA were also found predominately in the PD-1 hi CD4 T cell subsets in vaccine controllers compared with noncontrollers, albeit at 50- to 100-fold lower levels likely because of lower plasma viremia (data not shown). These data demonstrate that a significant fraction of PD-1 hi cells in the rectum are productively infected during uncontrolled SIV infection and actively support viral production in lymphoid sites known to highly contribute to viral persistence.

Higher antiviral CD8 T cells in the LN are associated with a reduction in CXCR5+ PD-1 hi CD4 T cells and better viral control

To understand the relationship between antiviral CD8 T cells and the frequency of PD-1 hi cells, we determined the frequency of Gag CM9 tetramer+ CXCR5+ PD-1 hi cells in the LN and rectum of vaccinated controllers and noncontrollers, albeit at 50- to 100-fold lower levels likely because of lower plasma viremia (data not shown). These data demonstrate that a significant fraction of PD-1 hi cells in the rectum are productively infected during uncontrolled SIV infection and actively support viral production in lymphoid sites known to highly contribute to viral persistence.

**FIGURE 5.** Phenotypic characterization of CCR5 expression and infection status of PD-1 hi CD4 T cells in the LN and rectum of chronically SIV-infected vaccine controller and noncontroller RMs. (A) Expression of CCR5 on PD-1 neg, int, and hi CD95+ CD4 T cells and the percentage of CCR5+PD-1 hi cells in the LN and rectum of healthy SIV naive (n = 6 and n = 12, respectively), SIV+ vaccine controller (n = 19 and 18, respectively), and SIV+ noncontroller (n = 17 and 18, respectively) RMs. (B) Total frequencies of CCR5+CD95+ CD4 T cells in the blood, LN, and rectum of healthy SIV naive (n = 9, 6, and 23, respectively), SIV+ vaccine controller (n = 19), and SIV+ noncontroller (n = 17, 18, and 16, respectively) RMs. (C) Infection status of PD-1 subsets in the LN and rectum of SIV-infected RMs. Scatter plots show Gag RNA and DNA copies/ng input RNA and DNA quantified by RT-PCR from FACS-sorted naive (CD95- ) and memory (CD95+) PD-1 neg, int, and hi CD4 T cells in the mesenteric LN (n = 9) and rectum (n = 6) and the ratio of RNA copies per DNA copies in PD-1 neg, int, and hi subsets (n = 4, 7, and 4, respectively). Scatter plots show the median. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. The Journal of Immunology 7

\[ Downloaded from http://www.jimmunol.org/ by guest on April 29, 2017 \]
Discussion

Depletion of CD4 T cells in the GALT is an important hallmark of SIV/HIV infection, and recent studies have sought to investigate the immune responses and cellular populations affected in the mucosal sites during pathogenic infection. In the early 1980s, germinal centers were considered as potential sites for long-lasting viral reservoirs and sanctuaries for viral recrudescence (43), but many questions were left unanswered. Recently, germinal centers of lymphoid sites and, in particular, CD4+ Tfh cells in the LN have emerged as an important population of interest during chronic HIV/SIV infection. In particular, several groups have described an increase in the frequency of CXCR5+PD-1hi Bcl-6+ Tfh cells in the LN during chronic SIV/HIV infection, and these cells have been speculated to significantly contribute to B cell dysfunction and hypergammaglobulinemia observed during chronic infection (29–31, 44). Despite the existing knowledge of Tfh cells in the LN during chronic SIV/HIV infection, the existence of these cells and their contribution to SIV pathogenesis have not been studied in the mucosal tissue, one of the most important sites of preferential viral replication and persistence.

Our data demonstrate that lymphoid follicles in the rectum of RMs with uncontrolled chronic SIV infection are highly enriched in actively proliferating PD-1hi CD4 T cells that retain survival potential and harbor a significant fraction of virus-infected cells. This enrichment is impressive considering the widespread depletion of memory CD4 T cells in the gastrointestinal tract following SIV infection. Furthermore, a significant fraction of these PD-1hi cells reside in B cell follicles of lymphoid aggregates in the rectum. Strategically, this seems to be an important mechanism by which these virus-infected PD-1hi CD4 T cells can contribute to ongoing viral replication and persistence while avoiding antiviral CD8 T cell responses, as it has been shown that germinal centers of LNs may act as viral sanctuaries during SIV infection. In addition, these cells maintained their ability to produce IL-21, which may support the uncontrolled or constant proliferation of memory B cells leading to B cell dysfunction and hypergammaglobulinemia commonly seen in chronic SIV and HIV infections (44).

With little known about PD-1hi CD4 T cells residing in the mucosal tissue of SIV naive and SIV-infected RMs, our study revealed some similarities and differences in immune responses in the rectal mucosa compared with the LN. In the absence of SIV infection, PD-1hi memory CD4 T cells are present at higher frequencies in the rectum compared with the LN. This may be because of either preferential depletion of PD-1int and PD-1neg cells or preferential differentiation to PD-1hi cells. Nevertheless, this happens in both compartments (LN and rectum). Although we only directly observed
an enrichment of PD-1hi CD4 T cells in the rectum during chronic uncontrolled SIV infection, we speculate that we would find a similar enrichment in other mucosal sites known to have a high density of GALT structures, such as the terminal ileum (41). In contrast to the LN, there was not an increase in the frequency of PD-1hi cells as a percentage of total lymphocytes in the rectum. This may likely be because of higher depletion of memory CD4 T cells in the GALT compared with LN during chronic uncontrolled HIV/SIV infection.

The increased frequency of PD-1hi memory CD4 T cells in the LN and rectum both consistently associated with viral control, but interestingly we observed an increase in the frequency of CCR5+ PD-1hi CD4 T cells and decrease in CXCR5+PD-1hi CD4 T cells in the rectum of vaccine controllers, an observation much less apparent in the LN. We can speculate that altered chemokine receptor expression may allow for differential homing of these PD-1hi CD4 T cells to regions close to the periphery of the germinal center or outside the B cell follicle. This change in localization may allow for increased immune pressure directed at PD-1hi CD4 T cells. In addition, because of the enrichment of these PD-1hi CD4 T cells in the rectum of SIV-infected noncontrollers, as a result of memory CD4 T cell depletion, these PD-1hi CD4 T cells seemed to express higher levels of Bcl-2, which was not observed in the LN. We can hypothesize that as a result of CD4 depletion, immune activation, and widespread epithelial damage in the GALT, these PD-1hi cells that localized to germinal centers of rectal aggregates may increase Bcl-2 expression to promote survival and persistence in the face of extensive immune dysregulation. In addition, in contrast to LN PD-1hi CD4 T cells in SIV noncontrollers that experienced decreased cytokine polyfunctional activity, PD-1hi CD4 T cells in the rectum seemed to retain their ability to produce IFN-γ and IL-2. PD-1hi cells in the rectum, compared with PD-1hi, and PD-1hi CD4 memory T cells, also seem to represent the most active cellular subset for ongoing viral production as they contained the highest RNA to DNA ratio per cell. These observations suggest that despite an observed aberrant enrichment of PD-1hi CD4 T cells in both the LN and rectum of SIV noncontrollers, site-specific immune response during chronic SIV infection may contribute to localized differences in PD-1hi CD4 T cell subsets.

A critical finding of our study is that the vaccine controllers do not show an enrichment of PD-1hi CD4 T cells in the B cell follicles of rectum or LN. We speculate that multiple mechanisms could have contributed to this outcome. We observed an increase in CCR5 expression and a decrease in CXCR5 expression on PD-1hi CD4 T cells in the controllers. This shift in chemokine receptor expression could promote T cell migration away from the germinal center area of B cell follicles toward T cell zones leading to enhanced killing by cytotoxic CD8 T cells. A recent study demonstrated that the Tfh cells move from one germinal center to another germinal center within a LN (48). Therefore, another plausible mechanism that prevents the aberrant enrichment of these PD-1hi cells is that before reaching the germinal center of lymphoid follicles, these PD-1hi cells are targeted and killed by antiviral CD8 T cells. In addition, the controllers maintained higher frequency of granzyme B+ antiviral CD8 T cells that correlated inversely with the frequency of PD-1hi CD4 T cells. It is possible that these CD8 T cells restrict virus replication in PD-1hi CD4 T cells through cytolytic as well as noncytolytic mechanisms. Furthermore, our ongoing work suggests that some of the antiviral CD8 T cells express CXCR5 and thus could migrate to B cell zone (data not shown).

Thus, our results highlight the importance of SIV-specific CD8 T cells at sites of ongoing viral replication and persistence. Our results also suggest the role of functional antiviral CD8 T cells in limiting the aberrant enrichment of SIV+PD-1hi CD4 T cells at lymphoid sites during chronic uncontrolled SIV infection. Finally, it is conceivable that manipulating the localization of Tfh and antiviral CD8 T cells to and from the germinal center may enhance immune-mediated control of HIV/SIV-infected target cells. These data highlight the importance of generating strong and potent antiviral CD8 T cells at sites of active viral replication and persistence and support the rationale for using targeted therapies that promote Tfh migration out of the GC to allow for increased clearance by antiviral CD8 T cells and enhanced viral control.

Acknowledgments
We thank the veterinary staff at Yerkes for animal care, Center for AIDS Research virology core for viral RNA and quantitative PCR analysis, and Center for AIDS Research immunology core for help with flow cytometry. Also, we thank the NIH AIDS Research and Reference Reagent Program for the provision of peptides.

Disclosures
R.R.A. and V.V. are co-inventors of PD-1 technology that has been licensed to Genentech by Emory University. All other authors have no financial conflicts of interest.

References
Supplemental Figure 1. (A) Kinetics of plasma viral load in the two cohorts of SIV infected vaccine controller (n=19) and non-controller (n=18) RM. (B) Percent of CD4 T cells as a % of total lymphocytes in the blood, LN and rectum of SIV naïve, SIV vaccine controller, and SIV non-controller RM. (C) Percentage of PD-1^hi, PD-1^int, and PD-1^neg CD4 T cells as a percent of lymphocytes in the blood, LN, and rectum of SIV naïve, SIV vaccine controller, and non-controller RM. Scatter Plots show median. *, P < 0.05; **, P < 0.01; ***, P <0.001; ****, P < 0.0001.
Supplemental Figure 2.

Histogram plot showing the expression of Bcl-2 on naïve (CD95-), and memory (CD95+) PD-1<sup>neg</sup>, PD-1<sup>int</sup>, and PD-1<sup>hi</sup> CD4 T cells in the LN and rectum of an SIV+ vaccine-controller and non-controller RM.
**Supplemental Table 1.** Cohort of SIV infected rhesus macaques used for sample collection

<table>
<thead>
<tr>
<th>Animal Name</th>
<th>Vaccination status</th>
<th>Set-Point Viral Load*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Controllers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RKc12</td>
<td>DM</td>
<td>579</td>
</tr>
<tr>
<td>RAq12</td>
<td>DM</td>
<td>1,200</td>
</tr>
<tr>
<td>RHn13</td>
<td>DM</td>
<td>280</td>
</tr>
<tr>
<td>RJp12</td>
<td>DMCD40L</td>
<td>&lt; 80</td>
</tr>
<tr>
<td>RHb12</td>
<td>DMCD40L</td>
<td>225</td>
</tr>
<tr>
<td>RKz12</td>
<td>DMCD40L</td>
<td>311</td>
</tr>
<tr>
<td>RJb12</td>
<td>DMCD40L</td>
<td>1,480</td>
</tr>
<tr>
<td>RWc13</td>
<td>DMCD40L</td>
<td>1,770</td>
</tr>
<tr>
<td>RHu12</td>
<td>DMCD40L</td>
<td>4,540</td>
</tr>
<tr>
<td>RAs12</td>
<td>DMRapa</td>
<td>&lt; 80</td>
</tr>
<tr>
<td>RJo12</td>
<td>DMRapa</td>
<td>&lt; 80</td>
</tr>
<tr>
<td>RFl12</td>
<td>DMRapa</td>
<td>&lt; 80</td>
</tr>
<tr>
<td>REd13</td>
<td>DMRapa</td>
<td>168</td>
</tr>
<tr>
<td>Req12</td>
<td>DMRapa</td>
<td>173</td>
</tr>
<tr>
<td>Riu12</td>
<td>DMRapa</td>
<td>1,460</td>
</tr>
<tr>
<td>RIt12</td>
<td>DMRapa</td>
<td>1,870</td>
</tr>
<tr>
<td>RMz11</td>
<td>DMRapa</td>
<td>3,050</td>
</tr>
<tr>
<td>RUp12</td>
<td>DMRapa</td>
<td>3,980</td>
</tr>
<tr>
<td>RFf12</td>
<td>DMRapa</td>
<td>4,520</td>
</tr>
<tr>
<td><strong>Non-Controllers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RDg12</td>
<td>DMCD40L</td>
<td>237,000</td>
</tr>
<tr>
<td>RRl12</td>
<td>DMRapa</td>
<td>10,000</td>
</tr>
<tr>
<td>RCv12</td>
<td>DMRapa</td>
<td>13,800</td>
</tr>
<tr>
<td>RJz12</td>
<td>DMRapa</td>
<td>20,400</td>
</tr>
<tr>
<td>RPn12</td>
<td>DMRapa</td>
<td>30,100</td>
</tr>
<tr>
<td>RNv12</td>
<td>DMRapa</td>
<td>39,000</td>
</tr>
<tr>
<td>RHy11</td>
<td>DMRapa</td>
<td>77,500</td>
</tr>
<tr>
<td>RTt12</td>
<td>DMRapa</td>
<td>91,600</td>
</tr>
<tr>
<td>RMn13</td>
<td>DMRapa</td>
<td>103,000</td>
</tr>
<tr>
<td>RBs12</td>
<td>DMRapa</td>
<td>179,000</td>
</tr>
<tr>
<td>RKt12</td>
<td>Unvaccinated</td>
<td>700,000</td>
</tr>
<tr>
<td>RMr13</td>
<td>Unvaccinated</td>
<td>27,700</td>
</tr>
<tr>
<td>RRe13</td>
<td>Unvaccinated</td>
<td>34,200</td>
</tr>
<tr>
<td>RDb13</td>
<td>Unvaccinated</td>
<td>115,000</td>
</tr>
<tr>
<td>REn13</td>
<td>Unvaccinated</td>
<td>186,000</td>
</tr>
<tr>
<td>RKi13</td>
<td>Unvaccinated</td>
<td>704,000</td>
</tr>
<tr>
<td>RUF13</td>
<td>Unvaccinated</td>
<td>759,000</td>
</tr>
<tr>
<td>RZn13</td>
<td>Unvaccinated</td>
<td>921,000</td>
</tr>
</tbody>
</table>

DM, DNA/MVA SIV vaccine; DMCD40L, CD40 ligand adjuvanted DNA/MVA vaccine; DMRapa, DNA/MVA vaccine with rapamycin treatment; * RNA copies/mL of plasma at week 24 post SIV infection.