Severe Depletion of CD4⁺ CD25⁺ Regulatory T Cells from the Intestinal Lamina Propria but Not Peripheral Blood or Lymph Nodes during Acute Simian Immunodeficiency Virus Infection[▽]

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CD4⁺ CD25⁺ regulatory T cells (Tregs) suppress the activation and proliferation of effector lymphocytes. In human immunodeficiency virus type 1 (HIV-1) infection, Tregs play a significant role in controlling the apoptotic loss of uninfected CD4⁺ T cells resulting from high levels of generalized immune activation. During acute HIV-1 infection, more than 50% of CD4⁺ T cells are depleted from the gastrointestinal lamina propria. To elucidate the role of Tregs in HIV-1-induced depletion of CD4⁺ T cells in the gut-associated lymphoid tissue (GALT), we first determine the distribution of Tregs in a setting of acute infection using the simian immunodeficiency virus (SIV)/pigtailed macaque model of HIV-1 disease. CD4⁺ T cells from the GALT, lymph nodes, and peripheral blood were isolated from SIV-infected pigtailed macaques on days 4, 14, and 114 postinoculation. Quantitative real-time reverse transcription-PCR was used to quantitate FOXP3 copy numbers in SIV-infected and uninfected control macaques. Expression of FOXP3 in the ileal lamina propria was significantly decreased at all stages of infection compared to levels in uninfected control macaques. In addition, functional analysis of ileal CD4⁺ T cells from SIV-infected macaques revealed a lack of suppressive activity suggestive of the absence of Tregs in that compartment. These results indicate that Tregs are rapidly depleted in the GALT of SIV-infected macaques, defining a role for the loss of Treg-mediated suppression in early events in the pathogenesis of the disease.

Significant and preferential depletion of mucosal CD4⁺ CCR5⁺ T cells has been documented during acute human immunodeficiency virus type 1 (HIV-1) infection in humans and in the simian immunodeficiency virus (SIV)/macaque model of HIV-1 infection (3, 4, 10, 19, 21–23, 29, 36). This depletion of CD4⁺ T cells occurs primarily at mucosal effector sites, particularly in the gut-associated lymphoid tissue (GALT), where a substantial portion of the CD4⁺ T cells in the body resides. A majority (87%) of the CD4⁺ T cells in the intestinal lamina propria (LP) express CCR5 (29, 36), a chemokine receptor that functions as a coreceptor for the most commonly transmitted form of HIV-1 and for SIV. Recent studies suggest that the massive depletion of CD4⁺ CCR5⁺ T cells at mucosal effector sites is mediated by direct viral infection and subsequent cell destruction via either viral cytopathic effects or cytotoxic T-lymphocyte-mediated cytotoxicity (21, 23).

Among the cells that express the coreceptor CCR5 are the CD4⁺ CD25⁺ regulatory T cells (Tregs), a unique population of T cells that suppress the activation and proliferation of effector lymphocytes (17, 18, 30–32, 34, 35, 37). Tregs specifically and exclusively express FOXP3, a transcription factor that plays a key role in their development and function (9, 14, 15). Ligation of CD80 or CD86 on effector T cells by the cell surface molecule CTLA-4 on Tregs results in the suppression

of the effector cell (30). In HIV-1-infected patients, Tregs may migrate to sites of infection, as the number of these cells is reduced in the peripheral blood and increased in the lymphoid organs (1, 16, 24). Tregs have been shown to be highly susceptible to productive HIV-1 infection in vitro (13, 25). In acute SIV infection of rhesus macaques, an early upregulation in the number of FOXP3⁺ Tregs is known to occur in lymphatic tissue (7).

Given that Tregs express CCR5, we asked whether Tregs were affected by the massive depletion of mucosal CD4⁺ CCR5⁺ T cells in acute infection. A decrease in the frequencies of Tregs has been shown to occur in the lymph nodes and colons of SIV-infected macaques during the terminal stages of the disease (26). To examine the dynamics of Treg expression and function in the GALT during acute infection, we used an accelerated and consistent SIV/pigtailed macaque model of HIV-1 disease (8, 20, 39, 40) in which all macaques develop AIDS within several months. We show here that in the early course of SIV-induced GALT depletion, there is a concomitant depletion of the resident Treg population in the intestinal LP. Furthermore, FoxP3 expression and functional activity were deficient at all stages of SIV infection in this site. Using a previously described mathematical model of viral and CD4⁺ T-cell dynamics, we predict that Treg depletion can negatively affect the total CD4⁺ cell count. Experimental results and modeling studies were consistent with the idea that the loss of Tregs early in SIV infection contributes to increased activation and loss of CD4⁺ T cells from the GALT, setting the stage for eventual immune failure.

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MATERIALS AND METHODS

Animal experiments. In a rapid, reproducible SIV/maaque model of AIDS, 12 previously healthy adult (mean age, 5.07 ± 0.18 years) male pigtailed macaques (*Macaca nemestrina*) were inoculated intravenously with an immunosuppressive viral strain, SIV/Delta B670 (50% animal infectious doses [AID₅₀]), and a macrophage-tropic molecular clone, SIV/17E-Fr (10,000 AID₅₀) (8, 20, 39). Macaques were euthanized at day 4 ($n = 3$), day 14 ($n = 3$), or day 114 ($n = 6$) postinoculation. Peripheral blood mononuclear cell (PBMC) samples were obtained at days 7, 10, 14, 21, 28, 35, 42, 56, 70, 84, and 98 and at necropsy (day 4, 14, or 114). Prior to necropsy, animals were perfused with sterile phosphate-buffered saline to remove blood from the tissues. FoxP3 was quantified from four locations: peripheral blood, intestinal lymph nodes (mesenteric and colonic), peripheral lymph nodes (axillary and inguinal), and LP lymphocytes (LPLs) isolated from the GALT (jejunum and ileum). All tissues listed above were obtained at necropsy. Five uninfected age- and gender-matched pigtailed macaques served as virus-negative controls, and the same tissues were obtained at necropsy.

Plasma viral load. Virion-associated SIV RNA in plasma was measured on postinoculation days 7, 10, 14, 21, 28, 35, 42, 56, 70, 84, and 98 as well as at terminal time points (day 4, 14, or 114) by real-time reverse transcription (RT)-PCR using an Applied Biosystems Prism 7700 sequence detection system (the TaqMan method), as previously described (40). Duplicate aliquots were separately reverse transcribed and amplified in triplicate. To control for DNA contamination, one reaction was processed and amplified without the addition of reverse transcriptase. Nominal copy numbers for test samples were then automatically calculated by interpolation of the experimentally determined threshold cycle values onto a regression curve derived from control transcript standards, followed by normalization for the volume of the extracted plasma specimen.

FOXP3 analysis. RNA was extracted from the indicated tissues with RNeasy (QIAGEN), and 300 ng of RNA was reverse transcribed using the Superscript III first-strand synthesis system (Invitrogen). Quantitative real-time RT-PCR was carried out using the TaqMan ABI Prism 7000 sequence detection system (Applied Biosystems). Standard curves were generated by cloning FOXP3 and 18S rRNA PCR products into plasmids using Platinum Pfx and the Zero Blunt TOPO PCR cloning kit (Invitrogen). The following primers were used: 5'-CCGTGGAATTCACGACCCATTCGAACGTCTG-3' (sense) and 5'-CTGATAGATCTCGAACCTCCGACTTTCTGTCT-3' (antisense) for 18S rRNA and 5'-GCACCTTCCCAATCCCAAGT-3' (sense) and 5'-GCAGGCAAGACAGTGGAAAC C-3' (antisense) for FOXP3. In vitro transcription was performed using the MEGAscript T7 kit (Ambion). For the quantitation of FOXP3 gene expression, we used a commercially available kit (TaqMan Assay-on-Demand; Applied Biosystems). To normalize for RNA input, the sample content of 18S rRNA was quantified with TaqMan reagents for 18S rRNA (Applied Biosystems).

Isolation of CD4⁺ T cells from tissues and peripheral blood. PBMCs were isolated by density gradient centrifugation. Lymph nodes were teased apart with needles to form single-cell suspensions. Intestinal sections of jejunum and ileum, approximately 15 cm in length, were cut into small pieces 3 cm in length. The tissues were then subjected to three sequential 45-min incubations, with medium changes in between, in $1 \times$ Hanks balanced salt solution plus EDTA (1 mM final) over a stir plate at room temperature. Liberated cells included intraepithelial lymphocytes, which were stored separately for further analysis. The pieces were then incubated for 180 min at 37°C over a stir plate in RPMI-collagenase medium to liberate lymphocytes of the intestinal LP. To remove mucus, cells were resuspended in a 25% Percoll solution and centrifuged at $600 \times g$ for 30 min. CD4⁺ T lymphocytes were then isolated via positive selection with nonhuman primate CD4 MicroBeads (Miltenyi).

Flow cytometry. For flow cytometric characterization of Tregs, CD4⁺ T cells were stained with directly conjugated monoclonal antibodies specific for CD4, CD25, CCR5, CTLA4, Ki67 (BD Pharmingen), and FoxP3 (clone PCH101; eBioscience) or with appropriate isotype controls. Flow cytometric analysis was performed by using a FACSCalibur apparatus with CellQuest software (Becton Dickinson).

Proliferation of CFSE-labeled CD4⁺ CD25⁺ and CD4⁺ CD25⁻ T cells. For indirect suppression assays, the proliferative responses of CD4⁺ T cells and CD4⁺ T cells depleted of the CD25⁺ subset by bead depletion (nonhuman primate CD25 MicroBeads; Miltenyi) were compared (12, 34). Prior to in vitro analysis, cell preparations were depleted of dead and dying cells with the Dead Cell Removal kit (Miltenyi). Populations of CD4⁺ CD25⁺ and CD4⁺ CD25⁻ T cells were incubated with 1 μ M carboxyfluorescein succinimidyl ester (CFSE) for 10 min at room temperature. Purified T-cell subsets (5×10^4 cells/well) were stimulated for 3 days in triplicate wells with 5×10^4 irradiated (3,000 rad) autologous PBMCs as antigen-presenting cells and 0.5 μ g/ml of anti-CD3 anti-

body in 96-well round-bottomed plates (Corning). On the fourth day, proliferation was measured by CFSE dilution using a FACSCalibur apparatus with CellQuest software (Becton Dickinson).

Assessment of FoxP3 expression by Western blotting. Total cellular lysate from 5×10^6 freshly isolated CD4⁺ T cells was run on 4 to 12% NuPAGE Bis-Tris gels (Invitrogen). Proteins were transferred onto Immobilon polyvinylidene difluoride membranes (Millipore), and the membranes were probed with affinity-purified anti-human FoxP3 monoclonal antibody (clone PCH101; eBioscience) followed by horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch); signal detection was achieved with Western Lighting chemiluminescent substrate (Perkin-Elmer Life Sciences). The amount of protein was quantified by densitometric determination of nonsaturated Western blots using the Alpha Innotech (San Leandro, CA) FluorChem 5500 imaging system.

Mathematical modeling of CD4⁺ T-cell dynamics. We applied a previously described model of T-cell dynamics in the setting of HIV-1 infection to gain insight into the functional consequences of Treg depletion in the GALT (5). Included in this model are resting (Q), activated (T), and productively infected (I) CD4⁺ T cells. We have slightly modified this model by approximating the plasma virus dynamics with those of productively infected cells. This is a reasonable approximation, which has been made previously (33), because the dynamics of free virions are closely approximated by the dynamics of cells that produce them. We assumed a constant inflow rate, λ , of resting CD4⁺ T cells from the periphery into the GALT. Resting CD4⁺ T cells die at a rate, δ_Q , and are activated at a rate, α_Q , to become activated cells. We assume that activated CD4⁺ T cells die at rate δ_T and proliferate at rate p or revert to a resting state at rate r . Activated cells may also become infected at a rate proportional to an infectivity constant, β . Productively infected cells die at rate δ_I . This model is described by a system of three ordinary differential equations:

$$\frac{dQ}{dt} = \lambda + rT - (\alpha_Q + \delta_Q)Q \quad (1)$$

$$\frac{dT}{dt} = \alpha_Q Q - rT - \beta TI \quad (2)$$

$$\frac{dI}{dt} = (\beta T - \delta_I)I \quad (3)$$

The steady-state solutions for equations 1 to 3 are as follows:

$$\bar{Q} = \frac{\lambda\beta + r\delta_I}{\beta(\alpha_Q + \delta_Q)} \quad (4)$$

$$\bar{T} = \frac{\delta_I}{\beta} \quad (5)$$

$$\bar{I} = \frac{\alpha_Q(\lambda\beta + r\delta_I)}{\beta\delta_I(\alpha_Q + \delta_Q)} - \frac{r + \delta_T - p}{\beta} \quad (6)$$

The total CD4⁺ T-cell number at steady state is thus $\bar{T}_{tot} = \bar{Q} + \bar{T} + \bar{I}$.

We simulated the depletion of Tregs with an increase in the activation rate from α_Q^0 to α_Q^∞ according to the equation $\alpha_Q = \alpha_Q^0 + (\alpha_Q^\infty - \alpha_Q^0)(1 - e^{-\theta t})$, where θ represents the first-order rate constant for Treg depletion. Likewise, we simulated the effect of Treg depletion on the proliferation rate of activated cells from p^0 to p^∞ as $p = p^0 + (p^\infty - p^0)(1 - e^{-\theta t})$. Calculations and simulations were performed with Matlab version 7.2.0.232.

Statistical analysis. The significance of all comparisons was calculated using a Student's two-tailed t test assuming unequal variance between uninfected and SIV-infected groups.

RESULTS

Decreases in CD4⁺ CCR5⁺ T cells and increases in CD4⁺ Ki67⁺ T cells among ileal LPLs from SIV-infected macaques with high viral loads. Massive depletion of CD4⁺ CCR5⁺ T cells from the intestinal mucosa occurs during acute HIV-1 infection in humans and during acute SIV infection in macaques (3, 4, 10, 19, 21–23, 29, 36). Recent findings suggest that massive viral infection of intestinal LP CD4⁺ T cells accounts for this depletion (21, 23).

To determine the frequencies of Tregs in the GALT during

TABLE 1. Tissue sampling schedule

No. of animals	Type of infection	Sampling time(s) for blood (days p.i.) ^a	Necropsy time (days p.i.)	Samples obtained at necropsy ^c
5	Control	NA	— ^b	Blood, inguinal LN, mesenteric LN, ileal LP
3	SIV	4	4	Blood, inguinal LN, mesenteric LN, ileal LP
3	SIV	7, 10, 14	14	Blood, inguinal LN, mesenteric LN, ileal LP
6	SIV	7, 10, 14, 21, 28, 35, 42, 56, 70, 84, 98, 114	114	Blood, inguinal LN, mesenteric LN, ileal LP

^a NA, not applicable.
^b Control animals sacrificed in this study were uninfected macaques that were age and gender matched to infected animals.
^c LN, lymph nodes.

acute SIV infection, a total of 12 male pigtailed macaques were euthanized at various time points following SIV infection (Table 1). In our model, acute SIV infection results in very high levels of plasma SIV RNA, with a day 7 geometric mean plasma viral load of 2.6×10^8 copies/ml (Fig. 1A). In addition, CD4⁺ T cells were severely depleted from the GALT during acute infection. By day 14 postinfection (p.i.), CD4⁺ T cells represented 10 to 15% of the total LP cellular population

FIG. 1. High plasma viral load and depletion of CD4⁺ T cells in ileal LPL samples. (A) Viral loads in SIV-infected pigtailed macaques before sacrifice at days 4 ($n = 3$), 14 ($n = 3$), and 114 ($n = 6$). Plasma viral load, in SIV RNA copies per ml of plasma, was measured with a quantitative real-time RT-PCR method (sensitivity, 100 copies/ml). (B) Percentage of ileal LPLs from an uninfected and an SIV-infected macaque that were CD4 positive before (left) and after (right) positive selection for CD4⁺ T cells. After CD4⁺ T-cell selection, the purity obtained was consistently 98 to 100%. Representative examples of fluorescence-activated cell sorter (FACS) profiles from at least three different uninfected or SIV-infected macaques are shown.

FIG. 2. Loss of CCR5-expressing CD4⁺ T cells among ileal LPLs of SIV-infected macaques. (A) CCR5 expression on CD4⁺ T cells from ileal LP, PMBCs, and MLNs. Expression decreased from 46.1% in a representative uninfected macaque (left) to 10.3% in a representative SIV-infected macaque at day 14 p.i. (right). A summary of the CCR5 expression on CD4⁺ T cells in uninfected macaques ($n = 4$) or in SIV-infected macaques sacrificed 14 days p.i. ($n = 3$) is provided in graph form. PE, phycoerythrin. (B) Ki67-expressing CD4⁺ T cells from ileal LP, PMBCs, and MLNs. Ki67-expressing ileal LP CD4⁺ T cells increased in SIV-infected macaques by day 14 p.i. ($P < 0.05$). A summary of Ki67-expressing CD4⁺ T cells in uninfected macaques ($n = 3$) or in SIV-infected macaques sacrificed at 14 days p.i. ($n = 3$) is provided in graph form. Significant differences in the percentages of either CCR5- or Ki67-expressing CD4⁺ T cells from the PBMCs or MLNs were not observed. Representative examples of FACS profiles from at least three different SIV-infected or uninfected macaques are shown. FITC, fluorescein isothiocyanate.

(versus 35 to 58% in uninfected macaques) and remained depleted throughout infection (Fig. 1B, left). In order to determine the effect of this depletion on CD4⁺ T-cell dynamics in the GALT, we positively selected CD4⁺ T cells from the ileal LP and further characterized them for the expression of FoxP3, CCR5, and Ki67. The purity of CD4⁺ T cells, positively selected from ileal LPLs, was consistently between 98 and 100% (Fig. 1B, right). CD4⁺ CCR5⁺ T cells were severely depleted from the GALT; among CD4⁺ T cells purified from ileal LPLs, the fraction of cells expressing detectable levels of CCR5 decreased from a mean level of 43.5% in uninfected macaques to a mean of 8.0% in SIV-infected macaques by day 14 p.i. ($P = 0.008$) (Fig. 2A). However, there was no detectable depletion of CD4⁺ CCR5⁺ T cells from PBMCs or mesenteric lymph nodes (MLNs) during acute infection. The frequency of LP CD4⁺ CCR5⁺ T cells had decreased even further by day 114 p.i. (data not shown). These results confirm the rapid loss of CCR5-expressing cells in this system and, given that CCR5 is expressed on ~80% of Tregs (25), raise the possibility of a virus-induced loss of Tregs in the intestinal LP. By day 14 p.i., the percentage of proliferating CD4⁺ T cells, as measured by intracellular staining for Ki67, was significantly increased in the ileal LP of infected animals ($P < 0.05$), demonstrating an increase in the activation and proliferation of lymphocytes from that compartment (Fig. 2B). No significant differences were detected in the percentages of proliferating CD4⁺ T cells from the PBMCs or MLNs.

Decreased levels of FoxP3 expression in the ileal LP of SIV-infected macaques. To determine the prevalence of Tregs in the GALT of SIV-infected and uninfected macaques, we quantified the expression of FOXP3 in GALT-derived CD4⁺ T cells by quantitative real-time RT-PCR as described previously (38). Tregs specifically and exclusively express FOXP3, a transcription factor that plays a key role in their development and function (9, 14, 15). FOXP3 mRNA levels were significantly lower ($P = 0.012$) in ileal LPLs from SIV-infected macaques than in those from uninfected macaques (Fig. 3A). FOXP3 copy numbers had decreased by day 4 p.i. and remained well below normal levels at day 114 in all SIV-infected macaques. In addition, FOXP3 was also depleted from all SIV-infected jejunal LPLs as well as jejunal and ileal intraepithelial lymphocyte samples (data not shown). In contrast, FOXP3 copy numbers in SIV-infected and uninfected macaques were compara-

ble in CD4⁺ T cells from the lymph nodes (inguinal and mesenteric) and peripheral blood (Fig. 3A).

Consistent with the observed decrease in FOXP3 mRNA, the expression of the FoxP3 protein in ileal LPLs, as determined by intracellular staining at day 14 p.i., was significantly decreased compared to that from uninfected macaques ($P < 0.05$). Depletion of Tregs, as assessed by FoxP3 protein expression in CD4⁺ T cells, was not apparent in the PBMCs or MLNs of the same SIV-infected macaques ($P = 0.16$ and 0.518 , respectively) (Fig. 3B). FoxP3 protein expression remained depleted in LP CD4⁺ T cells at day 114 p.i. (data not shown). We also examined the expression of high levels of CD25 (CD25^{hi}) on ileal LP CD4⁺ T cells from both uninfected and SIV-infected macaques at day 14 p.i. Regulatory T cells express high levels of CD25 and comprise about 10% of CD4⁺ T cells (18). All SIV-infected macaques analyzed at day 14 p.i. demonstrated a significant decrease ($P = 0.005$) in the percentage of CD4⁺ CD25^{hi} T cells from ileal LP CD4⁺ T cells compared to levels in uninfected macaques (Fig. 3C). In addition, intestinal CD4⁺ T-cell surface expression of CTLA-4, a functional mediator of Treg activity that is constitutively expressed on Tregs (30), decreased from a mean level of 23.5% in uninfected macaques to 8.7% in SIV-infected macaques on day 14 p.i. (data not shown).

In addition, Western blotting showed a strong decrease in FoxP3 protein expression in ileal LPL samples from SIV-infected macaques (Fig. 3D). Ileal expression of FoxP3 was decreased at day 14 and absent at day 114 p.i.; however, FoxP3 was readily detectable in the PBMCs, ileum, and MLN of an uninfected macaque. Normalization to α -vinculin showed a 20% decrease in FoxP3 expression by day 14 and a nearly total decrease by day 114 compared to the uninfected sample (Fig. 3D). FoxP3 protein expression in the PBMCs and MLNs from SIV-infected macaques did not differ from that in PBMCs and MLNs from uninfected macaques by Western blotting (data not shown).

Lack of Treg suppressor activity in SIV-infected GALT samples. Tregs suppress the activation and proliferation of effector cells. To provide a functional correlate for our findings of decreased ileal FoxP3 mRNA and protein levels, we measured the suppressor activity of Tregs from the ileal LP of SIV-infected and uninfected macaques using a previously described indirect suppression assay (34) (Fig. 4). For these assays, the

FIG. 3. Decreased FoxP3 mRNA and protein levels in CD4⁺ LPLs from SIV-infected macaques. (A) Quantitative real-time RT-PCR of FOXP3 expression in different tissues from uninfected (triangles) and infected (circles) macaques. FOXP3 expression was significantly decreased in ileal samples from SIV-infected macaques compared to that in ileal samples from uninfected macaques ($P = 0.012$). No obvious trend was observed in CD4⁺ T cells from the lymph nodes (inguinal and mesenteric) or peripheral blood. The copy number of FOXP3 in each sample was normalized to 18S rRNA. Samples were obtained at necropsy, days 4, 14, and 114 p.i. Horizontal lines represent the means for the SIV-infected macaques (red) and uninfected macaques (black). (B) Flow cytometric analysis of FoxP3-positive cells at day 14 p.i. Cells expressing the FoxP3 protein were depleted in SIV-infected ileal LPLs ($P < 0.05$) but present in the CD4⁺ T cells from the PBMCs and MLNs of the same macaques. At least three different SIV-infected or uninfected macaques were analyzed. Representative FACS profiles from day 14 p.i. are shown. A summary of the FACS profiles for all animals analyzed at this time point is provided in graph form. PE, phycoerythrin. (C) Flow cytometric analysis of the percentage of CD4⁺ CD25^{hi} T cells from ileal LP CD4⁺ T cells in uninfected macaques ($n = 3$) and SIV-infected macaques ($n = 3$) necropsied at day 14 p.i. (D) FoxP3 protein expression detected by Western blotting in CD4⁺ T cells from various tissues from humans and uninfected (uninf.) and SIV-infected macaques. FoxP3 protein expression was decreased in ileal CD4⁺ T cells isolated from SIV-infected macaques at days 14 ($n = 3$) and 114 ($n = 3$) p.i. In contrast, FoxP3 was readily detected in CD4⁺ T cells of PBMCs, MLN, and ileal LP from an uninfected macaque. To confirm the early depletion of FoxP3, Western blotting was repeated with all of the macaques at day 14 p.i. α -Vinculin was used as a loading control.

FIG. 4. Effect of depletion of CD25⁺ cells on the mitogenic response of ileal LPLs to T-cell receptor cross-linking. (A) CD4⁺ T cells isolated from the ileum of an uninfected macaque or an SIV-infected macaque at day 14 p.i. were labeled with CFSE and cultured with or without anti-CD3 stimulation and irradiated autologous PBMCs. Dilution of CFSE was measured by flow cytometry at day 4 poststimulation. A summary of the degree of suppression is provided in graph form (right). (B) Quantitative real-time RT-PCR for FOXP3 verifying that Tregs had been removed by CD25 depletion in both an uninfected macaque and an SIV-infected macaque. Prior to depletion, total ileal LP CD4⁺ T cells from the SIV-infected macaque contained significantly lower levels of FOXP3 than those from the uninfected macaque ($P < 0.01$). Data are representative of three separate experiments.

proliferative responses of CD4⁺ T cells and CD4⁺ T cells depleted of their CD25⁺ subset (CD4⁺ CD25⁻ T cells) were compared. Wells containing the CD25⁺ subset should include Tregs, which are expected to limit the division of naïve cells stimulated by T-cell receptor cross-linking. Using this assay, we found that suppressor activity was much greater in ileal CD4⁺ T cells from uninfected macaques than in ileal CD4⁺ T cells from SIV-infected macaques at day 14 p.i. (Fig. 4A). To ensure

that we had depleted Tregs from the ileal LP CD4⁺ T cells, quantitative real-time RT-PCR for FOXP3 was performed on the CD25-depleted populations from both uninfected and SIV-infected macaques (Fig. 4B). CD4⁺ T cells depleted of their CD25⁺ subset exhibited very low levels of FOXP3. In addition, prior to depletion, the expression of FOXP3 in total ileal CD4⁺ T cells from SIV-infected macaques at day 14 p.i. was significantly lower than that in total ileal CD4⁺ T cells

FIG. 5. Mathematical model of CD4⁺ T-cell dynamics in the GALT. (A) Schematic of the model. (B) Dependence of steady-state total GALT CD4⁺ T-cell numbers from SIV infection on the resting CD4⁺ T-cell activation rate, α_Q . (C) Time courses for total CD4⁺ T-cell number in the setting of low-level GALT CD4⁺ T-cell activation ($\alpha = 0.002$ day⁻¹) (black) and in the setting of rapid GALT depletion of Tregs on the background of low-level GALT CD4⁺ T-cell activation [$\alpha_Q = 0.002 + 0.018(1 - e^{-0.05t})$ day⁻¹] (blue). B and C are defined by equations 1 to 3, where λ equals 16,400 cells/day, r equals 0.009 day⁻¹, β equals 0.000015 cell⁻¹ day⁻¹, δ_Q equals 0.002 day⁻¹, and δ equals 0.693 day⁻¹. Ag, antigen; CTL, cytotoxic T lymphocyte.

from uninfected macaques, consistent with the lack of suppressor activity. These results suggest that the decreased expression of FoxP3 mRNA and protein in ileal CD4⁺ T cells from SIV-infected macaques is manifested as a functional loss of Treg-mediated suppression.

CD4⁺ T-cell depletion in the GALT can be explained by the depletion of Tregs. In sites of increased immune activation, such as the GALT during acute SIV infection, Tregs suppress polyclonal T-cell priming and/or expansion, thereby decreasing overall activation-induced cell death (Fig. 5A). Mathematical models have reproduced the clinically observed CD4⁺ T-cell

dynamics of HIV-1 infection and have offered additional insights into HIV-1 pathogenesis (2, 11, 27, 28). We applied a previously described mathematical model to determine whether Treg depletion in the GALT could lead to CD4 depletion in this site as a result of increased immune activation (5). We examined the relationship between the activation rate, α_Q , and the steady-state total CD4⁺ T-cell number, $\bar{T}_{tot}$. This model yields the interesting general prediction that despite the proliferation induced by T-cell activation, increased activation will result in greater CD4⁺ T-cell depletion in the setting of acute SIV infection (Fig. 5B). This result reflects the fact that

increased activation increases the availability of target cells, which become infected and succumb rapidly to viral cytopathic effects or cytotoxic T-lymphocyte-mediated cytolysis (Fig. 5A). The rapid loss of Tregs in the GALT results in a sharp decline in the steady-state $CD4^+$ T-cell number compared to macaques that control $CD4^+$ T-cell activation (Fig. 5C). The results suggest that Treg depletion augments $CD4^+$ T-cell depletion in the GALT.

DISCUSSION

Recent studies suggest that a massive depletion of $CD4^+$ T cells occurs during acute infection, particularly in the GALT, where a substantial portion of the $CD4^+$ $CCR5^+$ T cells in the body reside (3, 4, 10, 19, 21–23, 29, 36). As a result of frequent exposure to environmental antigens, the gastrointestinal mucosa is rich in recently activated cells and is thus an ideal site for viral replication. Massive viral replication may account for the extensive immune hyperactivation seen in the GALT during acute SIV and HIV-1 infection (21, 23), and there is a strong need to control the pool of activated target cells. A recent study by Mehandru et al. reported $CD4^+$ T-cell hyperactivation in the GALT concomitant with the massive $CD4^+$ T-cell depletion that is observed there during acute and early HIV-1 infection (23). Given that Tregs suppress the activation and proliferation of effector lymphocytes, their decline during acute HIV/SIV infection would in turn reduce the active suppression of conventional T cells and, hence, contribute to immune hyperactivation.

The findings presented here provide strong evidence that SIV-mediated GALT depletion affects the distribution of Tregs in the GALT. Our data demonstrate a marked decrease in FoxP3 mRNA and protein levels by day 14 p.i., which we find to be concomitant to the loss of functional suppressive activity in the GALT of SIV-infected macaques. $CD4^+$ T-cell destruction leads to the loss of critical regulatory functions and provides a continuous pool of activated T cells that maintain SIV replication. Tregs are not present to suppress the persistent inflammation resulting from continuous rounds of infection and activation of $CD4^+$ T cells, thereby providing an optimal site for SIV pathogenesis and $CD4^+$ T-cell depletion (Fig. 5A). Similarly, our mathematical simulations of $CD4^+$ T-cell dynamics predict that the rapid loss of Tregs contributes to a dramatic decrease in the steady-state number of GALT $CD4^+$ T cells in the setting of SIV infection due to the greater availability of activated target $CD4^+$ T cells. Once a state of $CD4^+$ T-cell hyperactivation has been achieved, there is an ensuing loss of effector cells, which ultimately manifests as increased susceptibility to opportunistic infections. Further work to examine the temporal relationship between gut-associated Treg depletion and activated $CD4^+$ T-cell depletion is needed to confirm the causative role of Treg loss in $CD4^+$ depletion.

The depletion of Tregs observed here may occur as a consequence of the same pathogenic process that depletes $CD4^+$ $CCR5^+$ T cells from the GALT. Given evidence for the extensive infection of GALT $CD4^+$ T cells during the acute phase (21, 23), it is possible that the Treg population in the GALT is depleted by direct viral cytopathic effects. We have shown that FoxP3⁺ Tregs from HIV-infected individuals are positive for

HIV-1 DNA by real-time PCR (our unpublished data). In addition, FoxP3 has recently been shown to enhance gene expression from the HIV-1 long terminal repeat (13), suggesting that infected Tregs may experience enhanced effects of HIV-1 gene transcription including the associated cytopathic effects.

Relocation to other anatomical sites could account for our observed loss of GALT Tregs. A recent study by Estes et al. showed an early increase in the frequency of Tregs in the LNs during acute SIV infection of rhesus macaques (7). In addition, a study by Nilsson et al. showed that FOXP3 mRNA expression was increased in LNs from SIV progressors relative to SIV nonprogressors (24). In our system, however, analysis of $CD4^+$ T cells from the peripheral blood as well as mesenteric and inguinal lymph nodes showed similar FoxP3 expression levels in SIV-infected macaques compared to those in uninfected macaques, suggesting that relocation from the GALT to other sites either does not occur or is very transient. Even if Treg relocation is occurring, the repopulation of ileal Tregs did not take place during the time frame of our studies. We cannot rule out the possibility that the rapid depletion of Tregs observed here is unique to the SIV/pigtailed macaque model of HIV-1 disease used in our study. In this model, animals consistently develop AIDS within several months. In addition, we found that the percentage of $CD4^+$ Ki67⁺ cells was high in the PBMCs of both uninfected and SIV-infected pigtailed macaques. Thus, an important caveat with studies using pigtailed macaques is that higher numbers of activated and proliferating cells could result in the acceleration of SIV infection and pathogenesis. A recent study of chronic HIV-1 infection by Epplé et al. showed increases in duodenal Tregs that subsequently normalize after highly active antiretroviral therapy. That study suggests that Tregs are able to repopulate the gastrointestinal tract later in infection, when levels of viral replication are much lower than during the acute phase (6).

In summary, we have shown that the GALT Treg population, crucial to controlling immune hyperactivation, is rapidly depleted in acute SIV infection. Mathematical simulations of $CD4^+$ T-cell dynamics suggest that their loss contributes directly to ongoing $CD4^+$ T-cell loss. Our findings underscore the need to implement therapeutic strategies designed to control immune hyperactivation early in acute HIV/SIV infection in order to limit GALT depletion of $CD4^+$ T cells.

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