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Expansion of FOXP3⁺ CD8 T Cells with Suppressing Potential in Colorectal Mucosa Following a Pathogenic Simian Immunodeficiency Virus Infection Correlates with Diminished Antiviral T Cell Response and Viral Control

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FOXP3⁺CD8⁺ T cells are present at low levels in humans; however, the function of these cells is not known. In this study, we demonstrate a rapid expansion of CD25⁺FOXP3⁺CD8⁺ regulatory T cells (Tregs) in the blood and multiple tissues following a pathogenic SIV infection in rhesus macaques. The expansion was pronounced in lymphoid and colorectal mucosal tissues, preferential sites of virus replication. These CD8 Tregs expressed molecules associated with immune suppressor function such as CTLA-4 and CD39 and suppressed proliferation of SIV-specific T cells in vitro. They also expressed low levels of granzyme B and perforin, suggesting that these cells do not possess killing potential. Expansion of CD8 Tregs correlated directly with acute phase viremia and inversely with the magnitude of antiviral T cell response. Expansion was also observed in HIV-infected humans but not in SIV-infected sooty mangabeys with high viremia, suggesting a direct role for hyperimmune activation and an indirect role for viremia in the induction of these cells. These results suggest an important but previously unappreciated role for CD8 Tregs in suppressing antiviral immunity during immunodeficiency virus infections. These results also suggest that CD8 Tregs expand in pathogenic immunodeficiency virus infections in the nonnatural hosts and that therapeutic strategies that prevent expansion of these cells may enhance control of HIV infection. The Journal of Immunology, 2010, 184: 1690–1701.

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ergulatory T cells (Tregs) have a fundamental role in suppressing autoimmune manifestations and maintaining tolerance in peripheral tissues (1–5). They exist in both CD4⁺ and CD8⁺ T cell compartments; however, CD4 Tregs have been better characterized. Multiple subsets of CD4 Tregs have been defined. These include natural Tregs, type 1 Tregs, and Th3 cells (6). Natural CD4 Tregs are characterized by constitutive expression of transcription factor FOXP3 and high levels of IL-2 receptor α-chain (CD25) (7–9). These cells act in an Ag-nonspecific manner by a cell-to-cell contact-dependent mechanism (10–13). In contrast, the type 1 Tregs and Th3 cells do not constitutively express FOXP3 and act via secretion of cytokines such as IL-10 and TGF-β, respectively (14, 15).

FOXP3 is thought to be the most specific marker for CD4 Tregs. In mice, FOXP3 is strictly correlated with regulatory activity. However, human-activated effector T cells have been shown to express FOXP3 following stimulation in vitro (16–19). These FOXP3 expressing activated T cells possess the classic phenotype of Tregs, however, may (20) or may not (17, 18) possess suppressive function. Thus, expression of FOXP3 alone by human T cells is not indicative of their suppressive capacity.

FOXP3⁺CD8⁺ T cells have not been reported in mice. However, a small fraction of CD8⁺ T cells in human blood express FOXP3 (21), and characterization of these cells ex vivo has been difficult because of their low frequency (22). Currently, it is not known how these cells develop in vivo. Many studies demonstrated the induction of FOXP3⁺CD8⁺ T cells in vitro following polyclonal activation or peptide-specific simulation of human PBMCs (20, 23–27). These in vitro-generated FOXP3⁺CD8⁺ T cells have been shown to suppress T cell activation by inducing a tolerogenic phenotype in APCs (23). Very little is known about the modulation of FOXP3⁺CD8⁺ T cells in vivo following an infection or a disease. Similarly, very little is known about their suppressive potential in vivo. A recent study demonstrated the expansion of FOXP3⁺CD8 Tregs with suppressive potential at the tumor site in patients with colorectal cancer (28). The expansion of FOXP3⁺CD8 Tregs with suppressive potential has not yet been reported following an infection in vivo. Tregs may play an important role in influencing viral control and disease progression during chronic HIV/SIV infections. On one hand, they may suppress antiviral cellular immunity and thereby diminish viral control. On the other hand, they may suppress hyperimmune activation, a strong predictor for rapid disease progression, and thus may prolong survival. Several studies evaluated the fate of CD4 Tregs during HIV (29–33) and SIV (34–37) infection and demonstrated a loss of these cells following infection, suggesting that these cells may not contribute for suppression of antiviral immunity or hyperimmune activation. Although these studies characterized CD4 Tregs, there are very little data available on the fate of CD8 Tregs during HIV and SIV infections. It is possible that HIV/SIV infections induce CD8 Tregs
to suppress antiviral immunity. Indeed, a few recent studies demonstrated a low-level expansion of FOXP3+ CD8+ T cells in the blood of SIV-infected cynomolgous macaques and HIV-infected humans (21, 38, 39). However, these studies did not evaluate the suppressive capacity of these cells. In addition, it is important to study the fate of Tregs in the lymphoid tissue and gut (the major reservoirs of HIV/SIV) and their influence on antiviral T cell response and hyperimmune activation in vivo. In view of the depletion of CD4 Tregs during SIV infection, we hypothesize that CD8 Tregs capable of suppressing antiviral immune responses may be induced following HIV/SIV infection.

In this study, we conducted a thorough evaluation of the magnitude of CD25+FOXP3+CD8+ T cells (CD8 Tregs) in multiple tissues including intestinal mucosa following SIV infection in rhesus macaques and studied their influence on antiviral T cell response in vitro and in vivo. Our results demonstrate the expansion of CD8 Tregs with suppressive capacity in blood and multiple tissues following infection. The expansion was higher at the preferential sites of virus replication such as lymphoid and colorectal tissue than in blood. Importantly, the early expansion of these cells correlated inversely with acute-phase antiviral CD4+ and CD8+ T cell response. Expansion of these cells was not observed in SIV-infected sooty mangabeys despite these animals having high viremia. These results demonstrate an important but previously unappreciated role for CD8 Tregs in suppressing antiviral immunity during immunodeficiency virus infections in their nonnatural hosts and suggest a direct role for hyperimmune activation and an indirect role for viremia in their induction.

Materials and Methods

Nonhuman primates and SIV infection

Young adult rhesus macaques and sooty mangabeys from the Yerkes breeding colony were cared for under 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. Rhesus macaques were infected with SIVmac251 either i.e.v. or intrarectally at a dose of 100 or 1000 of 50% tissue culture-infective dose, respectively. Dr. N. Miller at NIH (Bethesda, MD) provided the challenge stock. All animals were infected under these conditions. Some of the SIV-infected macaques were treated with antiretroviral drugs R-9-2-phosphonomethoxypropyl) adenine (20 mg/kg), (+)-2-deoxy-5-fluoro-3'-thiacytidine (30 mg/kg), Kaletra (Lopinavir, 12 mg/kg; Ritonavir, 3 mg/kg), and 3'-azido-3'-deoxythymidine (5 mg/kg) at ~18 wk following infection. Sooty mangabeys were housed in colonies of 50–60 animals, and SIVsm is endemic in this population. None of the animals used in the study was experimentally infected.

Human subjects

PBMCs obtained from uninfected (HIV negative) and untreated HIV-infected individuals were used for analyses. The characteristics of the HIV-infected individuals have been presented previously (40). All of the HIV-infected individuals were recruited at the Emory Center for AIDS Research (ECAR) of the Emory University. Enrollments of the Emory University Institutional Review Board approved this study. Signed informed consents were obtained from all individuals before enrollment in the study.

Cell isolation from blood and rectal biopsies

PBMCs were isolated from whole blood according to the standard Ficoll-Hypaque separation procedures as described before (41). Lymphocytes from pinch biopsies from the rectum were obtained as described before (42). Briefly, 10–20 pinch biopsies were collected in complete RPMI 1640 and washed twice with ice-cold HBSS. Biopsies were digested with 200 U/ml collagenase IV (Worthington, Lakewood, NJ) and DNase I (Roche, Indianapolis, IN), passed through decreasing sizes of needles of the Emory University. The Emory University Institutional Review Board approved this study. Signed informed consents were obtained from all individuals before enrollment in the study.

Suppressor assay

PBMCs were CFSE stained according to previously described protocol (45). Cells were then incubated with CD4 microbeads (Miltenyi Biotec) for 15 min at 37°C and were negatively selected on LD columns (Miltenyi Biotec). Column-bound CD4+ cells were reserved at 4°C for later usage. Cells from the flow through of LD columns (CD4−depleted cells) were incubated with CD25 microbeads (Miltenyi Biotec) for 15 min at 37°C and were positively selected on MS columns (Miltenyi Biotec). Column-bound CD4+ cells were considered as CD4 T cells, and CD3+CD8−CD4+ cells were considered as CD8 T cells. These CD4 or CD8 T cells were then gated for cytotoxic-positive cells.

Staining for FOXP3+ cells

FOXP3+CD25+ T cells were measured directly ex vivo. Two million PBMCs or 1 million cells from colorectal mucosa were resuspended in 100 μl RPMI 1640 plus 10% FBS. Cells were stained with LIVE/DEAD marker (Invitrogen, Carlsbad, CA), followed by surface staining with Abs against CD3 (BD Pharmingen), CD4 (PerCP, clone L200; BD Pharmingen), CD8 (Qdot 655, clone SK1; BD Biosciences, San Jose, CA), CD25 (PE, clone 4E3; Miltenyi Biotec, Auburn, CA), CD95 (Paclue, clone DX2; Caltag Laboratories, Burlingame, CA), and CD28 (PE-Cy7, clone CD28.2; eBioscience, San Diego, CA) for 30 min at 4°C. Cells were washed with PBS with 2% FBS and fixed with 1× fix/perm buffer (Biolegend, San Diego, CA) for 20 min at room temperature. Cells were then washed once with permeabilization solution (Biolegend) and incubated in this solution for 15 min. Cells were washed once with PBS with 2% FBS and incubated for 30 min with mAb to FoxP3 (Alexa Fluor 488, clone 206D; Biolegend). Cells were then washed twice with PBS with 2% FBS and resuspended in 1% formalin in PBS. Samples were acquired on LSRII (BD Immunometry Systems, San Jose, CA) and analyzed using FlowJo software (Tree Star, Ashland, OR). Lymphocytes were identified based on live cells, followed by scatter pattern, and CD3−CD8−CD4+ cells were considered as CD4 T cells, and CD3−CD8−CD4+ cells were considered as CD8+ T cells (Fig. 1A). The gates for FOXP3+ cells were defined based on the lack of FOXP3 expression on non-CD3+ cells within the same sample.

For analysis of the expression of intracellular Ki-67 (clone B56, BD Pharmingen), surface CD127 (clone R34.34; Beckman Coulter, Fullerton, CA) and surface CTLA-4 (clone BN13; BD Pharmingen), intracellular granzyme B (clone GB11; BD Biosciences), and intracellular perforin (PF344; Mabtech, Cincinnati, OH), a similar procedure was used, except that PE anti-CD25 Ab was replaced with Ab against the respective marker. For analysis of tetramer-positive cells, cells were stained with Ab against human CD3, CD5, and FOXP3, and Gag CM9 tetramer (43).

Intracellular cytokine staining analysis

Intracellular cytokine production was assessed as previously described with few modifications (44). Briefly, 2 million PBMCs were stimulated in 200 μl RPMI 1640 with 10% FBS in a 5-ml polypropylene tube. Peptide pools (15-mer overlapping by 11) specific for SIV Gag and Env were used for stimulations at a final concentration of 1 μg/ml. Staphylococcal enterotoxin B (SEB) was used as a positive control at 1 μg/ml. Stimulations were performed in presence of anti-CD28 and anti-CD49d Abs (1 μg/ml; BD Pharmingen). Cells were incubated at 37°C in the presence of 5% CO2 for 6 h. Brefeldin A (10 μg/ml) was added after 2 h of incubation. At the end of stimulation, cells were washed once with PBS containing 2% FBS, surface stained for 30 min at 4°C with anti-human CD4 (clone L200; BD Pharmingen), anti-human CD3 (clone SP34-2; BD Pharmingen), and anti-human CD8 (clone SK1; BD Biosciences). Cells were fixed with cytotoxic/cytopermin (BD Pharmingen) for 20 min at 4°C and permeabilized with 1× permeabilization buffer (BD Pharmingen) for 15 min at 4°C. Cells were then incubated with anti-human IFN-γ Ab, washed twice with 1× permwash, once with 2% FBS in PBS, and resuspended in 1% formalin in PBS. Approximately 500,000 lymphocytes were acquired on the LSRII (BD Immunometry Systems) and analyzed using FlowJo software (Tree Star). Lymphocytes were identified based on their scatter pattern and CD3+CD8−CD4+ cells were considered as CD4 T cells, and CD3+CD8+CD4− cells were considered as CD8+ T cells. These CD4 or CD8 T cells were then gated for cytokine-positive cells.
(Invitrogen), anti-CD3 (Allophycocyanin-Cy7, clone SP34-2; BD Pharminigen), anti-CD4 (PerCP, clone L200; BD Pharmingen), anti-CD8 (Qdot 655, clone SK1; BD Biosciences), and intracellularly for anti-Ki-67 (PE, clone B56; BD Pharmingen). Proliferation status was assessed by CFSE dilution and expression of Ki-67 nuclear protein.

Quantitation of SIV RNA plasma load

The SIV copy number was determined using a quantitative real-time PCR as described previously (44). All specimens were extracted and amplified in duplicates, and the mean results are reported.

Statistical analysis

The Wilcoxon signed-rank test was used to compare the frequency of FOXP3+ T cells between blood and colorectal tissue and between different time points after SIV infection. The Wilcoxon rank-sum test was used to compare the frequency of FOXP3+CD8 T cells in humans and sooty mangabeys. The Spearman’s rank correlation coefficient ($r_s$) or the Pearson’s product moment correlation coefficient ($r$) was used to assess the relationship between viral load, percentage of FOXP3+ cells, and percentage of antiviral T cells. If the data followed the parametric assumptions, Pearson’s method was used; otherwise, Spearman’s method was used. A two-sided $p < 0.05$ was considered statistically significant. Statistical analyses were performed using software program S-PLUS 7.0.

Results

FOXP3+ CD8 T cells are present at low levels in blood and colorectal tissue of normal macaques

We measured the steady-state levels of CD4+ and CD8+ T cells that coexpress FOXP3 and CD25 in blood and colorectal mucosal tissue of normal (SIV negative) macaques to understand the relative levels and distribution of regulatory T cells. FOXP3+CD25+CD4+ T cells (referred to as CD4 Tregs hereafter) were readily detected in peripheral blood as well as colorectal mucosa (Fig. 1). The frequencies of CD4 Tregs were generally similar in the two compartments with a geometric mean frequency of 4.8% of total CD4+ T cells (Fig. 1A, 1B). In contrast to CD4+ T cells, only a small fraction of CD8+ T cells coexpressed FOXP3 and CD25 (referred to as CD8 Tregs hereafter) (Fig. 1A, 1B). The frequencies of these CD8 Tregs in normal macaques had geometric mean of 0.2% in blood and 0.12% in the colorectal mucosal tissue. These results demonstrate that in normal macaques CD8 Tregs are present at much lower levels than CD4 Tregs (Fig. 1B) and suggest that the latter play a greater role in maintaining T cell homeostasis and tolerance to self-Ags. In addition, the differences in frequencies could also point to differences in mechanisms used by the two cell types.

Rapid expansion of CD8 Tregs following SIV251 infection: higher expansion in colorectal mucosa than blood

To understand the role of CD8 Tregs in SIV infection, we studied the frequencies of these cells both in blood and colorectal mucosa of 17 rhesus macaques that were infected with SIVmac251. Analyses were performed before and at 2, 3, 6, 12, and 24 wk following infection. The frequencies of CD8 Tregs expanded in both blood and colorectal mucosal tissue following SIV infection. In blood, CD8 Tregs peaked between 2 and 6 wk and ranged from 0.4 to 1.5% of total CD8+ T cells (Fig. 2B). Peak levels were ~2.5-fold higher than their prechallenge levels ($p = 0.003$) and remained fairly constant over the next 18 wk (data not shown). Similar or higher increases were also observed for the absolute number of CD8 Tregs in blood following SIV infection (Fig. 2C).

A greater expansion of CD8 Tregs (4.3-fold) was observed in the colorectal mucosa than in blood following SIV infection ($p < 0.001$) (Fig. 2A, 2B). In the colorectal mucosa, CD8 Tregs generally peaked at week 6 and then remained fairly constant during

![FIGURE 1. FOXP3+ T cells in normal rhesus macaques. A, Gating pattern for CD4 and CD8 FOXP3+ cells in peripheral blood (PBMCs) and colorectal mucosa. T cells were identified based on live/dead cell marker exclusion, followed by side scatter (SSC) and CD3, followed by forward scatter (FSC) and CD3. CD3+CD4+CD8+ cells were identified as CD4 T cells, and CD3+CD4−CD8+ cells were identified as CD8 T cells. FOXP3+CD25+CD4+ T cells were identified as CD4 Tregs, and FOXP3+CD25−CD8+ T cells were identified as CD8 Tregs. B, Summary of the frequency of FOXP3+ CD4 and CD8 T cells as a percentage of respective total cells in blood and colorectal mucosa of normal macaques. The horizontal lines represent geometric mean for the group. These experiments were repeated at least twice, with eight macaques or more per experimental group.](http://www.jimmunol.org/Downloadedfrom)
the next 18 wk (data not shown). The peak levels of these cells ranged from 0.5 to 6.7% of total CD8+ T cells and were \( \sim 14 \)-fold higher than the levels observed prior to infection \( (p, 0.001) \). A similar pattern was also observed for the absolute number of CD8+ Tregs in the colorectal mucosal tissue (Fig. 2D).

To assess whether expansion of CD8 Tregs is a reflection of global T cell activation that occurs during viral infections, we compared the magnitude and kinetics of expansion of CD8 Tregs and total activated CD8+ T cells (CD8+CD25+) both in blood and colorectal mucosal tissue (Fig. 2C, 2D). In colorectal tissue, the expansion of total-activated CD8+ T cells occurred by 2 wk, whereas in blood, it occurred by 6–12 wk (Fig. 2D). These levels were generally 10-fold higher compared with the magnitude of CD8 Tregs in the respective tissue. In addition, the expansion of total activated CD8+ T cells peaked later in blood and earlier in colorectal tissue compared with CD8 Tregs.

To further understand whether FOXP3+CD8+ T cells are generated following activation in vitro, we stimulated PBMCs for 5 d with anti-CD3 and anti-CD28 and stained for FOXP3 and CD25. The percentages on the FACS plots represent the frequency of FOXP3+CD25+CD8+ T cells as a percentage of total CD8+ T cells. Each symbol represents an individual macaque \( (n = 17) \). C, Summary of absolute number of CD8+CD25+FOXP3+ or CD8+CD25+ T cells per milliliter of blood. D, Summary of number of CD8+CD25+FOXP3+ or CD8+CD25+ T cells per 100,000 lymphocytes in gut following SIV challenge. E, Representative FACS plot for detection CD8+CD25+FOXP3+ T cells after culture for 5 d with or without anti-CD3 and anti-CD28 stimulation \( (left panels); \) summary of CD8+CD25+FOXP3+ and CD8+CD25+FOXP3+ T cells poststimulations \( (right panels) \) \( (n = 3) \). F, Representative FACS plot for detection of CD8+CD25+FOXP3+ T cells in uninfected and HIV–1–infected humans. Cells were gated as described in Fig. 1 \( (left); \) summary of frequency of CD8+CD25+FOXP3+ T cells in uninfected and HIV–1–infected humans \( (right) \) \( (n = 10) \). Each symbol represents an individual animal in the experimental groups. Error bars represent means ± SEM. *p < 0.01; **p < 0.001.

FIGURE 2. Expansion of CD8+FOXP3+ T cells post-SIV infection in rhesus macaques. A, Representative FACS plots for detection of CD8+CD25+FOXP3+ T cells following SIV infection in peripheral blood and colorectal mucosa. CD8+ T cells were gated as described in Fig. 1 and analyzed for expression of FOXP3 and CD25. B, Summary of the frequency of CD8+CD25+FOXP3+ T cells expressed as a percentage of total CD8+ T cells. Each symbol represents an individual macaque \( (n = 17) \). C, Summary of absolute number of CD8+CD25+FOXP3+ or CD8+CD25+ T cells per milliliter of blood. D, Summary of number of CD8+CD25+FOXP3+ or CD8+CD25+ T cells per 100,000 lymphocytes in gut following SIV challenge. E, Representative FACS plot for detection CD8+CD25+FOXP3+ T cells after culture for 5 d with or without anti-CD3 and anti-CD28 stimulation \( (left panels); \) summary of CD8+CD25+FOXP3+ and CD8+CD25+FOXP3+ T cells poststimulations \( (right panels) \) \( (n = 3) \). F, Representative FACS plot for detection of CD8+CD25+FOXP3+ T cells in uninfected and HIV–1–infected humans. Cells were gated as described in Fig. 1 \( (left); \) summary of frequency of CD8+CD25+FOXP3+ T cells in uninfected and HIV–1–infected humans \( (right) \) \( (n = 10) \). Each symbol represents an individual animal in the experimental groups. Error bars represent means ± SEM. *p < 0.01; **p < 0.001.

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ggesting that the activation of macaque CD8+ T cells through TCR alone does not induce FOXP3 expression on these cells in vitro. Collectively, these results demonstrate that expansion of CD8 Tregs occurs independent of total-activated CD8+ T cells and suggest that CD8 Tregs may not represent a subset of activated CD8+ T cells.

To further assess the expansion of CD8 Tregs in HIV infection, we compared the levels of these cells in the blood of HIV-infected and uninfected individuals (Fig. 2F). CD8 Tregs were present at low levels (geometric mean frequency of 0.014% of total CD8+ T cells) in HIV-negative humans. However, these cells were readily detectable and were 14-fold higher (geometric mean frequency of 0.2% of total CD8+ T cells; \( p < 0.001 \)) in HIV-infected individuals. These results demonstrate that CD8 Tregs also expand following HIV infection in humans.

Selective expansion of CD8 Tregs in intestinal mucosa following SIV infection

We next determined the distribution of CD8 Tregs in various tissues of SIV-negative and SIV-positive macaques (Fig. 3). Tissues analyzed included blood, gut-associated lymph nodes, spleen, thymus, liver, genitalia, and various intestinal mucosa. In normal macaques, low levels of CD8 Tregs were detected in blood,
lymphoid tissues, liver, and genitalia, with highest levels detected in lymph nodes (~1.5% of total CD8+ T cells). Similarly, in SIV-infected macaques, CD8 Tregs were present in blood, lymphoid tissues, liver, and genitalia (Fig. 3). However, the levels of these cells tended to be higher in the lymphoid tissue of SIV-infected macaques than uninfected macaques.

Interestingly, CD8 Tregs were present mostly below our detection limit (<0.01%) in intestinal mucosal tissue of normal macaques (Fig. 3B). However, in contrast to SIV-negative macaques, CD8 Tregs were readily detected in intestinal mucosal tissue of SIV-infected macaques, with the highest levels in the large intestine (colon and rectum, ranging from 0.5 to 6% of total CD8+ T cells; p < 0.05) (Fig. 3B). These results demonstrate a selective expansion of CD8 Tregs in the intestinal mucosal tissue of rhesus macaques following SIV infection.

CD8 Tregs express molecules associated with regulatory function

We next sought to characterize CD8 Tregs during chronic SIV infection (weeks 12–24 post-SIV infection) for the expression of phenotypic markers that predict their function (Fig. 4A, 4B) such as CTLA-4 and ATP ectonucleotidase (CD39). At 24 wk postinfection, ~40% of CD8 Tregs expressed CTLA-4, which is known to participate in downregulating immune responses (46) (Fig. 4A, 4B). This phenotype was stable even 1 y postinfection (data not shown). Approximately 30% of CD8 Tregs expressed CD39 (Fig. 4A, 4B), which degrades ATP into cAMP, and has been identified as a regulatory T cell marker (Fig. 4A, 4B) (47, 48). ATP is critical for actively proliferating cells, and thus, degradation of this molecule creates a microenvironment that is not conducive for proliferation of effector cells. To assess whether CD8 Tregs have cytolytic capacity, we characterized these cells for expression of granzyme B and perforin. CD8 Tregs expressed lower levels of granzyme B and perforin than FOXP3+CD8+ T cells, suggesting that these cells may not possess killing potential (Fig. 4A, 4B). Similar to human CD4 Tregs (49, 50), CD8 Tregs in rhesus macaques also expressed low levels of CD127 (Fig. 4A, 4B). We next characterized the proliferation status of CD8 Tregs in vivo by staining for intranuclear expression of Ki-67 (Fig. 4A, 4B). Consistent with their expansion following infection, ~60% of CD8 Tregs were actively proliferating at 12 wk postinfection.

To study whether these CD8 Tregs produce IFN-γ, we stimulated cells isolated from mesenteric lymph nodes with PMA/ionomycin and stained for expression of IFN-γ and FOXP3 (Fig. 4C). About 10% of total FOXP3+ cells coexpressed IFN-γ that was marginally lower than the levels expressed by FOXP3+CD8+ T cells demonstrating that these CD8 Tregs can produce IFN-γ following stimulation (Fig. 4C). It is important to note that the mean fluorescence intensity of FOXP3 on IFN-γ-producing cells was lower compared with IFN-γ+ FOXP3+ cells. However, when cells were stimulated with a SIV Gag peptide pool, none of the FOXP3+CD8+ T cells coexpressed IFN-γ. Furthermore, none of the FOXP3+CD8+ T cells were positive for Gag-CM9 tetramer that binds to CD8+ T cells specific for the immunodominant epitope CM9 in Mamu A*01+ animals (43) (Fig. 4D). Collectively, these data suggest that these CD8 Tregs may not be specific for SIV Ags.

CD8 Tregs suppress antiviral T cell response in vitro

To assess whether the CD8 Tregs are capable of suppressing T cell proliferation, we depleted these cells and compared proliferation of total and CD8 Treg depleted cells in response to either SIV Ags or a mitogen, SEB (Fig. 5A). Selective depletion of CD8 Tregs was achieved by a sequential depletion of CD4+ cells followed by CD25+ cells and reconstitution with CD4+ cells as outlined in Fig. 5A. This procedure yielded a near complete depletion of CD8+CD25high Tregs (Fig. 5B). Selective depletion of CD8 Tregs either from PBMCs or cells obtained from colonic lymph nodes resulted in enhanced proliferation (2- to 33-fold; p < 0.05) of CD8+ T cells in response to stimulation with SIV Ags (Gag or Env) suggesting that these CD8 Tregs have suppressive potential (Fig. 5C, 5D). In two of the five samples tested, proliferation prior to CD8 Treg depletion was below our detection level (0.01%). However, after depletion of CD8 Tregs, proliferation was clearly detectable at ~2% of total CD8+ T cells (Fig. 5D). It is important to note that
this enhancement in proliferation was observed despite the presence of CD4 Tregs in the culture. This enhancement in proliferation in the absence of CD8 Tregs was not consistently observed following stimulation with SEB (Fig. 5C,5D).

To determine a direct evidence that CD8 Tregs exert suppression, in some experiments, we added these cells back to CD8 Treg-depleted cells at a CD8 Treg/total PBMC ratio of 1:12.5 and evaluated their ability to suppress proliferation of CD8+ T cells in response to stimulation with SIV Gag peptide pool. Addition of CD8 Tregs resulted in near total loss of proliferation of CD8+ T cells (p, 0.05) (Fig. 5D), indicating that these cells are highly potent at suppressing antiviral specific responses.

CD8 Tregs suppress antiviral T cell response in vivo

To assess whether CD8 Tregs had a potential role in suppressing the expansion of antiviral T cells in vivo, we compared the magnitude of SIV-specific cellular immune response and CD8 Tregs early following infection in blood (Fig. 6A). We observed an inverse correlation between the frequency of SIV-specific IFN-γ-producing CD8+ T cells (p = 0.043) or CD4+ T cells (p < 0.01) and the levels of CD8 Tregs at 2–3 wk postinfection (Fig. 6A).

To further understand the relationship between CD8 Tregs and antiviral CD8+ T cell response in the colorectal tissue, we compared the magnitude and kinetics of expansion of CD8 Tregs and CD8+ T cells specific for the immunodominant Gag-CM9 epitope in Mamu A*01+ animals (Fig. 6B). In agreement with the suppression of antiviral T cells in blood, we also observed an inverse relationship for the kinetics of expansion and contraction between CD8 Tregs and the Gag CM9-tetramer–positive T cells in the colorectal tissue. Importantly, in three of the four animals studied, the magnitude of CD8 Tregs was comparable to the magnitude of Gag CM9-tetramer–specific CD8+ T cells, suggesting that ratios of responder:Treg ratios similar to those frequently used for in vitro suppression assays can be achieved in vivo. Similar results were also observed in an additional six Mamu A*01+ rhesus macaques (data not shown). These results suggest an important role for CD8 Tregs in suppressing virus-specific T cell response during early stages of SIV infection in macaques.

High levels of CD8 Tregs correlate with high levels of viremia in rhesus macaques

To comprehend the relationship between the levels of CD8 Tregs and viral load during SIV infection, we compared the levels of these cells and viral load during chronic phase of SIV infection (week 10 or 12 postinfection) (Fig. 7A). A direct correlation between the plasma viral load and levels of CD8 Tregs in blood (p < 0.001) was observed (Fig. 7A), suggesting that high levels of viral RNA induce high levels of CD8 Tregs following infection. This correlation was true for both percent CD8 Tregs (Fig. 7A) and absolute number of CD8 Tregs (data not shown).
In contrast to CD8 Tregs, and as shown previously (34–37), a decline in the absolute number of CD4 Tregs was observed following SIV infection (Fig. 7B). This depletion was observed both in peripheral blood ($p < 0.01$) and colorectal mucosa ($p < 0.001$) and had occurred early following infection (Fig. 7B). Furthermore, depletion of CD4$^+$ Tregs was greater in colorectal mucosa than in blood (Fig. 7B). Depletion was also observed in other tissues such as gut-associated lymph nodes, spleen, and small intestine (data not shown). Consistent with the loss of CD4 Tregs, $\sim$25% of these cells expressed the viral coreceptor CCR5 (data not shown). An inverse correlation was observed between viremia and levels of CD4 Tregs in colorectal tissue at 10–12 wk postinfection ($p < 0.01$) (Fig. 7C). Collectively, these results suggest that the loss of CD4 Tregs might occur because of the cytopathic effects of viral infection as well as possible direct killing by the virus and indicate that CD4 Tregs play minimal role in modulating antiviral T cell responses during SIV infection.

To further understand the influence of viral load on CD8 Tregs, we evaluated their levels in SIV-infected macaques before and after antiretroviral therapy (ART) (Fig. 7D). Suppression of viral load with ART resulted in reduction in the frequency and absolute numbers of CD8 Tregs (data not shown) of CD8 Tregs in the colorectal mucosa ($p < 0.001$). In contrast, suppression of viral load with ART resulted in an increase in the frequency of CD4 Tregs in the colorectal mucosa ($p \leq 0.01$), indicating that reduction in viral load as a result of ART treatment can restore CD4$^+$ Treg levels. This may be a reflection of total CD4$^+$ T cell reconstitution that occurs after ART treatment. Interestingly, in the blood, ART did not influence the frequency or absolute number of either total CD8 or CD4 Tregs (Fig. 7D). However, we observed a significant reduction in the frequency of FOXP3$^+$ central memory CD8$^+$ T cells following ART (data not shown).

No expansion of CD8 Tregs in SIV-infected sooty mangabeys

To better understand whether the expansion of CD8 Tregs is a common feature of immunodeficiency viral infections, we sought to quantify these cells in SIV-infected sooty mangabeys (Fig. 8). Sooty mangabeys rarely progress to disease despite the presence of high viremia. The lack of disease progression in SIV-infected sooty mangabeys has been attributed to the absence of high levels of hyperimmune activation, which is normally seen in SIV-infected rhesus macaques and HIV-infected humans (51). We assayed eight sooty mangabeys chronically infected with SIVsm that
maintained persistent set point viral loads for >5 y (Fig. 8A). In SIV-negative sooty mangabeys, CD8 Tregs were present at low levels (<0.2% of total CD8+ T cells) both in the rectum and blood (Fig. 8B). Interestingly, the levels of these cells were similar between the SIV-infected and uninfected sooty mangabeys (Fig. 8B), demonstrating that CD8 Tregs did not expand during chronic SIV infection of the natural host. These results demonstrate that expansion of CD8 Tregs is not observed in all immunodeficiency virus infections and suggest that hyperimmune activation in addition to level of viremia may influence their expansion.

**Discussion**

Our study evaluating the magnitude of FOXP3+ CD8 Tregs following a pathogenic SIVmac251 infection in rhesus macaques clearly demonstrates a rapid expansion of these cells in the colorectal mucosal tissue, a preferential site of virus replication. These CD8 Tregs highly expressed molecules associated with immune suppressive function and suppressed proliferation of virus-specific T cells in vitro. In addition, our results demonstrate a role for these cells suppressing virus-specific T cell response early following infection in vivo. Collectively, these results suggest an important role for CD8 Tregs in regulating antiviral immunity and viral control following a pathogenic immunodeficiency virus infection.

Our study is the first to demonstrate suppressive capacity of CD8 Tregs in an infectious disease setting. The in vivo-generated CD8 Tregs following SIV infection suppressed antiviral T cell responses in vitro. In agreement with their suppressive capacity in vitro, we also observed an inverse correlation between the frequency of CD8 Tregs and antiviral T cells during the acute phase of infection suggesting their suppressive role in vivo. We did not consistently observe suppression of T cell responses following in vitro stimulation with SEB. This may be due to higher ratios of SEB-specific T cells:CD8 Tregs in comparison with relatively lower ratios of SIV-specific T cells:CD8 Tregs.

One of the interesting aspects of our study is that the expansion of CD8 Tregs is not observed in all immunodeficiency virus infections and suggest that hyperimmune activation in addition to level of viremia may influence their expansion.

**FIGURE 6.** Association between CD8 Tregs with antiviral T cell response in SIV-infected rhesus macaques in vivo. A, Association between peak (week 2 or 3) antiviral-specific CD8+ or CD4+ T cell response with CD8 Tregs post-SIV infection in blood (n = 22). B, Relationship between CD8 Tregs and CM9-Gag tetramer-positive T cells in Mamu A*p01 macaques in colorectal mucosa.
suggesting that these cells might have been infected and killed by the virus. Consistent with this hypothesis, previous studies (32, 38) as well as our study demonstrated that CD4 Tregs express viral coreceptor CCR5 and can be infected by the virus. Our results suggest that because human and simian immunodeficiency viruses infect CD4 Tregs, these viruses may use CD8 Tregs as a mechanism to mediate suppression of antiviral immunity during acute and chronic phases of infection.

Tregs are known to exert their suppressive effects through various mechanisms. These include cell-to-cell contact-dependent mechanisms using molecules such as CTLA-4 (5, 11, 52) and glucocorticoid-induced TNF receptor (53), degradation of ATP (54), secretion of cytokines such as IL-10 (55) and TGF-β (56), and sequestration of IL-2. The CD8 Tregs described in our study expressed CTLA-4 and CD39. CTLA-4 binds to the same ligands as costimulatory molecule CD28 and inhibits T cell activation. CD39 is an ectoenzyme that converts ATP into cAMP. Expression of CD39 by CD4 Tregs has been shown to be associated with their suppressive function (47, 48). We are yet to quantify the levels of cAMP harbored by these CD8 Tregs. However, our results suggest

**FIGURE 7.** Relationship between CD8 and CD4 Tregs with viral load in SIV-infected macaques and effect of ART of Tregs. A, Correlation between frequency of CD8 Tregs and plasma viral RNA during chronic infection (week 10 or 12 post-SIV) (n = 17). B, Number of CD4⁺CD25⁺FOXP3⁺ T cells per milliliter of blood (left); number of CD4⁺CD25⁺FOXP3⁺ T cells per 100,000 lymphocytes in colorectal mucosa (right) (n = 17). C, Correlation between absolute numbers of CD4 Tregs and plasma viral RNA during chronic infection (week 10 or 12 post-SIV) (n = 17). D, Influence of ART on CD8 and CD4 Tregs in blood and colorectal mucosa and viral load in blood. Each symbol represents an individual macaque (n = 6). ***p < 0.001; **p < 0.01; *p < 0.05.

**FIGURE 8.** CD8 Tregs in sooty mangabeys. A, Plasma viral load in sooty mangabeys during the course of 5 y. B, Frequency of CD8 Tregs in blood and colorectal mucosa of normal (n = 8) and SIV-infected sooty mangabeys (n = 8).
that CD8 Tregs in SIV-infected macaques may use CTLA-4 and CD39-dependent mechanisms for their suppressive function. CD8 Tregs generated in vitro have been shown to suppress T cell proliferation using their cytolytic activity (57). This raises the possibility that CD8 Tregs in our study might be cytolytic. But only a small fraction of CD8 Tregs in our study expressed molecules associated with cytolytic function such as granzyme B and perforin, suggesting that killing may not be a preferred mechanism of their suppressive function.

Understanding the mechanisms by which SIV infection induces these CD8 Tregs could have therapeutic benefits. It has been shown that SIV/HIV induces increased expression of IDO, an immunosuppressive enzyme that is capable of inducing CD4 Tregs (58). This increase in IDO expression is detected in lymphoid and intestinal mucosal tissues as early as 7 d after SIV infection (30, 31, 37, 59). Similarly, TGF-β has also been shown to induce expression of FOXP3 by mouse naive CD8+ T cells following stimulation with anti-CD3 and anti-CD28 (60). SIV infection has been shown to increase expression of TGF-β (37). Collectively, these results suggest that IDO and TGF-β may be playing a role in the induction of CD8+ Tregs in rhesus macaques. We observed a direct correlation between the frequency of CD8 Tregs and viral load, suggesting that viral load influences the magnitude of these cells either directly or indirectly. Analysis of CD8 Tregs in normal and SIV-infected sooty mangabeys revealed no increase in these cells either in blood or rectum. These results strongly suggest that the observed increase in SIV-infected rhesus macaques may be due to indirect effects of high viral load. SIV-infected rhesus macaques and sooty mangabeys differ significantly for the level of hyperimmune activation (61), with the former inducing much higher levels than the latter. In addition, SIV has been shown to induce IFN-α production by plasmacytoid dendritic cells from rhesus macaques but not sooty mangabeys (62). It is possible that activation of plasmacytoid dendritic cells and/or hyperimmune activation may be responsible for induction of CD8 Tregs in the rectum of SIV-infected rhesus macaques.

The origin and specificity of these CD8 Tregs is not clear. Consistent with their expansion in vivo, a significant proportion of CD8 Tregs expressed the proliferation marker Ki-67. This suggests that the expansion in the number of CD8 Tregs could be due to the division of existing cells rather than induction of this phenotype in Treg precursors. Recent studies have demonstrated that human CD8+ T cells express FOXP3 following stimulation with anti-CD3 in the presence of either IL-2 or IL-15 but not IL-7 in vitro (24). This raises the possibility that the CD8 Tregs in SIV-infected animals could be SIV specific. However, the failure to produce IFN-γ and bind to an immunodominant epitope-specific tetramer, an inverse relationship with expansion of antiviral T cell response, suggests that these CD8 Tregs might not be specific for the virus. It is possible that virus-specific Tregs may produce immunosuppressive cytokines such as TGF-β (63) and IL-10 in response to SIV Ag stimulation. It is also possible that the CD8 Tregs express CD8α rather than CD8αβ and thus may not bind tetramers. In the absence of these analyses, it is difficult to conclude their SIV Ag specificity.

We observed that the expansion of CD8 Tregs during SIV infection occurs in the lymphoid compartments and in the large intestine. The expansion of CD8 Tregs in large intestine might indicate that these cells are suppressing antiviral T cells at the preferred site of viral replication, whereas expansion in lymphoid compartments indicates that these cells may also be suppressing generation of new antiviral T cells at site of T cell activation.

It has been suggested that high levels of Tregs during acute HIV/SIV infection could be advantageous for the host by suppressing the hyperimmune activation that has been shown to play a role in progression to disease (61, 64, 65). However, prior reports of FOXP3+ CD8+ T cells in HIV or SIV infections (21, 38, 39) did not demonstrate suppressive activity of these cells, leaving open the question whether FOXP3 expression on CD8+ T cells is a true marker of regulatory activity or activation of CD8+ T cells. Our data suggest that hyperimmune activation is inducing CD8 Tregs capable of suppressing SIV-specific CD4+ and CD8+ T cell responses and that the high levels of CD8 Tregs present during acute infection may be deleterious rather than advantageous to the host. In conclusion, our results demonstrate a rapid expansion of FOXP3+ CD8 Tregs at the preferential sites of virus replication following pathogenic SIV infection and provide evidence that these cells contribute to the suppression of antiviral T cell responses and poor viral control. Our results suggest that therapeutic approaches that block the induction of these cells may improve viral control.

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