Increased Loss of CCR5\(^+\) CD45RA\(^-\) CD4\(^+\) T Cells in CD8\(^+\) Lymphocyte-Depleted Simian Immunodeficiency Virus-Infected Rhesus Monkeys\(^\dag\)

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Previously we have shown that CD8\(^+\) T cells are critical for containment of simian immunodeficiency virus (SIV) viremia and that rapid and profound depletion of CD4\(^+\) T cells occurs in the intestinal tract of acutely infected macaques. To determine the impact of SIV-specific CD8\(^+\) T-cell responses on the magnitude of the CD4\(^+\) T-cell depletion, we investigated the effect of CD8\(^+\) lymphocyte depletion during primary SIV infection on CD4\(^+\) T-cell subsets and function in peripheral blood, lymph nodes, and intestinal tissues. In peripheral blood, CD8\(^+\) lymphocyte-depletion changed the dynamics of CD4\(^+\) T-cell loss, resulting in a more pronounced loss 2 weeks after infection, followed by a temporal rebound approximately 2 months after infection, when absolute numbers of CD4\(^+\) T cells were restored to baseline levels. These CD4\(^+\) T cells showed a markedly skewed phenotype, however, as there were decreased levels of memory cells in CD8\(^+\) T cells compared to controls. In intestinal tissues and lymph nodes, we observed a significantly higher loss of CCR5\(^+\) CD45RA\(^-\) CD4\(^+\) T cells in CD8\(^+\) lymphocyte-depleted macaques than in controls, suggesting that these SIV-targeted CD4\(^+\) T cells were eliminated more efficiently in CD8\(^+\) lymphocyte-depleted animals. Also, CD8\(^+\) lymphocyte depletion significantly affected the ability to generate SIV Gag-specific CD4\(^+\) T-cell responses and neutralizing antibodies. These results reemphasize that SIV-specific CD8\(^+\) T-cell responses are absolutely critical to initiate at least partial control of SIV infection.

Retroviruses that cause AIDS in primates including the human immunodeficiency virus (HIV) and its close relative the simian immunodeficiency virus (SIV) belong to the group of lentiviruses (“lenti” means slow) (26). This is because overt clinical disease is usually seen only after a prolonged period of a relatively stable, chronic infection (clinical latency), which would suggest a slow but progressive decline of the immune system. However, detailed immunologic and virologic investigations in recent years have shown that infection with these viruses is a two-phased process, with an initial phase of an exceedingly high viral replication accompanied by a massive loss of memory CD4\(^+\) T cells, particularly in the gastrointestinal tract and other mucosal tissues (8, 24, 48, 54, 73). This is followed by a chronic phase of variable, but typically incomplete, virus control with eventual progression to AIDS (46).

Correlative studies have suggested that CD8\(^+\) T-cell responses play a major role in the early containment of HIV or SIV replication (6, 39, 42). In primary infection, strong HIV- and SIV-specific CD8\(^+\) T-cell responses are generally detected in close temporal proximity to peak viremia during a period when antibody responses are either weak or undetectable. In addition, robust neutralizing antibody responses are generally only observed weeks, if not months, after the initial decline of primary viremia (10, 28). These observations cast doubt as to whether humoral immune responses significantly contribute to virus containment during primary viremia (68). In addition to adaptive immune responses, it is possible that innate immune responses or nonimmune factors could also contribute to the decline of early viremia. However, currently available data suggest that adaptive immune responses generated by CD8\(^+\) T cells are the major force in the containment of primary viremia (46). The best evidence for this comes from multiple studies in nonhuman primates, demonstrating that in vivo depletion of CD8\(^+\) lymphocytes results in enhanced AIDS virus replication during both primary and chronic infection (1, 12, 36, 37, 49, 50, 52, 64, 66, 67, 76).
The major target cells for HIV and SIV replication in primary infection are memory CCR5+ CD4+ T cells (13, 19). Several laboratories have demonstrated that extensive infection and depletion of these cells occur in primary HIV or SIV infection at mucosal sites, particularly in the gastrointestinal tract (8, 53–55, 73, 74). It is estimated that about half of the CD4+ T cells in the body reside in lymphoid tissue in the gastrointestinal tract and that the vast majority of these cells express the HIV or SIV coreceptor CCR5 (20, 40, 58, 72). Therefore, it is not surprising that this particular subset, which is a preferred target cell population for the viruses, undergoes a drastic decline during primary HIV or SIV infection of humans and nonhuman primates.

Although it is now generally accepted that CD4+ CCR5+ T cells are rapidly and selectively eliminated in primary HIV or SIV infection (61) and that AIDS virus-specific CD4+ T cells are particularly targeted and destroyed by HIV (7, 21), the mechanisms underlying the loss of CD4+ T cells are still debated (2, 13, 18, 20, 24, 25, 29, 30, 65). Some studies have proposed that direct cytopathic effects of viral infection of these CD4+ CCR5+ T cells are primarily or solely responsible for the massive decline of these cells, whereas others have hypothesized that “bystander” apoptosis may substantially contribute to elimination of these cells. In fact, over the last 2 decades a number of possible mechanisms have been proposed that could explain even the loss of uninfected CD4+ T cells. The most prominent mechanisms include (i) apoptosis through either bystander activation-induced cell death mediated by Fas/Fas-ligand and/or TRAIL/DR5 interactions (3, 5, 23, 27, 31, 32, 48) or AIDS viral proteins (11, 47) and (ii) homeostatic deterioration of the regenerative capacity of CD4+ T cells (20, 56, 60, 62). However, it has also been hypothesized that virus-specific CD8+ T-cell responses may contribute to the decline of a significant number of CD4+ T cells by direct killing of infected CD4+ T cells expressing viral proteins or peptides (4, 22, 77, 78). Thus, virus-specific CD8+ T cells could play both a protective and pathological role in HIV or SIV infection by eliminating virus-producing cells and yet contributing to the overall loss of CD4+ T cells that become infected before, or in spite of, the development of virus-specific CD8+ T-cell responses.

To determine how SIV-specific CD8+ T-cell responses may influence the marked, early loss of intestinal CD4+ T cells in SIV infection, we examined the effects of CD8+ lymphocyte depletion in primary SIV infection of rhesus monkeys on changes in the percentage, phenotype, and function of CD4+ T cells in both peripheral and mucosal lymphoid tissues. Here, we demonstrate that CD8+ lymphocyte depletion in primary SIV infection results in higher virus replication, impaired development of antiviral CD8+ T-cell responses, and an increased loss of CCR5+ CD45RA+ CD4+ T cells in the intestinal tract and lymph nodes. Furthermore, the consequences of the impaired CD8+ T-cell responses include the inability to generate SIV-specific CD4+ T-cell responses, humoral immune responses, and a more rapid progression of disease, presumably all as a consequence of less effective control of viral replication. Finally, we show in the accompanying report that CD8+ lymphocyte depletion in primary SIV infection results in significantly reduced immune pressure, as evidenced by delayed viral escape from dominant SIV Tat and Gag cytotoxic T-lymphocyte epitopes (38).

**MATERIALS AND METHODS**

**Virus.** SIVmac251, a pathogenic biological isolate, was originally isolated from peripheral blood lymphocytes of a rhesus monkey (animal Mm 251-79) that had developed a malignant lymphoma and an AIDS-like disease (17). Since its original isolation, the uncloned virus used for the present study was expanded four times in human peripheral blood lymphocytes. The virus stock was negative for foamy and type D viruses. Challenged animals received 1 ml of the SIVmac251 culture supernatant diluted with phosphate-buffered saline (PBS) to yield 0.15 ng/ml SIV p27 Gag antigen.

**Animal inoculations and in vivo CD8+ lymphocyte depletion.** Twelve male rhesus monkeys (Macaca mulatta) of Indian origin and expressing the major histocompatibility complex class I allele Mamu-A*01 were included in the study; Mamu-A*01 typing was performed by PCR as described previously (41). Eight monkeys were transiently depleted of CD8+ lymphocytes by the administering of a chimeric anti-human CD8 monoclonal antibody (mAb), e-mT807, at 10 mg/kg of body weight subcutaneously on day 0 (the day of SIV infection) followed by 5 mg/kg intravenously on days 3 and 7. A chimeric mAb against respiratory syncytial virus (chimeric MAb 1D10) was administered at the same dose to a control group of four monkeys. For all procedures, animals were sedated with ketamine HCl. All animals were housed and maintained in accordance with the Guide for the Care and Use of Laboratory Animals (59), and all studies and procedures were reviewed and approved by the Institutional Animal Care and Use Committees of both Tulane University and Harvard University.

**Lymphocyte immunophenotyping and SIV Gag p11C tetramer staining.** EDTA-anticoagulated whole blood specimens were stained for flow cytometry using a whole-blood staining technique as previously described (41). Lymph node and endoscopic intestinal biopsies of the jejunum were collected from macaques and processed to yield single-cell suspensions as previously described (43, 70). For all three tissues, cells were immunophenotyped by flow cytometry using the following antibody panels: (i) anti-CD3(FN18)-fluorescein isothiocyanate (FITC) (FN18 was a gift from David Neville, NIH), anti-CD8-phycocerythrin (PE) (DK25; Dako, Carpenteria, CA), anti-CD20-energy-coupled dye (Beckman Coulter, Miami, FL), and anti-CD4-allophycocyanin (APC) (BD Biosciences, San Jose, CA); (ii) anti-CD95-FITC (DX2), anti-CD28-PE (CD28.2), anti-CD8-peridinin chlorophyll protein (SK-1), and CD4-APC (all from BD Biosciences); and (iii) anti-CD45RA-FITC (2H4; Beckman Coulter), anti-CCR5-PE (3A9), anti-CD8-peridinin chlorophyll protein (SK1), and CD4-APC (BD Biosciences). For detection of SIV Gag p11C-specific CD8+ T cells, tetramer complexes were prepared as described previously (41), and the following panel was utilized: anti-CD3-FITC, anti-CD8-energy-coupled dye (Beckman Coulter), and SIV Gag p11C tetramer complex-APC. Erthrocytes were lysed from blood specimens using the Immunoprep Reagent System and a T-Prep Workstation (Beckman Coulter), washed, fixed in PBS–1% formalin, and analyzed on a Becton Dickinson FACS Calibur four-color flow cytometer. Absolute lymphocyte counts on blood specimens were obtained using an automated hematology analyzer. Lymph node and intestinal cell suspensions were similarly stained with the lysing step omitted.

**Determination of plasma virus levels.** Plasma SIV levels were determined by a real-time reverse transcription-PCR assay (Quantitative Molecular Diagnostic Section, AIDS Vaccine Program, NCI-Frederick, Frederick, MD) (limit of detection as used in the present studies, 60 RNA copies/ml) (16).

**CD4+ T-cell immune assay.** Peripheral blood mononuclear cells (PBMC) were separated from whole blood by Ficoll density gradient centrifugation. PBMC (107) were incubated at 37°C in a 5% CO2 atmosphere for 6 h in the presence of RPMI 1640–10% fetal calf serum medium alone (unstimulated), a pool of 15-mer SIV Gag peptides (2 μg/ml each peptide; AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH, Germantown, MD), or staphylococcal enterotoxin B (5 μg/ml, Sigma-Aldrich, St. Louis, MO) as a positive control. All cultures contained monensin (GolgiStop; BD Biosciences) as well as 1 μg/ml anti-CD49d (BD Biosciences). The cultured cells were stained with the following antibody panel prior to fixation: CD3(FN18)-FITC and CD4-PE (SK3; BD Biosciences). The PBMC were washed twice with PBS–2% fetal calf serum and then fixed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences). Cells were then stained with anti-beta interferon (IFN-γ)-APC (BD Biosciences). Cells were washed twice with Perm/Wash buffer and then fixed in 1.5% formaldehyde–PBS. Samples were collected on a FACS Calibur four-color flow cytometer and analyzed with CellQuest software (BD Biosciences). Approximately 100,000 to 300,000 events...
in the lymphocyte gate were acquired. The background level of cytokine staining varied from sample to sample but was typically <0.04% of CD4+ T cells. The only samples considered positive were those in which the percentage of cytokine-staining cells was at least twice that of the background and in which there was a distinct population of cytokine-brightly positive cells.

**Measurements of neutralizing antibody responses.** Antibody-mediated neutralization of SIVmac251 was assessed in a CEMx174 cell-killing assay as previously described (57). The cell-free stocks of the T-cell line-adapted (TCLA) SIVmac251 prepared in H9 cells were added to serial dilutions of test serum in 100 μl of RPMI 1640–12% fetal bovine serum–50 μg of gentamycin in triplicate in 96-well culture plates. After incubation for 1 h at 37°C, CEMx174 cells (5 x 10^5 cells in 100 μl) were added to each well. Infection led to extensive synctium formation and virus-induced cell killing in approximately 4 to 6 days in the absence of antibodies. Neutralization was measured by staining viable cells with Fainter’s neutral red in poly-L-lysine-coated plates. Percent protection was determined by calculating the difference in absorption (ΔA492) between test wells (cells plus serum sample plus virus) and virus control wells (cells plus virus), dividing this result by the difference in absorption between control cell wells (cells only) and virus control wells, and multiplying by 100. Neutralization was measured at a time when virus-induced cell killing in virus control wells was greater than 70% but less than 100%. Neutralizing titers are given as the reciprocal dilution required to protect 50% of cells from virus-induced killing.

**SIV in situ hybridization.** Formalin fixed, paraffin-embedded tissues were stained for SIV RNA utilizing a method previously described (33). Briefly, the sections were deparaffinized, pretreated with proteinase K, and hybridized overnight at 50°C with either sense or antisense SIVmac239 digoxigenin–UTP-labeled riboprobe. The hybridized sections were washed with posthybridization buffers and RNAse solutions and blocked with 3% normal sheep serum followed by sheep anti-digoxigenin-alkaline phosphatase (1:500; Roche Molecular Diagnostic, Pleasanton, CA) for 1 h. The sections were reacted with BCIP/NBT (5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium) (Vector Laboratories, Ltd., Burlingame, CA) for 10 h, rinsed with distilled water, counter-stained with nuclear fast red (Sigma-Aldrich), and examined with a Zeiss Axioscope microscope (Carl Zeiss MicroImaging Inc., Thornwood, NY). Negative controls included mock hybridization, sense probe applied to infected tissues, and antisense probe on uninfected tissues.

**Confocal microscopy.** Tissue sections were stained for viral SIV RNA as outlined above with the following modifications. All samples were treated with a methonolic-hydrogen peroxide solution to quench endogenous peroxidase before the addition of sheep anti-digoxigenin–horseradish peroxidase (Roche Molecular Diagnostics) followed by tyramide-FITC signal amplification (Perkin Elmer, Waltham, MA). Samples were then incubated with rabbit polyclonal anti-CD3 antibodies (DAKO, Carpinteria, CA) and Ki-67 (DAKO) nuclear activation marker. The anti-CD3 antibody was detected with goat anti-rabbit immunoglobulin G-Alexa594 (Invitrogen). Ki-67 was detected with a biotinylated anti-mouse secondary antibody (Vector Laboratories, Ltd., Burlingame, CA) for 10 h, rinsed with distilled water, counter-stained with nuclear fast red (Sigma-Aldrich), and examined with a Zeiss Axio Imager Z-1 instrument (Carl Zeiss MicroImaging Inc., Thornwood, NY). Negative controls included mock hybridization, sense probe applied to infected tissues, and antisense probe on uninfected tissues.

**RESULTS**

**CD8+ lymphocyte depletion significantly reduces the survival of SIVmac251-infected rhesus monkeys.** Previously, we observed that CD8+ lymphocyte depletion during primary SIV infection resulted in significantly reduced survival of rhesus monkeys (67). Also, the length of survival was dependent on the relative duration of CD8+ lymphocyte depletion. In this study, we investigated a total of 12 Mamu-A*01-positive rhesus monkeys. Four rhesus monkeys were treated with the control antibody, and eight received the anti-CD8+ lymphocyte-depleting antibody cM-T807. The difference in survival of CD8+ lymphocyte-depleted animals and control antibody-treated animals approached significance (P = 0.06) (Fig. 1A). Similar to our previous observations, half of the CD8+ lymphocyte-depleted animals showed a relatively short-term depletion of CD8+ lymphocytes for ~21 days (CD8+ lymphocytes reappeared between days 14 and 21 after infection), and the other half showed a relatively long-term depletion of CD8+ lymphocytes for ~28 days (CD8+ lymphocytes reappeared between days 28 and 35 after infection) (Fig. 1B). The longest survival was observed in three of the control antibody-treated animals, an intermediate range of survival was seen in short-term lymphocyte-depleted animals, and the fastest progression was seen in long-term lymphocyte-depleted animals (Kruskal-Wallis test, P = 0.035).

Since the number of animals presented in both the previously published study and the present study was relatively small, we decided to combine the survival data and include additional animals that will be described elsewhere in an unrelated publication (K. Williams et al., unpublished data). Thus, a total of 10 control antibody-treated animals and 25 CD8+ lymphocyte-depleted animals that all were at least 3.5 years of age and received the same strain and inoculum of SIVmac251 were compared. A highly significant difference was then observed when CD8+ lymphocyte-depleted animals were compared to control antibody-treated animals (P = 0.0006) (Fig. 1C). Stratifying the CD8+ lymphocyte-depleted animals into two groups of short-term lymphocyte-depleted versus long-term lymphocyte-depleted animals compared to controls resulted in a highly significant difference between all three groups (P < 0.0001) (Fig. 1D). All long-term CD8+ lymphocyte-depleted animals had to be euthanized before 150 days after SIVmac251 infection due to clinical signs of AIDS. At day 250, about 70% of the control animals were still surviving. However, only 30% of the short-term lymphocyte-depleted animals survived until day 250 postinfection.

**CD8+ lymphocyte depletion efficiently eliminates CD8+ T cells from peripheral blood and lymph nodes but not from intestine.** As previously shown, CD8+ lymphocytes are very efficiently depleted from peripheral blood following anti-CD8 antibody administrations. As shown in Fig. 2, on day 12 after the initiation of the anti-CD8 antibody treatment and SIVmac251 infection, virtually no CD8+ lymphocytes remained in the peripheral blood (Fig. 2A and B). Also, the number of CD8+ lymphocytes in lymph nodes was markedly reduced at this time point (Fig. 2C and D). However, CD8+ lymphocyte depletion in the intestine was surprisingly incomplete, as the majority of CD8+ T cells persisted in this compartment (Fig. 2E and F). A large percentage of intestinal CD8+ T cells persisted through 12 days of infection/antibody treatment (and throughout the entire time of peripheral blood CD8+ lymphocyte depletion [data not shown]), but the staining intensity (median fluorescence intensity) was significantly reduced, suggesting that the cell surface-bound anti-CD8 antibody cM-T807 was partially blocking the binding of the anti-CD8 antibody used for phenotyping T cells and/or that the CD8
molecule was partially down-modulated upon binding of the anti-CD8 antibody to CD8^+ lymphocytes.

In one macaque, we also investigated whether increasing the dose of anti-CD8 antibodies would potentially increase the efficacy of CD8^+ lymphocyte depletion in the gastrointestinal tract. However, even performing repeated injections at 50 mg/kg of body weight did not change the efficacy of depletion of intestinal CD8^+ lymphocytes (data not shown).

Administration of cM-T807 delays or inhibits the generation of SIV Gag-specific CD8^+ T cells in peripheral blood, lymph nodes, and intestine. Strong SIV Gag-specific CD8^+ T-cell responses are usually detected in peripheral blood coincident with peak viremia in acute infection. A detailed demonstration of the peripheral blood SIV Gag and Tat tetramer responses of the 12 animals investigated here is shown in the accompanying report (38). As shown for a representative animal (Fig. 3A and B), SIV Gag tetramer responses were simultaneously detected in peripheral blood, lymph nodes, and jejunum in the control antibody-treated group. In contrast, SIV Gag tetramer responses were delayed in peripheral blood, lymph nodes, and jejunum in the short-term CD8 lymphocyte-depleted animals (representative data shown in Fig. 3C and D) and either further delayed or persistently undetectable in long-term lymphocyte-depleted animals (representative data shown in Fig. 3E and F). Thus, the relative length of CD8^+ lymphocyte depletion in peripheral blood was inversely correlated with the generation of tetramer responses in peripheral blood and lymphatic and gastrointestinal tissues.

SIV Gag-specific CD4^+ T-cell responses and TCLA SIVmac251 neutralizing antibody responses are significantly impaired in CD8^+ lymphocyte-depleted animals. Next, we examined whether CD8^+ lymphocyte depletion would influence the generation of SIV-specific CD4^+ T-cell responses and/or humoral immune responses. We performed intracellular cytokine staining to detect SIV Gag-specific CD4^+ T-cell responses by examining IFN-γ production from SIV Gag peptide pool-stimulated lymphocytes. We were able to readily detect CD4^+ T-cell responses in control antibody-treated animals but found essentially undetectable levels of IFN-γ responses by CD4^+ T cells in both groups of anti-CD8 antibody-treated animals.

FIG. 1. Kaplan-Meier survival curves of CD8^+ lymphocyte-depleted and control antibody-treated SIVmac251-infected rhesus monkeys. (A and B) Survival data of 12 rhesus monkeys presented in the current study. (C and D) Combined survival data of 35 rhesus monkeys obtained from three independent CD8^+ lymphocyte depletion studies using the same challenge stock of SIVmac251 and MAbs for in vivo treatment. Graphs in panels A and C compare survival rates of CD8^+ lymphocyte-depleted animals to control antibody-treated animals. Graphs shown in panels B and D compare survival rates of control antibody-treated animals with rates of short-term and long-term CD8^+ lymphocyte-depleted animals.
differences in Gag-specific CD4+ T-cell IFN-γ responses between the three groups of monkeys were highly significant (P < 0.05) by a Kruskal-Wallis test at each time point depicted in Fig. 4A. This suggested that the anti-CD8 antibody treatment significantly impaired the ability of the immune system to generate SIV-specific CD4+ T-cell responses, which was most likely due to the increased viremia observed in CD8+ lymphocyte-depleted animals.

Neutralizing antibodies to TCLA SIVmac251 are generally observed within 4 weeks of SIVmac251 infection. In the present study, on day 28 after infection the median titer of TCLA SIVmac251 neutralizing antibodies in the control group was 24,000, which was more than 2 orders of magnitude higher than in both groups of anti-CD8 antibody-treated animals (Fig. 4B). Short-term lymphocyte-depleted animals were slowly able to generate high titers of neutralizing antibodies, reaching levels similar to the control animals 75 days after infection. However, long-term lymphocyte-depleted animals never generated significant levels of neutralizing antibodies. Significant differences (P < 0.05) were detected between the three groups of animals by a Kruskal-Wallis test at each time point depicted in Fig. 4B.

CD4+ T-cell subset changes are predictive for disease progression. To determine the effect of CD8+ lymphocyte depletion in peripheral blood, lymph node, and jejunum using the anti-CD8 MAb cM-T807. The top row depicts flow cytometry graphs generated for specimens collected 7 days before SIVmac251 infection (day -7) and anti-CD8 antibody treatment. The bottom row depicts samples obtained on day 12 after SIVmac251 infection. The anti-CD8 antibody treatment was given on days 0, 3, and 7.

FIG. 2. Efficacy of CD8+ lymphocyte depletion in peripheral blood, lymph node, and jejunum using the anti-CD8 MAb cM-T807. The top row depicts flow cytometry graphs generated for specimens collected 7 days before SIVmac251 infection (day –7) and anti-CD8 antibody treatment. The bottom row depicts samples obtained on day 12 after SIVmac251 infection. The anti-CD8 antibody treatment was given on days 0, 3, and 7.

FIG. 3. Percentages of CD8+ T cells and SIV Gag p11C tetramer+ CD8+ T cells in peripheral blood (top row) and lymph node and jejunum (bottom row) for representative control antibody-treated, short-term CD8+ lymphocyte-depleted, and long-term CD8+ lymphocyte-depleted rhesus monkeys.
of animals investigated in each group (n = 4), statistical analysis only approached significance (P = 0.07, Kruskal-Wallis test).

**Loss of CCR5+ CD45RA− CD4+ T cells in intestine and lymph nodes predicts faster SIV disease progression.** It was hypothesized that CD8+ T cells help control viral replication and yet contribute to CD4+ T-cell depletion by direct killing of infected CD4+ T cells (78). To address the influence of SIV-specific CD8+ T cells on the loss of gastrointestinal tissue-associated CD4+ T cells, we collected intestinal biopsies prior to SIV infection and at multiple time points during the first 100 days of infection from animals in both the control antibody-treated and anti-CD8 antibody-treated groups. During acute SIV infection, the numbers of intestinal CD4+ T cells were markedly reduced in the control group and also in the CD8+ lymphocyte-depleted groups (Fig. 6A). Interestingly, we did not detect a significant difference in percentages of total intestinal CD4+ T cells following infection between the three groups investigated. This observation is probably due to the incomplete depletion of the gut CD8+ T cells, which makes a relative evaluation (percentage) of the number of CD4+ T cells difficult.

We along with others have also previously reported that SIV selectively targets and eliminates memory CCR5+ CD4+ T cells in virtually all lymphoid tissues very early after SIV or HIV infection (24, 44, 48, 53, 74). This depletion is most pronounced in intestinal tissues as mucosal sites harbor most of the memory CD4+ CCR5+ T cells of the body (75). Here, we observed that all animals from all three groups had a significant and profound reduction of these target cells in the intestine following SIV infection. However, the control antibody-treated animals were able to preserve larger numbers of these cells than the anti-CD8 antibody-treated animals (representative animals from each group are shown in Fig. 6B). Both short-term and long-term lymphocyte-depleted animals showed a
much more precipitous decline in target cells than control animals, showing that the generation of SIV-specific CD8+ T-cell responses was correlated with a less pronounced decline of intestinal CCR5+ CD4+ T cells, the target cells for SIV. Nevertheless, we cannot rule out the contribution of nonspecific killing mechanisms by CD8+ T cells and other cell subsets (e.g., by Fas/Fas-ligand) to (some) CD4+ T-cell loss in gastrointestinal tissues. Control animals eventually demonstrated a partial rebound of these cells, yet the CD8+ lymphocyte-depleted animals never demonstrated any attempt to repopulate these cells. Interestingly, the control animal, which had the lowest peak viremia and subsequent set point viremia, experienced the least decline in this CD4+ T-cell subset and was able to maintain large percentages of these cells throughout infection.

Next, we focused on the time span during primary infection that is known to show the most precipitous decline of CD4+ T cells during primary viremia (days 10 to 21). During this time span, SIV-specific CD8+ T-cell responses were almost completely absent in the CD8+ lymphocyte-depleted animals in contrast to the control antibody-treated animals. As shown in Fig. 6C, on day 12 after infection the median percentage of CCR5+ CD45RA− CD4+ T cells in the jejunum was significantly higher in the control group than the combined values from both CD8+ lymphocyte-depleted groups (P = 0.0162, Mann-Whitney test). The nadir of CCR5+ CD45RA− CD4+ T cells in the intestine of control animals occurred at day 21 after infection (no significant difference was detected between the control group and the combined values from both CD8+ lymphocyte-depleted groups; P = 0.1091, Mann-Whitney test). As shown in Fig. 6B, however, CD8+ lymphocyte-depleted animals were never able to recover from the loss of SIV target cells, whereas the CCR5+ CD45RA− CD4+ T cells in control antibody-treated animals partially recovered.

Before infection, the percentage of memory CD4+ T cells (CCR5+ CD45RA− CD4+) in lymph nodes was much lower than in the intestine. However, similar to what was observed in the intestine, the loss of CCR5+ CD45RA− CD4+ T cells in lymph nodes was significantly higher in CD8+ lymphocyte-depleted animals than in control animals (in a comparison of the control group with all combined CD8+ lymphocyte-depleted animals, P = 0.004 for day 12 and P = 0.006 for day 21 values; Mann-Whitney test) (Fig. 6D).

Interestingly, we did not find significant differences in the percentages of CCR5+ CD45RA− CD4+ T cells in peripheral blood following SIV infection between the three groups (data not shown). This may be in part due to the fact that we used a relatively simple phenotypic panel to investigate the CCR5+ CD4+ T cells when we performed a four-color flow cytometry analysis. Compared to the intestine and lymph nodes, the CCR5+ CD4+ T cells are relatively rare in the blood, and increased proliferation and homing of these cells through the blood may obscure losses of these cells when baseline percentages are sparse. A more complex polychromatic phenotyping panel (14, 45, 63) may reveal differences in selective CCR5+ CD4+ T-cell subsets that we could not detect. Also, polychromatic flow cytometry that was not available to us at the time of these investigations would likely help to further dissect memory-associated T-cell subsets in lymphatic and gastrointestinal tissues.
SIV replication in terminal SIV infection is relatively high in lymph nodes and spleen and occurs to a lesser extent in jejunum. To examine the effect of CD8⁺ lymphocyte depletion on the distribution of SIV-infected cells in tissues in terminal SIV infection, we performed SIV-specific in situ hybridization on lymph nodes, spleen, and jejunum tissues obtained at necropsy from animals euthanized with clinical signs of AIDS. The animals examined consisted of one of four control antibody-treated animals, three of four short-term CD8⁺ lymphocyte-depleted animals, and four of four long-term CD8⁺ lymphocyte-depleted animals. At necropsy, a relatively high amount of virus replication was observed in lymph nodes and spleen in the CD8⁺ lymphocyte-depleted animals, but overall, smaller amounts of virus-infected cells were detected in the jejunum (Table 1 and Fig. 7). However, multiple areas of the jejunum did display foci with high numbers of infected cells, often associated with solitary lymphoid follicles (Fig. 7C and D) and sometimes in effector sites (lamina propria) (Fig. 7A and B). In contrast, the sacrificed control antibody-treated animal showed a relatively lower level of viral replication in all the tissues examined (data not shown).

Since our flow cytometric studies demonstrated severe depletion of CCR5⁺ CD4⁺ T cells, we next sought to determine which cellular subsets were the major sources of SIV viremia. In recent studies of SIVsm-infected macaques with a rapid disease course similar to the progress of disease in these animals, tissue macrophages were identified as the major source of SIV (9). Since the number of actively SIV-producing cells in the jejunum was lower than in the lymph nodes, we focused our investigations on the latter. We performed confocal microscopy for SIV in combination with anti-CD3 and HAM56.

### Table 1. SIV-specific in situ hybridization of terminal tissues from SIVmac251-inoculated macaques

<table>
<thead>
<tr>
<th>Experimental group and animal</th>
<th>Relative level of SIV infection by tissue type&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td>LN</td>
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<tr>
<td>Long-term CD8-depleted</td>
<td></td>
</tr>
<tr>
<td>NS13</td>
<td>3+</td>
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<tr>
<td>BR06</td>
<td>3+</td>
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<td>BP92</td>
<td>3+</td>
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<tr>
<td>E324</td>
<td>1+</td>
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<td>Short-term CD8-depleted</td>
<td></td>
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<tr>
<td>T224</td>
<td>2+</td>
</tr>
<tr>
<td>AP53</td>
<td>2+</td>
</tr>
<tr>
<td>BF63</td>
<td>2+</td>
</tr>
<tr>
<td>Control antibody-treated, AH88</td>
<td>1+</td>
</tr>
</tbody>
</table>

<sup>a</sup> 1, rare SIV-positive cell per high-power (×40) field; 2, 1 to 5 SIV-positive cells per high-power field; 3, 5 to 10 SIV-positive cells per high-power field; Neg, no SIV-positive cells detected in entire section. LN, lymph node; GI, gastrointestinal tissue.
identify T cells and macrophages, respectively). In the three animals investigated, the majority of SIV-expressing cells co-expressed CD3 (68 to 78%) and thus were presumably CD4 T cells (Table 2), with a minority of macrophages also expressing SIV (data not shown). Confocal microscopy with a combination of anti-CD3 and anti-Ki-67 antibodies showed that 61% to 83% of these T cells were Ki-67-positive T cells, indicating that most of the virus-producing T cells were proliferating memory T cells (Table 2).

**DISCUSSION**

Here, we have demonstrated that CD8 lymphocyte depletion of macaques in early SIV infection in contrast to control antibody treatment results in a more significant perturbation of the number and function of CD4 T cells in peripheral blood, lymph nodes, and the intestine. Furthermore, a more profound loss of SIV target cells, the CCR5 CD45RA CD4 T-cell subset, was observed in the intestine and lymph nodes of CD8 lymphocyte-depleted macaques, suggesting that SIV-specific CD8 T-cell responses preserve mucosal memory CD4 T cells, presumably by limiting the viral replication. In addition, reduced function of CD4 T cells in CD8 lymphocyte-depleted rhesus monkeys was evidenced by the decreased ability to generate SIV-specific IFN-γ CD4 T-cell responses. Also, the delay to nearly total inhibition of TCLA SIVmac251 neutralizing antibody responses provided indirect evidence that CD4 T-cell responses were severely affected in the CD8 lymphocyte-depleted animals.

Similar to recent observations (67), our results indicated that CD8 lymphocyte depletion during primary SIV infection of rhesus monkeys was variable, with about half of the animals experiencing a relatively long-term depletion of peripheral blood CD8 lymphocytes and the other half showing a relatively short-term depletion. In addition, the relative length of peripheral blood CD8 lymphocyte depletion significantly correlated with survival of the animals. Surprisingly, administration of the anti-CD8 MAb did not deplete CD8 T cells in the intestine. In fact, only a small percentage of CD8 T cells was eliminated from the intestine, indicat-

**TABLE 2. Confocal microscopy and SIV in situ hybridization with CD3 and Ki-67 to identify SIV-expressing cells in CD8-depleted terminally infected macaques**

<table>
<thead>
<tr>
<th>Animal (n)</th>
<th>No. of SIV-expressing cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>BR06 (14)</td>
<td>203</td>
</tr>
<tr>
<td>BP92 (9)</td>
<td>69</td>
</tr>
<tr>
<td>NS13 (9)</td>
<td>137</td>
</tr>
</tbody>
</table>

* n, number of fields counted on ×63 field.
ing that the mechanisms of depletion of this systemically administered antibody may be organ specific. This finding could indicate that large molecules, like the antibody used, may have difficulties in reaching intestinal mucosal sites through some sort of blood-intestinal barrier. However, it is probably more likely that additional molecules (e.g., complement proteins) or cell subsets (e.g., NK cells, granulocytes, and/or monocytes/macrophages) that may deliver the cytotoxic effect to anti-CD8 antibody-covered CD8+ lymphocytes are present at various levels and may also function differently depending on the particular organ environment.

Despite the lack of intestinal CD8+ T-cell depletion, binding of the anti-CD8 MAb to CD8+ T cells in vivo may have also affected the ability of these cells to mount SIV-specific T-cell responses since we have previously observed that binding of the anti-CD8 MAb CM-T807 to CD8+ T cells in in vitro cultures completely inhibited the expansion of SIV Gag-specific CD8+ T cells by SIV Gag peptides (69). Thus, investigations of primary SIV infection using a CD8+ lymphocyte depletion strategy permit investigations into the effects of hampered SIV-specific immune responses by depletion of CD8+ lymphocytes and inhibition of the generation of SIV-specific CD8+ T-cell responses through steric hindrance of the function of the anti-CD8 antibody-covered CD8 molecule. Nevertheless, the fact that a considerable number of CD8+ T cells remained in the intestine suggests that the remaining CD8+ T cells could still potentially interfere with the course of the disease by nonspecific immune mechanisms (e.g., secretion of cytokines following tissue damage or Fas/Fas-ligand interaction).

As expected, peripheral blood CD4+ T cells in control antibody-treated animals showed a typical, progressive decline. In contrast, CD8+ lymphocyte-depleted animals exhibited a more rapid CD4+ T-cell decline during primary infection, followed by a short rebound at about 2 months after infection, essentially reaching levels observed prior to infection. This finding of “normal” CD4+ T-cell counts following primary viremia was surprising, particularly since these animals produced significantly higher amounts of virus than control animals. However, phenotypic investigations showed that the CD4+ T-cell population in these animals had a more pronounced loss of memory CD4+ T cells, suggesting that the increased viremia clearly had a more detrimental impact on memory CD4+ T-cell subsets, which confirms recent findings by others (62). In addition, the functional analysis of the peripheral blood CD4+ T cells showed that CD8+ lymphocyte-depleted animals had a significantly impaired ability to generate Gag-specific CD4+ T-cell responses. We also observed a delay or complete abrogation of the ability to generate TCLA SIVmac251 neutralizing antibodies, which was likely a consequence of the profound loss of memory CD4+ T cells.

Although we did not detect a difference in the magnitude of CD4+ T-cell responses between short- and long-term CD8+ lymphocyte-depleted animals, the differences observed in their abilities to generate humoral immune responses suggested that short-term CD8+ lymphocyte-depleted animals were still able to mount some SIV-specific CD4+ T-cell responses that were not detected in the intracytoplasmic cytokine assays employed here. With a significantly increased viremia in CD8+ lymphocyte-depleted animals, in particular, in long-term CD8+ lymphocyte-depleted animals, it is very likely that SIV-specific CD4+ T-cell responses were even more efficiently inhibited or eliminated. This has recently been suggested for HIV infection, where HIV-specific CD4+ T cells showed a higher infection rate than memory CD4+ T cells in general (21).

Several studies have now confirmed that memory CCR5+ CD4+ T cells are selectively and profoundly depleted in primary HIV or SIV infection, particularly in mucosal tissues. Interestingly, we did not detect significant differences in the total percentages of CD4+ T cells remaining in intestinal biopsies of CD8+ lymphocyte-depleted or control animals after infection. However, as CD8+ T cells were to a variable degree incompletely depleted in the intestine, the percentage of intestinal CD4+ T cells in CD8+ lymphocyte-depleted animals may not truly reflect the loss of these cells. Also, admittedly it is difficult to determine the effects of the remaining CD8+ lymphocytes on the intestinal environment that did not contain SIV-specific CD8+ T cells as long as peripheral blood CD8+ lymphocytes were eliminated. However, the fact that significant differences in the percentages of target cells (memory CCR5+ CD4+ T cells) were clearly evident between CD8+ lymphocyte-depleted and control antibody-treated animals indicated that the CD8+ lymphocyte depletion did have a significant, albeit negative, impact on mucosal tissues. A major objective of this study was to determine how systemic SIV-specific CD8+ T-cell responses might influence the magnitude of the destruction of memory CCR5+ CD4+ T cells in the intestine. Both groups of anti-CD8-treated animals experienced a much more dramatic loss of these memory CD4+ T cells in the intestine and in the lymph nodes than control antibody-treated animals, which were able to partially repopulate these cells after the typical nadir of depletion was reached at day 21 after infection (61).

Interestingly, the animal in the control group with the best control of virus replication showed the least loss of these SIV target T cells, which supports our earlier observation that the frequency of these intestinal target cells indirectly correlates with viral loads (74). However, it is also important to highlight that the loss of these cells was very similar in both groups of CD8+ lymphocyte-depleted animals and, therefore, could not be utilized as a surrogate marker to discriminate between the two groups of anti-CD8 antibody-treated monkeys, which showed a considerable difference in viremia and survival.

As true for all tools used for in vivo manipulations, the CD8+ lymphocyte depletion model has its drawbacks. The CD8+ lymphocyte depletion may result in reactivation of other viral infections including latent herpes virus infections like cytomegalovirus, which could potentially result in a significantly exacerbated course of disease. In addition to depleting CD8+ T cells, the anti-CD8 MAb also eliminates other CD8+ lymphocytes, such as NK cells. While the role of NK cells in controlling SIV replication remains unclear (15), emerging data suggest that they may play a significant role in HIV infection of humans (51). In addition to depletion of cell subsets other than CD8+ T cells, CD8+ lymphocyte depletion may also transiently increase the number of viral targets by nonspecific activation of CD4+ T cells through release of enzymes/inflammatory mediators from dying cells or homeostatic mechanisms. However, it is still unclear whether such effects contribute to
the increased pathogenicity observed in CD8+ lymphocyte-depleted and SIV-infected animals (L. J. Picker, personal communication).

In summary, depletion of CD8+ lymphocytes in SIV-infected rhesus monkeys has helped to unravel major correlates of early immune control of viral replication and the pathogenesis of infection. SIV-specific CD8+ T-cell responses provide critical help in limiting AIDS virus replication in early infection, and the studies here suggest that they also play a role in preserving CD4+ T cells and, thereby, indirectly assist in the generation of virus-specific CD4+ T-cell and B-cell responses. In turn, it is also highly likely that these immune responses may have a supportive role in the generation of robust memory CD8+ T-cell responses. Although primary CD8+ T-cell responses can be generated without CD4+ T-cell help, sustained robust memory CD8+ T-cell responses depend on CD4+ T-cell help (35, 71). It is conceivable that in AIDS virus-infected hosts, CD8+ T cells may also exert a negative effect on the immune system by promoting the development or activation of memory CD4+ CCR5+ cells, which serve as targets for additional viral replication. However, in naïve and very likely in vaccinated individuals, even partially effective immune control of a pathogenic AIDS virus infection appears to be impossible without virus-specific CD8+ T-cell responses.

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REFERENCES


